GENETIC ABNORMALITIES IN PAEDIATRIC GLIAL TUMOURS

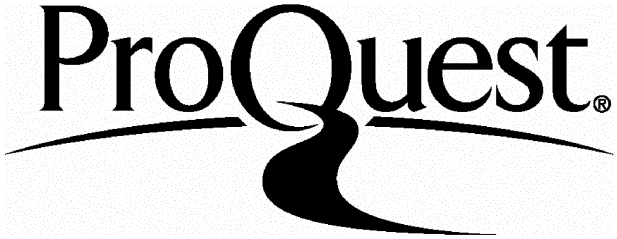
Presented by

Samantha Jane Ward

A thesis submitted in part fulfillment of the requirements for the degree of Doctor of Philosophy of the University of London

Neuro-Oncology Group
Department of Molecular Pathogenesis
Institute of Neurology
Queen Square
London WC1N 3BG

March 2003



ABSTRACT

Primary central nervous system tumours account for 9-20% of all malignancies in the paediatric age group with astrocytoma being the most common tumour type and ependymoma the third. Little in known about the genetic aberrations that occur in paediatric glial tumours, and there are no definitive genetic markers that can determine outcome in children with these tumours. The aims of the present study were to identify consistent regions of genetic loss and gain in paediatric ependymoma and astrocytoma using comparative genomic hybridisation (CGH) and to determine whether any chromosome abnormalities can be used as prognostic markers.

CGH was used to analyse 51 ependymoma and 64 astrocytoma from 110 patients for regions of genomic imbalance. A large proportion of the samples (65.5%) had normal karyotypes. In both ependymoma and astrocytoma, gain of 4q was the most frequent region of gain and loss of chromosome 19 was the most frequently deleted region. With a few exceptions loss of 1p was seen independently of gain of 1q in ependymoma, allowing the ependymoma to be classified into three groups based on CNAs. The study showed the alterations found in paediatric astrocytoma to differ from those seen in adult tumours, suggesting a different pathway leads to their formation. *MDR1* expression was observed in 65% of ependymoma suggesting this gene may have a role in the drug resistant phenotype of some ependymoma.

A number of potential prognostic markers were identified, including increased survival in ependymoma patients with monosomy 19. Gain of 7q and loss of 12q were associated with recurrent ependymoma. Loss of 16q was seen more frequently in primary tumours that had recurred than those with no tumour recurrence. The presence of a normal karyotype was associated with increased survival in patients with low-grade astrocytoma. Decreased survival was seen in astrocytoma patients with either gain of 2q, 5q and 7 or loss of 16p.

CONTENTS

List of Figures

Chapter 4: Genetic analysis of ependymoma

Chapter 5: Genetic analysis of astrocytoma

Chapter 7: Future work

List of Tables

ABBREVIATIONS

AA	Anaplastic astrocytoma
ACNU	3(4-amino-2-methyl-5-pyrimidinyl)methyl-1-(chloroethyl)-1-nitrosourea hybdrochloride
AE	Anaplastic ependymoma
AR	Androgen receptor
ATM	Ataxia telangiectasia-mutated
BB	Baby brain
BBB	Blood brain barrier
BCNU	1,3-bis-(2-chloroethyl)-1-nitrosourea
CAS	Capillary assay solution
CCSG	Childrens cancer study group
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
CDK4	Cyclin dependent kinase 4
CDK6	Cyclin dependent kinase 6
CDKN2A	Cyclin dependent kinase inhibitor 2A
CDKN2B	Cyclin dependent kinase inhibitor 2B
CGH	Comparative genomic hybridisation
CNA	Copy number aberration
CNS	Central nervous system
CSF	Cerebral spinal fluid
CT	Computerised tomography
DA	Diffuse astrocytoma
DAPI	4,6-diamidinophenylindole
DCAI	Desmoplastic cerebral astrocytoma of infancy
dH_2O	Distilled water
DMBT1	Deleted in malignant brain tumours 1
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOP-PCR	Degenerate oligonucleotide primed polymerase chain reaction
EDTA	Ethylene diamine tetraacetic acid
EGFR	Epidermal growth factor receptor
FCS	Foetal calf serum
FISH	Fluorsecent In Situ Hybridisation
GBM	Glioblastoma multiforme
GKRS	Gamma knife radiosurgery
GST	Glutathione S-transferase
HBSS	Hanks buffered saline solution
HDCT	High dose chemotherapy
ICE	Ifosfamide, carboplatin and VP-16
i(17q)	Isochromosome 17q
kD	Kilo Daltons
LGG	Low grade glioma
LN_2	Liquid nitrogen
LOH	Loss of heterozygosity
MDM2	Human homolog of mouse double minute 2
MDR	Multidrug resistance

MDR1	Multidrug resistance 1
MEN1	Multiple endocrine neoplasia type 1
MGMT	O^6- methylguanine-DNA methyltransferase
MMAC1	Mutated in multiple advanced cancers
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MT	Metallothionein
MXI1	MAX-interacting protein 1
NF1	Neurofibromatosis 1
NF2	Neurofibromatosis 2
NP-40	Nonidet P-40
PA	Pilocytic astrocytoma
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PFS	Progression free survival
PGP	P-Glycoprotein
PHA	Phytohaemaglutin
PNET	Primitive neuroectodermal tumour
PTEN	Phosphatase and tensin homolog
PXA	Pleomorphic xanthoastrocytoma
RB	Retinoblastoma
RNA	Ribonucleic acid
RT	Radiotherapy
SCOMP	Single cell comparative genomic hybridisation
SDS	Lauryl sulfate
SE	Subependymoma
SEGA	Subependymal giant cell astrocytoma
SSC	Sodium chloride/Sodium citrate
SV40	Simian virus 40
TAE	Tris Acetate
Tag	Tumour antigen
TCR	Transcriptional control regions
TE	Tris EDTA
Topo IIα	DNA topoisomerase IIα
TSC	Tuberous sclerosis complex
ULS	Universal linkage system
VCR	Vincristine
VP-16	Etoposide
WHO	World Health Organisation

CHAPTER 1
INTRODUCTION

Incidence and classification of paediatric brain tumours

Primary central nervous system (CNS) tumours are the most common solid tumour in childhood, accounting for 9-20% of all malignancies in the paediatric age group (Bigner and Schrock, 1997; Karnes and Raffel, 1993; Packer, 1994). Approximately 300 children are diagnosed each year with a primary brain tumour in the United Kingdom (http://www.nottingham.ac.uk). The distribution of the different subtypes of brain tumour in children can be seen in Figure 1.1. In adults the most common brain tumours are malignant gliomas, meningioma, schwannoma, pituitary tumours and metastases, yet these tumours are rare in the paediatric age group. Low grade astrocytoma and PNET account for a higher percentage of brain tumours in children than adults (Pollack, 1999).

There is also a difference between adults and children in the location of tumours. In adults most neoplasms are located supratentorially whereas most paediatric CNS tumours arise in the posterior fossa. In adults the majority of tumours will arise within the cerebral hemispheres, whereas approximately 50% of paediatric brain tumours will arise infratentorially (Pollack, 1994; Pollack, 1999).

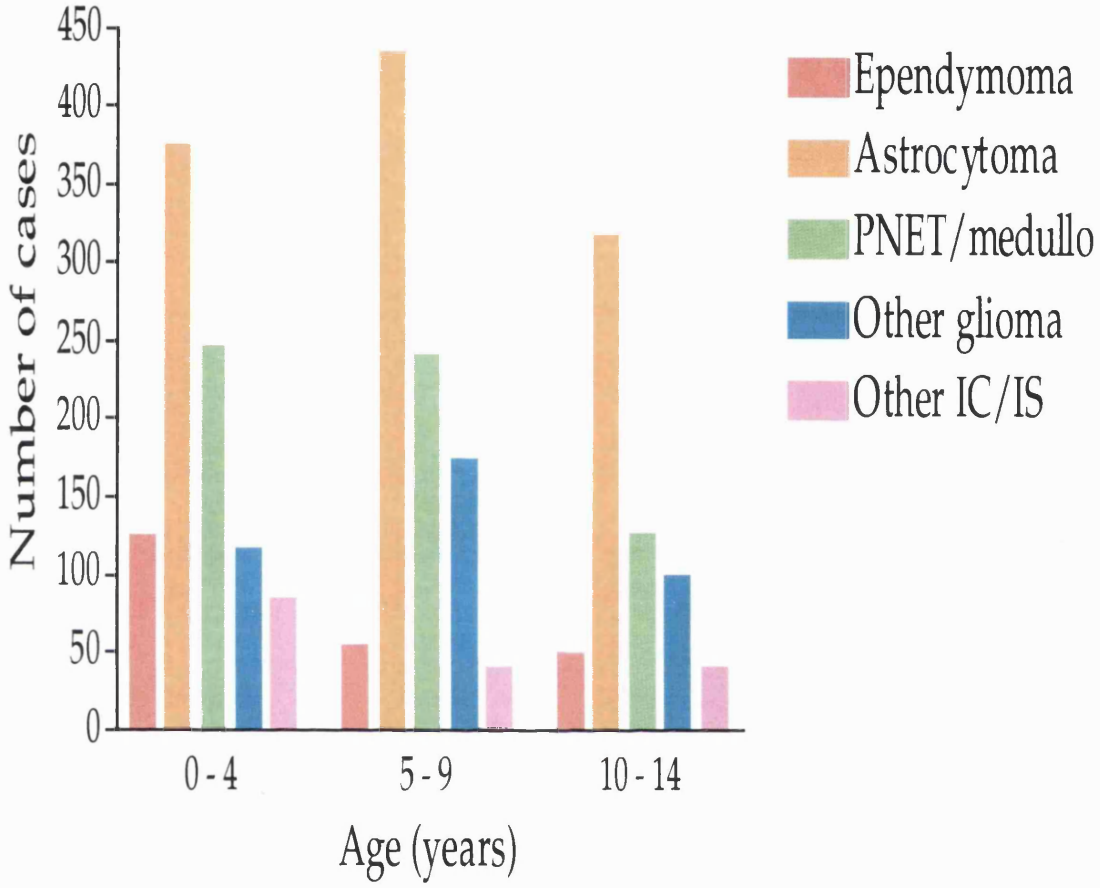


Figure 1.1: Age distribution of paediatric brain tumours in the UK.
Data compiled from the Regional Cancer Registries of Scotland, Northern Ireland, East Anglia, SouthEast England, NorthWest England, West Midlands, Trent, North Yorkshire and Oxford for the years 1983 - 2000. Reported cases are split into three age groups: 0-4 years, 5-9 years and 10-14 years and represent the number of reported cases of ependymoma, astrocytoma, PNET/medulloblastoma, other gliomas (including oligodendroglioma) and other intracranial/intraspinal lesions.

Ependymoma

Ependymoma are tumours that arise from the ependymal cells that line the ventricles of the central nervous system, the central canal of the medulla and the filum terminale (Dohrmann et al, 1976; Verstegen et al, 1997). Although ependymoma may occur at any site in the ventricular system they most commonly present in the posterior fossa, followed by the lateral ventricle and the spinal cord (Schiffer and Wiestler, 1997). In children, ependymoma represents 6-12% of intracranial tumours and 30% of these tumours occur in children under three years of age (Schiffer and Giordana, 1998). In adults, ependymoma represents less than 3% of CNS tumours (Allen et al, 1998). For the age distribution of ependymoma see Figure 1.2. As many as 90% of paediatric ependymoma are intracranial tumours and only 10% spinal (Allen et al, 1998). Ependymoma may show different growth patterns, such as expansion toward the adjacent nervous tissue or infiltration from the solid tumour to the normal nervous system. Isolated tumour islands may be found in adjacent tissue. The tumour may spread via the cerebral spinal fluid (CSF) especially if it is located in the posterior fossa or if it is anaplastic (Pierre-Khan et al, 1983; Salazar, 1983). There have been reports of ependymoma metastasizing to the lungs in several cases (Schiffer and Wiestler, 1997).

The WHO recognises five categories of ependymal tumours: ependymoma, anaplastic or malignant ependymoma, myxopapillary ependymoma, subependymoma and ependymoblastoma (Gonzales, 1995).

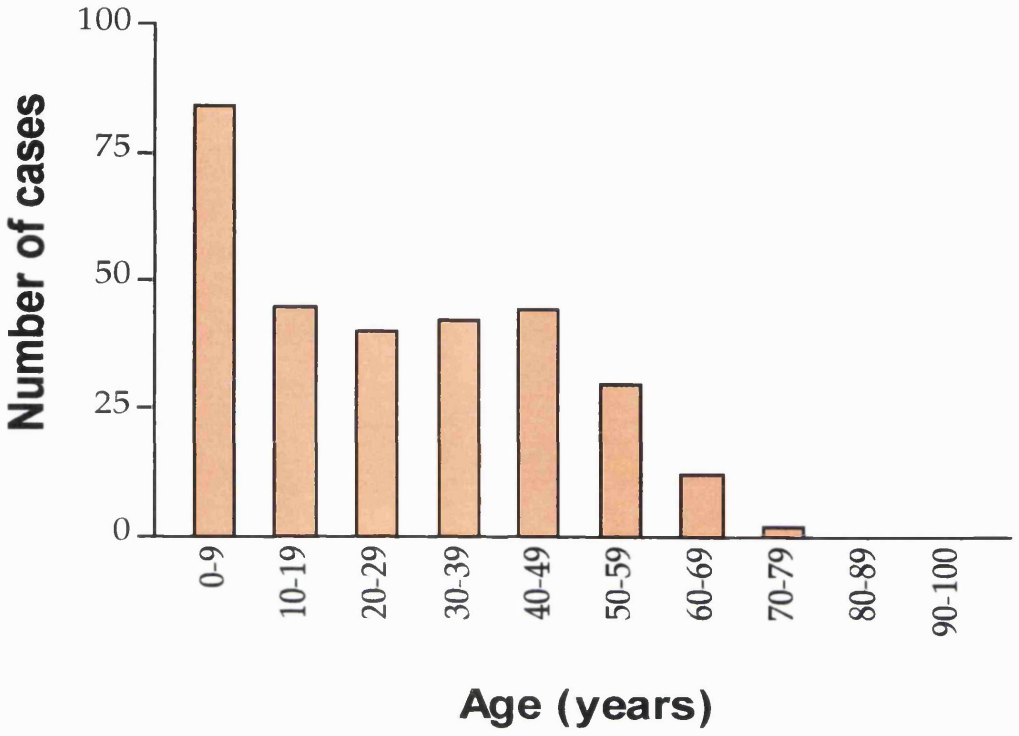


Figure 1.2 Age distribution of ependymoma.
Data based on 298 cases (Schiffer et al, 1991).

Classic Ependymoma: Ependymoma occurs in all age groups. In children the mean age at manifestation is 6.4 years (Schiffer and Wiestler, 1997). These tumours occur at any site in the ventricular system, with the most common being the posterior fossa, followed by the lateral ventricles and the spinal cord (Wiestler et al, 2001b). Ependymoma are classified as WHO grade II tumours. The current WHO classification recognises four histological variants of classic ependymoma: cellular, papillary, clear cell and tancytic (Schiffer and Wiestler, 1997).

Anaplastic Ependymoma: Anaplastic (malignant) ependymoma is an aggressive tumour that corresponds histologically to WHO grade III. The incidence of these tumours varies from institution mostly due to the uncertainty regarding the histological criteria for malignancy (Wiestler et al, 2001a). Anaplastic ependymoma are more likely to occur intraventriculary than spinally. They account for 25% of all ependymoma and have a greater tendency to invade adjacent brain structures and metastasize via the CSF than lower grade tumours (Schiffer and Wiestler, 1997). Anaplastic ependymoma are recognised by their increased cellularity, increased mitotic activity and the presence of endothelial proliferation (Applegate and Marymont, 1998; Lopes et al, 1995).

Myxopapillary Ependymoma: These are a distinct variant of ependymoma, limited to the region of the cauda equina/filum terminale. They rarely invade nerve roots and even more rarely erode sacral bone. They are occasionally found at other locations such as the cervical-thoracic spinal cord, the lateral ventricle and the brain parenchyma (Liu et al, 1976; Sonneland et al, 1985; Warnick et al, 1993). These slow growing tumours are biologically benign and correspond to WHO grade I. They are more commonly seen in adults (age 30-40 years) than in children, with the average age of incidence being 36.4 years. (Lopes et al, 1995; Schiffer and Wiestler, 1997).

Subependymoma: Subependymoma account for approximately 2% of ependymoma and correspond histologically to WHO grade I (Schiffer and Wiestler, 1997). Subependymoma seldom gives rise to symptoms during life but is usually an incidental finding at autopsy (Verstegen et al, 1997). This type of tumour is relatively uncommon in children and is seen most frequently in middle aged and elderly males. (Lopes et al, 1995; Schiffer et al, 1991; Schiffer and Wiestler, 1997). The most frequent site is the fourth ventricle (50-60%) followed by the lateral ventricles (30-40%) (Schiffer and Wiestler, 1997). Other locations are the septum

pellucidum, and foramen of Monro. Spinal cord subependymoma develop as either cervical intramedullary or extramedullary growths (Jallo et al, 1996).

Astrocytoma

Astrocytic tumours are derived from astrocytes which are the most abundant cellular component of the connective tissue of the brain (Paulus et al, 1996). The World Health Organization (WHO) recognises five clinicopathological groups of astrocytic tumour.

Diffuse astrocytoma: Diffuse astrocytoma may arise anywhere within the CNS but are most often located in the cerebral hemispheres. Irrespective of histological grade these tumours infiltrate adjacent and distant brain structures and have a tendency to progress to a more malignant phenotype. Diffuse astrocytoma are the most frequent intracranial neoplasms, accounting for 60% of all primary brain tumours (Whittle et al, 1989). They are more prevalent in adults than children.

There are three distinct entities within diffuse astrocytoma:

• ***Low grade diffuse astrocytoma***: These tumours are characterised by a high degree of cellular differentiation, slow growth and infiltration of surrounding brain structures. They correspond to WHO grade II and represent 25% of all astrocytoma occurring in the cerebral hemispheres. The peak incidence is in young adults aged between 30 and 40 years (see Figure 1.3). These tumours most commonly develop supratentorially in the cerebrum. This class of tumours can be subdivided further into fibrillary astrocytoma, gemistocytic astrocytoma and protoplasmic astrocytoma (Lu et al, 2001).

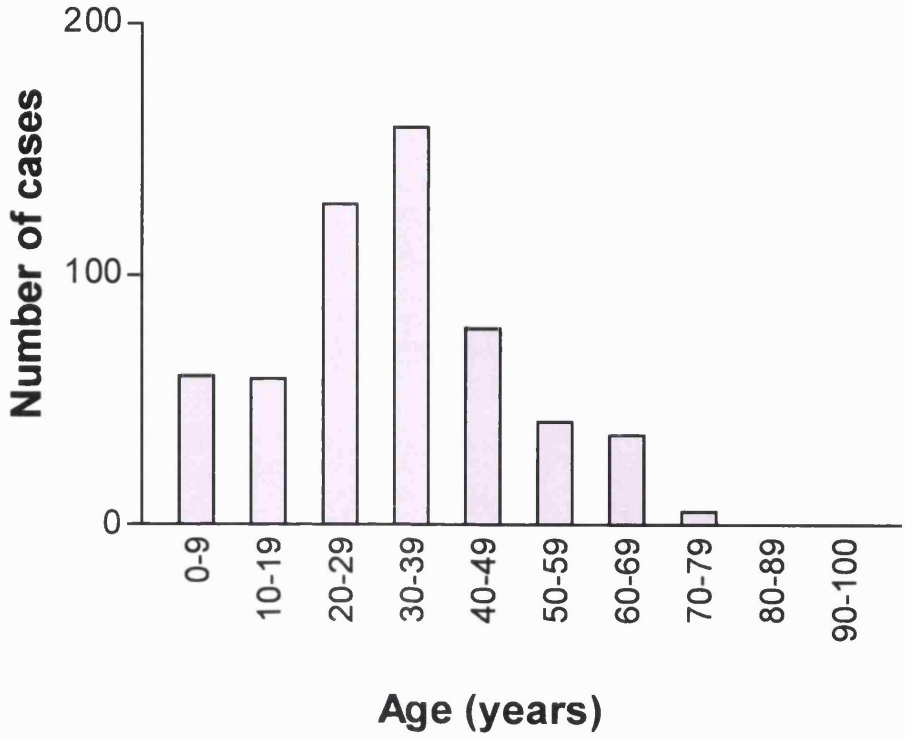


Figure 1.3: Age distribution of diffuse astrocytoma (WHO grade II). Figures based on the biopsies of 529 patients from the Tumour Registry of the University of California, San Francisco, USA and the Institute of Neuropathology, University Hospital, Zurich (Kleihues et al, 2001).

• ***Anaplastic astrocytoma***: Anaplastic astrocytoma can arise from low grade astrocytoma but can also be diagnosed at the time of first surgery. These tumours have a tendency to progress to glioblastoma multiforme (GBM). They correspond to WHO grade III. The age distribution of anaplastic astrocytoma is shown in Figure 1.4.

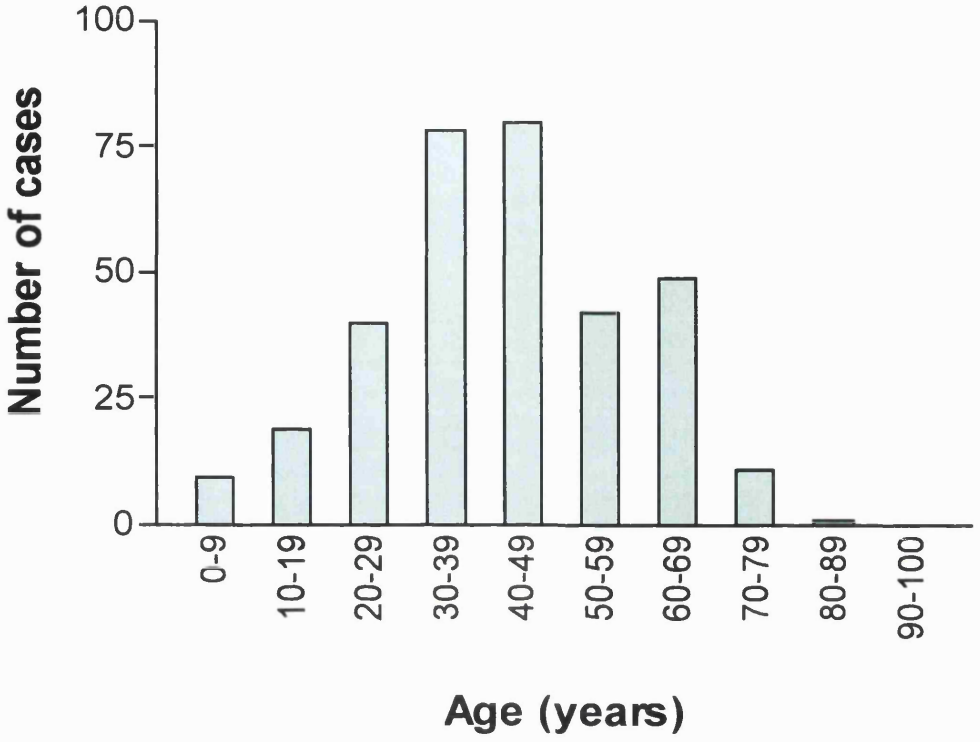


Figure 1.4: Age distribution of anaplastic astrocytoma

Data based on the biopsies of 319 patients treated at the University Hospital, Zurich (Kleihues et al, 2001)

• ***Glioblastoma multiforme (GBM)***: This is the most malignant of the astrocytic tumours and corresponds to WHO grade IV. These tumours are composed of poorly differentiated neoplastic astrocytes with areas of vascular proliferation and/or necrosis. GBM is more common in adults than children with a peak incidence between 45 and 70 years (see Figure 1.5). In the adult population, such tumours may develop from diffuse or anaplastic astrocytoma. There are several variants of GBM: multi-focal GBM, primary GBM, and secondary GBM (Chakrabarty et al, 1999).

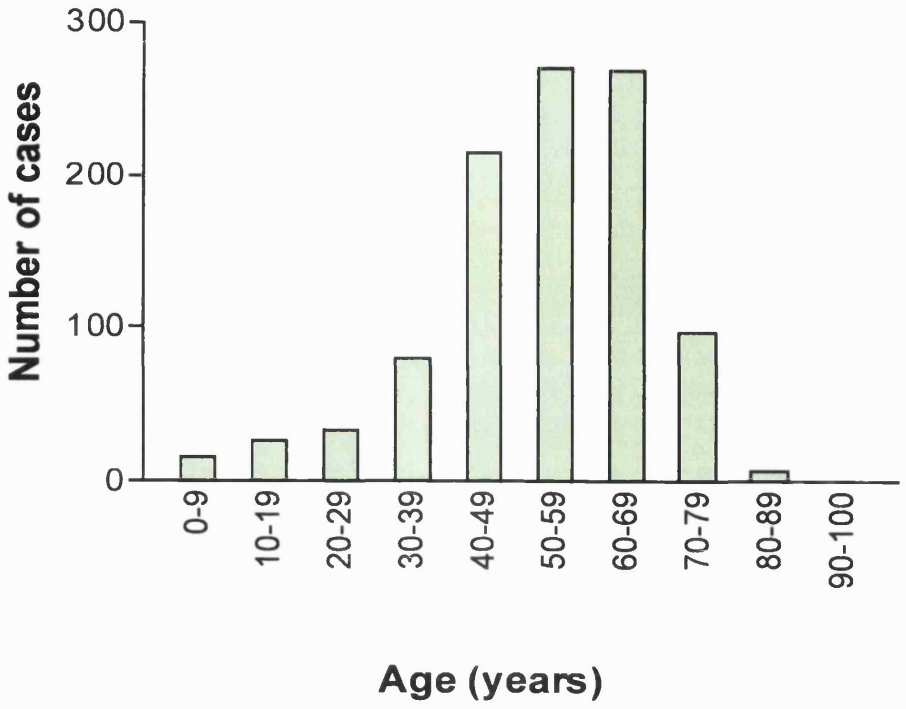


Figure 1.5: Age distribution of glioblastoma multiforme
Data based on the biopsies of 1003 patients treated at the University Hospital, Zurich (Kleihues et al, 1993a).

Primary GBM develop *de novo* without any evidence (clinical, radiological or morphological) of a less malignant precursor lesion. In comparison, secondary GBM arise through progression from low grade or anaplastic astrocytoma (Kleihues and Ohgaki, 1999). Primary GBM is characterised by a rapid development of clinical symptoms, with 50% of patients typically having a history of less than three months. In secondary GBM, the progression from a lower grade tumour varies from case to case, with time intervals of less than a year to more than ten years being reported (Ohgaki et al, 1999; Watanabe et al, 1997). One study has shown no difference in survival for patients with secondary GBM compared to those with primary GBM (Dropcho and Soong, 1996). In recent years strong evidence has accumulated for the existence of different genetic pathways in GBM pathogenesis (see Figure 1.6).

Despite some overlapping features the pathogenesis of both types of GBM is markedly different, suggesting they are two different diseases. The most frequent genetic alterations in primary GBM are *EGFR* amplification/overexpression, *MDM2* amplification/overexpression, *CDKN2A/B* deletion and *PTEN* mutations. In secondary GBM, p53 mutations are the most common alteration. The main differences between the two kinds of GBM is shown in Table 1.1.

	Primary GBM	Secondary GBM
Preoperative clinical history	1.7 months (Watanabe et al, 1996)	53 months from low grade astrocytoma (Watanabe et al, 1996) 25 months from anaplastic astrocytoma (Watanabe et al, 1996)
Sex ratio (M/F)	1/4 (Watanabe et al, 1996)	0/8 (Watanabe et al, 1996)
Age at diagnosis (years)	55.5 (Kleihues et al, 1993b; Watanabe et al, 1996)	40 (Kleihues et al, 1993b; Watanabe et al, 1996)
P53 mutation	2/19 (11%) (Watanabe et al, 1996)	20/30 (67%) (Watanabe et al, 1996)
MDM2 amplification	8/104 (7.5%) (Biernat et al, 1997; Reifenberger et al, 1993)	0/27 (0%) (Biernat et al, 1997)
EGFR amplification	11/28 (39%) (Klein, 1987)	0/22 (0%)
P16 deletion	10/28 (36%) (Biernat et al, 1997)	1/23 (4%) (Biernat et al, 1997)
PTEN mutation	9/28 (32%)	1/25 (4%)

Table 1.1: Synopsis of clinical and genetic data of primary and secondary GBM. Data from Kleihues & Ohgaki (1999).

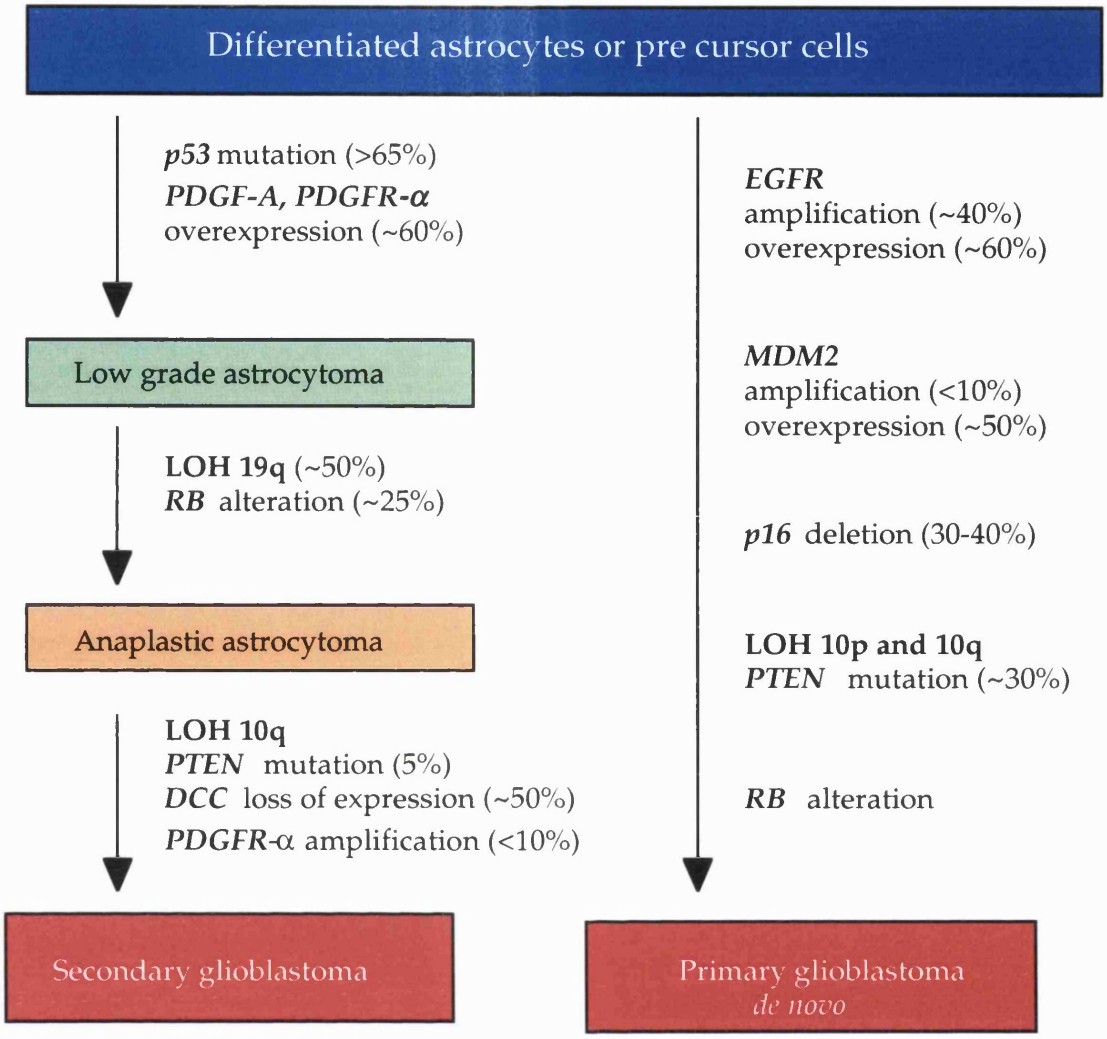


Figure 1.6: Genetic pathways operating in the evolution of GBMs (Kleihues and Ohgaki, 1999).

Pilocytic Astrocytoma: This type of astrocytic tumour generally occurs in children and young adults (see Figure 1.7). The tumours are composed of variable portions of spongy and compact tissue and correspond to WHO grade I. Pilocytic astrocytoma (PA) arise throughout the neuraxis, with the most common sites being the optic nerve, optic chiasm, thalamus and basal ganglia, cerebral hemispheres and the brain stem (Burger, 1996; Clark et al, 1985; Hayostek et al, 1993; McGirr et al, 1987; Rodriguez et al, 1990). Spinal cord tumours are less frequent but not uncommon (Minehan et al, 1995). PA are slow

growing tumours that may stabilise or even regress. These tumours rarely prove to be fatal, although there are few long term follow up studies outlining the ultimate outcome of patients. PA are the main cerebral nervous system neoplasm associated with neurofibromatosis type 1 (NF1). Approximately 15% of patients with NF1 will develop PA, usually of the optic nerve (Burger et al, 1993; Gilles et al, 2000).

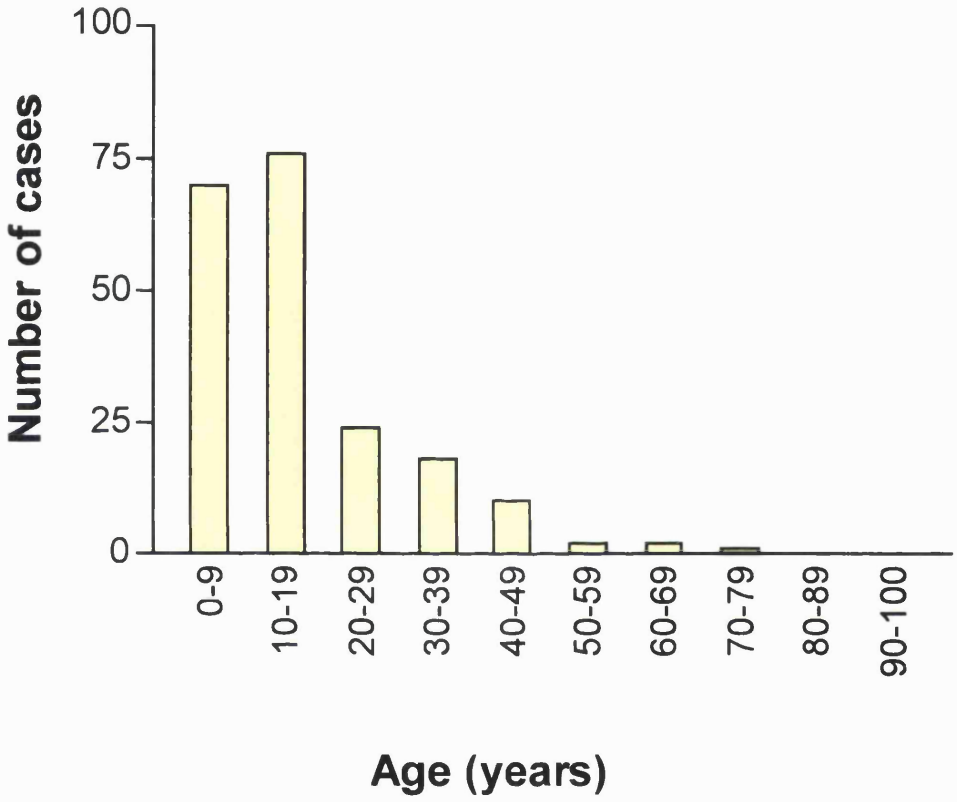


Figure 1.7: Age distribution of pilocytic astrocytoma.
Data based on the biopsies from 205 patients treated at the University Hospital, Zurich (Burger et al, 2001).

Pleomorphic Xanthoastrocytoma: This tumour is most often seen in children and young adults and 60% of patients are under 18 years of age (see Figure 1.8). Pleomorphic xanthoastrocytoma (PXA) account for less than 1% of all astrocytic tumours (Rostomily et al, 1997). They have a superficial location in the cerebral hemispheres and there is often

involvement of the meninges. The temporal lobe is the more favoured site, though there have been reports of spinal tumours (Herpers et al, 1994; Kepes et al, 1993). Most pleomorphic xanthoastrocytoma are classified as WHO grade II although some may have malignant features, in particular marked nuclear pleomorphism. Some tumours may undergo malignant transformation to high-grade glioma or GBM (Chakrabarty et al, 1999).

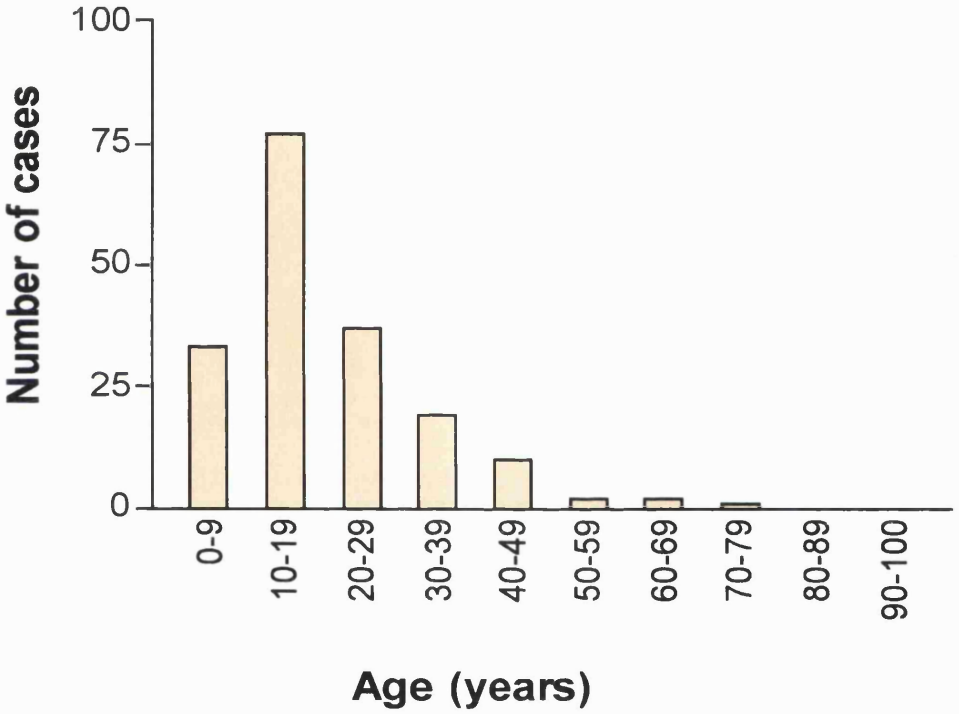


Figure 1.8: Age distribution of patients with pleomorphic xanthoastrocytoma (Kepes et al, 2000).

Desmoplastic cerebral astrocytoma of infancy (DCAI): These tumours are often cystic and slow growing. They are rare neoplasms that are quite large and often involve the superficial cortex and leptomeninges where they attach to the dura. They are WHO grade I tumours and have a good prognosis after surgical resection. DCAI occur more

frequently in males than females, with a peak age incidence at 5-6 months of age (Eberhart et al, 2002; Taratuto and Rorke, 1997).

Subependymal giant cell astrocytoma (SEGA): This form of astrocytoma is most often seen in the first two decades of life, usually in association with the tuberous sclerosis complex (TSC). SEGA is the most common CNS neoplasm in TSC and the incidence ranges from 6% to 16% of confirmed cases of TSC (Shepherd et al, 1991). These tumours are quite often nodular solid tumours that can be well delineated from the underlying brain parenchyma. They most commonly arise from the wall of a lateral ventricle and less commonly from the third ventricle. Calcification and signs of previous haemorrhage can be present (Lopes et al, 1993; Wiestler et al, 1997).

Clinical Manifestations

Children with brain tumours exhibit a number of clinical manifestations, which may include irritability, listlessness, vomiting, failure to thrive, and progressive macrocephaly (Pollack, 1994). Such symptoms are due in part to raised intracranial pressure which may arise as a result of the mass effect of the tumour itself or from ventricular obstructions (Berger, 1996; Kadota et al, 1989).

Treatment

Progress in the treatment of brain tumours has been relatively poor compared to that achieved in other forms of childhood cancer. This is due in part to the differing tumour types occurring in different areas of the brain and also to the low incidence of these tumours making it difficult for meaningful therapeutic trials to be conducted (Finlay et al, 1987). Historically the standard approach to the treatment of childhood brain tumours has been either surgery alone or surgery followed by radiation therapy. If the tumour recurred following treatment with

surgery and radiotherapy it was often felt there was no advantage to subjecting the child to unproven chemotherapeutic agents. Pioneering trials in the 1970's led to the use of vincristine and nitrosureas in the treatment of paediatric brain tumours and also led to the establishment of multicentre co-operative group trials for the testing of adjuvant chemotherapy in the treatment of children with newly diagnosed brain tumours (Rosenstock et al, 1976; Shapiro, 1975).

Surgery

The main aims of surgery are to establish a histological diagnosis, to restore the CSF pathway and reduce the tumour burden (Pollack, 1999; Punt, 1995; Tomita, 1998). Craniotomy provides the largest samples for pathological examination whereas stereotactic or ventriculoscopic biopsies are less invasive forms of surgery but have a lower diagnostic value for heterogeneous tumours (Tomita, 1998). For example, gliomas originating in the brain stem may be a mix of benign and malignant astrocytoma therefore a small sample of tissue taken from one area of the tumour may not be representative of the entire tumour. Obtaining maximum cytoreduction and neural decompression enhances tumour sensitivity to adjuvant therapy (either radiotherapy and/or chemotherapy) – the smaller the tumour the greater the response to therapy. This also means a lower dose of radiation and less aggressive chemotherapy can be used (Tomita, 1998). Tumour resection is usually accomplished by ultrasonic aspiration, which aids internal debulking of the tumour. In most tumours there is no well defined margin between tumour and normal brain and this infiltration of the surrounding brain tissue is the limitation to complete resection (Pollack, 1999).

The increased use of CT and MRI techniques has allowed earlier diagnosis of tumours and have provided the surgeon with a better delineation of the location and contours of the tumour and its relation to surrounding structures, effectively providing a "road map" to the tumour (Finlay et al, 1987). Stereotactic biopsy using the data provided by MRI and CT scans has become important in the neurosurgical

management of children with brain tumours. It enables the pathological diagnosis of lesions within brain structures that were once considered to be inaccessible, such as the brain stem, basal ganglia, hypothalamus and pineal body (Finlay et al, 1987). The stereotactic aspiration of tumours allows palliative management of patients with recurrent tumours and can be repeated if necessary (Finlay et al, 1987).

Surgery is the most effective mode of treatment for primary ependymoma. Complete resection is associated with a five year progression free survival (PFS) of 60-80% compared to 0-30% in patients undergoing incomplete resection (Hukin et al, 1998; Pollack et al, 1995). Incomplete tumour removal is common among patients with 4th ventricle ependymoma. This is due to the infiltration of tumour along the ventricle into the brain stem. In patients with supratentorial lesions the degree of resection is dependent upon the size and extent of the tumour. Total removal of tumour has been reported in up to 70% of intramedullary ependymoma and 90% of cauda equina tumours (Kun et al, 1988). In a recent study ten patients with ependymoma have been treated with total surgical resection and no adjuvant therapy. Of these patients only one has experienced a recurrence (Tomita, 1998). A prospective study investigated survival in ten paediatric patients with intracranial ependymoma treated by surgery alone. All patients had radiologically confirmed gross total resection. Seven of the ten patients remained in remission 48 months after surgery and three patients underwent repeat surgical resections for recurrent or residual disease. Seven of the eight supratentorial ependymoma were in remission 48 months after gross total resection (Hukin et al, 1998). Twelve patients with supratentorial ependymoma were treated with radical surgery alone in an Italian study (Palma et al, 2000). Three of these patients suffered a recurrence, three patients died within 11 months of surgery and the remaining patients were in remission. This study showed that true radical excision cannot always be achieved in supratentorial ependymoma. In most cases surgery is usually applied in conjunction

with radiotherapy and/or chemotherapy for the treatment of all grades of ependymoma.

Resection is the treatment of choice for patients with gliomas. Children with pilocytic astrocytoma who have undergone total surgical resection have 10-20 year survival rates of between 90 and 100% (Reddy and Packer, 1999). Three patients with pilocytic astrocytoma were treated with surgery alone and were free of disease at a mean of 37 months postoperatively (Vinchon et al, 2001). In a study of thirty five children with low grade gliomas who had no postoperative treatment after surgical resection the overall survival at ten years was 97% and 75% of the patients were also disease free at ten years (Pollack et al, 1995). Twenty-four patients with glioma underwent gross total resection. None of these patients developed a recurrence of their tumour and had a ten year survival of 83% (Fisher et al, 2001). Patients with benign astrocytoma of the cerebellum have excellent survival if there is no brain stem involvement. In a study involving 165 patients with this tumour type who underwent surgical excision and had no postoperative treatment the ten year overall survival was 100% for patients with the cerebellar form and 80% for those patients with brain stem involvement (Pencalet et al, 1999).

Reduction of intracranial pressure using steroids and mannitol is the first step in the treatment of malignant astrocytoma. Radical surgical resection of newly diagnosed anaplastic astrocytoma and GBM in children is a favourable predictor of outcome if followed by radiotherapy and chemotherapy (Finlay and Wisoff, 1999). One patient with anaplastic astrocytoma was treated with surgery alone and died postoperatively. Two patients with GBM underwent surgery followed by chemotherapy. One patient died 12 months after diagnosis from recurrence and the other patient was alive and disease free 64 months after diagnosis (Vinchon et al, 2001). Three patients with GBM and five with anaplastic astrocytoma were treated with surgery alone. Median

survival was 11 months for GBM and 15 months for anaplastic astrocytoma (Raco et al, 1997).

Radiotherapy

Improvements in imaging and dose planning techniques have meant that irradiation can be tailored to the geometry of a patient's individual tumour. The most controversial aspect of radiation treatment is the volume of tissue that should be irradiated. Radiation applied to the whole brain is known to be toxic in young children and can lead to serious intellectual deficit and emotional disturbance (Helseth et al, 2001; Hukin et al, 1998; Tomita, 1998). Craniospinal irradiation can lead to short stature, due to impaired growth hormone secretion or the direct effects of radiation on the growing spine. Posterior fossa irradiation can lead to hearing impairments (Tomita, 1998). A quality of life study in children who had undergone radiotherapy for a brain tumour showed major focal neurological deficits in 33% of children less than 2 years of age compared to 22% in children aged 2-4 years. Major visual defects were present in 35% of children less than 2 years old compared to 18% of 2-4 year olds. Although uncommon, blindness occurred more frequently in patients younger than age 2 (19%) than in 2-4 year olds (4%). No difference was seen in hearing or schooling. Radiation treatment was associated with a cure in 1 of 3 patients, however quality of life for survivors was not good due to the focal neurological defects present from the time of first treatment (Jenkin et al, 1998).

In ependymoma radiotherapy is used to prevent subarachnoid seeding by eradicating tumour cells in the CSF and to prevent tumour recurrence by removing the postoperative residual tumour and possible metastases. The addition of postoperative irradiation has improved 5-year survival from approximately 20% to the range of 35-60% when combined with maximum resection (Kun et al, 1988).

Strategies are based on patient age, the tumour location and grade and the status of the subarachnoid space and CSF (Verstegen et al, 1997). A summary of treatment protocols for paediatric ependymoma can be seen in Table 1.2. Even after tumour resection and adjuvant radiotherapy there is nearly always recurrence at the primary tumour site rather than distantly within the neuraxis (Hodgson et al, 2001; Kovalic et al, 1993; McLaughlin et al, 1998; Paulino, 2001; Paulino and Wen, 2000; Stafford et al, 2000). A number of studies have shown survival to be increased using treatment doses of 45Gy or more. Paulino and Wen (2000) reported a 5 year survival of 71.5% in patients treated with 45Gy to the primary site. Survival of 46-70% was reported in a retrospective study of ependymoma treated with 45-55Gy to the primary site (Kun et al, 1988). Kovalic et al, (1993) reported superior survival in tumours treated with cranial radiation doses of 50Gy. Treatment duration of less than 50 days was also reported to increase survival in patients treated with 45Gy (Paulino and Wen, 2000).

In the treatment of low grade astrocytoma radiotherapy can result in shrinkage of the tumour and may improve the duration of disease free survival in children who have undergone partial tumour resection (Reddy and Packer, 1999). The use of irradiation is often reserved for children with recurrent or symptomatic astrocytoma (Tarbell and Loeffler, 1996). The role of radiotherapy in preventing recurrence of cerebellar astrocytoma is controversial. Some studies suggest that postoperative radiotherapy is beneficial after incomplete resection in maintaining local tumour control, delaying the time to recurrence and therefore improving the survival time (Dewit et al, 1984; Dirven et al, 1997; Ilgren and Stiller, 1986). In comparison, other studies have shown a recurrence rate of around 34% in spite of radiation doses of 40Gy (Garcia and Fulling, 1985; Marsa et al, 1973; Wallner et al, 1988).

No. Patients	Diagnosis	Dose	Area	Survival	Reference
14	Ependymoma	35-40Gy	Craniospinal axis + boost to posterior fossa	10 year = 35%	(Evans et al, 1996)
9	PF ependymoma	39-40Gy 18-36Gy	Local RT Craniospinal	5 year = 28%	(Helseth et al, 2001)
1	ST ependymoma	51Gy 30Gy	Local RT Craniospinal	5 year = 0%	
3	IS ependymoma	54Gy 32-54Gy	Local RT Spinal	5 year = 100%	
47	Ependymoma	33Gy 35Gy	Spinal Whole brain	5 year = 46%	(Goldwein et al, 1990)
20 12	Ependymoma AE	45-54Gy 23-36Gy	Primary site Craniospinal	5 year = 64%	(Robertson et al, 1998)
8 9 7 7 20	Ependymoma Ependymoma AE AE AE	36-45Gy 36-45Gy 36-45Gy 36-45Gy 30-40Gy	Partial brain Whole brain Partial brain Whole brain Craniospinal	4-10years = 12% 3-10years =67% 5 years = 8% 5 years = 8% 5 years = 47%	(Salazar et al, 1983)
18 25	Sp AE Sp Ependymoma	50Gy 50-55Gy	Entire CNS Spinal RT	5 years = 68%	(Whitaker et al, 1991)
22	IT Ependymoma	51Gy 32Gy	Primary site Craniospinal	10 years = 45%	(McLaughlin et al, 1998)
10	ST Ependymoma	35-58Gy 36Gy	Primary site Craniospinal	10 years = 20%	
9	Sp Ependymoma	45-52Gy	Spinal	10 years = 100%	
12	RT Ependymoma	12-24Gy	Primary site (SRS)	3 year = 68%	(Stafford et al, 2000)
35	IT Ependymoma	36Gy 39Gy 54Gy	Craniospinal Whole brain Primary site	5 year = 71% 5 year = 60% 5 year = 80%	(Paulino and Wen, 2000)
53	IT Ependymoma	35Gy 20Gy	Neuroaxis PF boost	3 year = 75.6%	(Timmermann et al, 2000)
28	Ependymoma	12.5Gy	Primary site (SRS)	3 year = 29%	(Hodgson et al, 2001)
30	Ependymoma	47.5Gy 50.8Gy	Local field Whole brain	10 year = 75% 10 year = 28%	(Kovalic et al, 1993)

Table 1.2: Summary of radiotherapy protocols used in the treatment of paediatric ependymoma. PF = posterior fossa; IT = intracranial; ST = supratentorial; Sp = spinal; AE = anaplastic ependymoma; SRS = stereotactic radiosurgery; RT = recurrent

Chemotherapy

Since the early 1990s chemotherapy has been widely used in the management of brain tumours in young children. Vincristine was the first agent to have clinical efficacy in childhood brain tumours (Rosenstock et al, 1976). It was originally used as a single agent but is now more often used in combination with other agents to treat astrocytoma. Other single agent treatments have included carboplatin in the treatment of progressive or low-grade astrocytoma (Friedman et al, 1992), high dose cyclophosphamide for the treatment of disseminated pilocytic astrocytoma (McCowage et al, 1996) and oral etoposide for the treatment of recurrent cerebellar pilocytic astrocytoma (Chamberlain, 1997).

The majority of children with intracranial ependymoma will die from tumour progression despite treatment with surgery and radiotherapy (Bouffet and Foreman, 1999). The poor prognosis for these patients has led to the use of chemotherapy in the management of children with ependymoma. A number of protocols have been used to treat children with ependymoma, a summary of these can be seen in Table 1.3. Multi-agent chemotherapy (carboplatin, procarbozine, etopside, cisplatin, vincristine and cyclophosphamide) has been used in the treatment of children with both posterior fossa and supratentorial ependymoma (Grill et al, 2001). Progression or relapses were diagnosed in fifty-three patients 2 to 45 months after the initiation of chemotherapy. The 4 year PFS was 22%, with an overall survival rate of 59%. This study suggests that a proportion of children with ependymoma may be able to avoid treatment with radiation by the administration of adjuvant chemotherapy. Deferring irradiation did not appear to compromise the overall survival of the patient population in this particular study, but survival was not that different from that seen in studies using surgery and radiotherapy. The Children's Cancer Study Group initiated a study to assess the value of adjuvant chemotherapy (using vincristine

followed by maintenance chemotherapy consisting of CCNU, vincristine and prednisone) in children with posterior fossa ependymoma after treatment with radiotherapy. A control group was treated with radiotherapy alone. There was no difference in outcome between the two groups, suggesting chemotherapy is not effective in improving the outcome in children with ependymoma (Evans et al, 1996). The eight-drugs-in-1-day regimen has been used to treat children with ependymoma and anaplastic ependymoma (Robertson et al, 1998). The eight agents used were methylprednisone, vincristine, CCNU or BCNU, procarbozine, hydroxyurea, cisplatin, cytarabine, and cyclophosphamide given in two courses, fourteen days apart, followed by the initiation of craniospinal irradiation. PFS was 45% after a mean follow up of 24.7 months.

No. patients	Diagnosis	Agents	No. cycles	Survival	Reference
22	E	Vincristine CCNU Prednisone	8	10 years 40%	(Evans et al, 1996)
20 12	E AE	Vincristine CCNU Prednisone or 8-drugs-in-1-day	8 10	5 year 64%	(Robertson et al, 1998)
48	E	Vincristine Cyclophosphamide Cisplatin Etoposide	2	2 years 74%	(Duffner et al, 1993)
60 13	AE E	Carboplatin Procarbozine Etoposide Cisplatin Vincristine Cyclophosphamide	7	4 year 59%	(Grill et al, 2001)
11	AE	Ifosfamide VP16 Carboplatin	10	10 years 39%	(Fouladi et al, 1998)

Table 1.3: Summary of chemotherapy protocols used in the treatment of paediatric ependymoma. E = ependymoma; AE = anaplastic ependymoma

The impact of adjuvant chemotherapy on the survival of patients with anaplastic ependymoma has been investigated. Eleven patients with anaplastic ependymoma underwent surgical resection followed by chemotherapy (Fouladi et al, 1998). The regimen, known as ICE, consisted of ifosfamide, VP-16 and carboplatin for two cycles prior to radiotherapy and eight cycles after radiation. Patients less than 2 years of age did not undergo radiotherapy and had 10 cycles of ICE only. Patients undergoing adjuvant ICE chemotherapy had a seven year survival of 39%. This regimen did not confer an overall survival advantage. A recent study investigated the use of pre-irradiation "sandwich" chemotherapy in children with anaplastic ependymoma (Timmermann et al, 2000). Fifty-five patients were treated; fifteen with maintenance chemotherapy and forty with sandwich chemotherapy. Maintenance chemotherapy was started during irradiation with the administration of vincristine once a week. Six weeks after the end of irradiation eight cycles of chemotherapy were given every six weeks comprising cisplatin, CCNU and vincristine. Sandwich chemotherapy consisting of ifosfamide, etoposide, methotrexate, citrovorum-factor (CF rescue), cisplatin and cytarabine was administered 14 days after surgery. If there was progression of disease during chemotherapy radiotherapy was started immediately. Children having maintenance chemotherapy had a 3 year PFS rate of 61.9% and those on the sandwich chemotherapy regimen had a 3 year PFS of 63.7%. The administration of chemotherapy to the patients in this study did not alter their prognosis. Sixty children with anaplastic ependymoma were treated according to a protocol described by Grill et al. (2001). The four-year overall survival for these patients was 61%. No data was available on PFS for these patients. The data on the effects of chemotherapy on survival in patients with ependymoma is comparable to that reported for patients who have received radiotherapy and no chemotherapy (Goldwein et al, 1990; Nazar et al, 1990; Shuman et al, 1975). This suggests that chemotherapy does not increase outcome compared to that achieved with surgery and radiotherapy.

Various combinations of chemotherapeutic agents have been used in the treatment of children with astrocytoma, a summary of which can be seen in Table 1.4. Carboplatin and etoposide have been used to treat nineteen patients with incompletely resected astrocytoma and resulted in an overall survival of 58% at 60 months post-diagnosis (Castello et al, 1998). Cisplatin and vincristine have been used to treat both pilocytic and diffuse astrocytoma and resulted in PFS of 87.5% at 96 months post-diagnosis (Strojan et al, 1999).

No. patients	Diagnosis	Agents	No. cycles	Survival	Reference
22	GBM	Trofosfamide Etoposide	2	4 years 22%	(Wolff et al, 2000)
7 3	AA GBM	Carboplatin Ifosfamide Etoposide	4	53 months 70%	(López-Aguilar et al, 2000)
13	LGA	Etoposide Carboplatin	4	5 years 58%	(Castello et al, 1998)
8	LGA (ST)	Cisplatin Vincristine	1-3 1-7	10 years 87.5%	(Strojan et al, 1999)
14	BSA	Cyclophosphamide Vincristine Cisplatin Etoposide	2	2 years 42%	(Duffner et al, 1993)
18	MA	Cyclophosphamide Vincristine Cisplatin Etoposide	2	2 years 65%	(Duffner et al, 1993)
13 15	AA GBM	CCNU Vincristine Prednisone	9	5 year 46%	(Sposto et al, 1989)
6 3 9	GBM AA HGA	Vincristine Cyclophosphamide Cisplatinum VP16	2	5 year 43%	(Duffner et al, 1996)

Table 1.4: Summary of chemotherapy protocols used in the treatment of paediatric astrocytoma. LGA = low grade astrocytoma; BSA = brain stem astrocytoma; MA = malignant astrocytoma; ST = supratentorial; AA = anaplastic astrocytoma; GBM = glioblastoma multiforme.

Surgery for treatment of high-grade astrocytoma is ineffective because of the infiltrative nature of the tumour over neural tissue. Chemotherapy is used routinely in the post operative treatment of children with malignant brain tumours and un-resectable low grade astrocytoma (Nazar et al, 1990; Pollack, 1999). A study in 1976 by the Children's Cancer Study group (CCSG) was the first to demonstrate the efficacy of chemotherapy in the treatment of high-grade astrocytoma of childhood. This study involved the use of CCNU, vincristine and prednisone in addition to radiotherapy. Five year survival for patients receiving both chemotherapy and radiotherapy was 43% compared to 18% for those only receiving radiotherapy (Sposto et al, 1989). A 5 year survival rate of 50% was seen in children with malignant astrocytoma treated with prolonged postoperative chemotherapy (two cycles of cyclophosphamide and vincristine) and delayed irradiation (Tassi et al, 2001). Another early study investigated the use of vincristine as a single agent to treat recurrent paediatric malignant astrocytoma (Rosenstock et al, 1976). Nine patients were treated with vincristine for a period of 12 weeks and survival was 37.5% at 72 months post-diagnosis. Lopez-Aguilar et al. (2000) used ifosfamide, carboplatin, and etoposide, which enhances the effect of carboplatin, to treat eleven paediatric patients with anaplastic astrocytoma and GBM. This treatment resulted in partial response in 60% of patients and complete response in 30% of patients. Patient survival was also increased (survival at 53 months was 70%). Patients in this study with brain stem tumours still had a very poor survival rate. Twenty-two patients with newly diagnosed GBM were treated postoperatively with irradiation and chemotherapy (Wolff et al, 2000). The treatment protocol involved the administration of trofosfamide and VP-16. The four year overall survival rate was 22% and the progression free survival at four years was 4%. None of the surviving patients were free of disease. This study did not show a relevant benefit for patients treated with this combination of agents. In another study patients with anaplastic astrocytoma and GBM were treated with postoperative radiotherapy and chemotherapy (Held-

Feindt et al, 2002). The chemotherapy administered was vincristine, carboplatin and CCNU started within 48 hours of surgery and repeated every 6-8 weeks for 18 months. The median survival was 22 months for GBM and 30 months for AA. In this case chemotherapy did not improve survival compared to patients treated with surgery and radiotherapy. Eighteen patients with GBM and anaplastic astrocytoma and five patients with brain stem glioma were treated with postoperative chemotherapy, using a combination of vincristine, cyclophospahamide, cisplatin and etoposide, started 2 to 4 weeks after surgery (Duffner et al, 1993). None of the patients with brain stem gliomas responded to the treatment whereas 60% of the GBM and anaplastic astrocytoma patients showed either a complete or partial response. The 2 year PFS for the brain stem gliomas was 28% compared to 54% for patients with anaplastic astrocytoma and GBM. This chemotherapy regimen allowed radiotherapy to be delayed and therefore resulted in a reduction in neurotoxicity. A study by the "Baby Pediatric Oncology Group" investigated the response of eighteen patients, aged less than three years, with malignant astrocytoma (6 GBM, 3 AA and 9 with unclassified malignant glioma) to treatment with vincristine, cyclophosphamide, VP-16 and cisplatinum (Duffner et al, 1996). Children less than 2 years received 24 months of chemotherapy and children aged 2-3 years received chemotherapy for 1 year. PFS at 5 years was 43%, with an overall survival at 5 years of 50%. This study suggests that malignant astrocytoma in infants may be sensitive to chemotherapy and these patients will have a good prognosis.

Recently there has been an interest in the use of high dose, myeloablative chemotherapy using autologous stem cell rescue for the treatment of patients with poor risk tumours such as PNET, high grade glioma and relapsing tumours of any type (Bouffet and Foreman, 1999; Kalifa et al, 1999; Pollack, 1999). The use of high dose chemotherapy (HDCT) can increase the distribution of anticancer agents in the brain,

and thus the tumour. Stem cell rescue is used to overcome the myelosuppression associated with HDCT. Bone marrow involvement is rare in patients with brain tumours and there is, therefore, a low risk of tumour contamination of re-infused bone marrow cells. This makes myeloablative chemotherapy an attractive agent for investigation in CNS tumours (Kalifa et al, 1999). Two patients with GBM, one with anaplastic astrocytoma and two with ependymoma were treated with HDCT followed by bone marrow transplant (Busca et al, 1997). Patients with GBM and ependymoma showed no evidence of disease 3-67 months (mean 20 months) after bone marrow transplantation. The patient with anaplastic astrocytoma died 15 months after treatment from disease progression. This preliminary study suggests that HDCT and bone marrow transplantation may be effective in children with malignant brain tumours (Busca et al, 1997).

Tamoxifen, a protein kinase C inhibitor, is currently being investigated for use in the treatment of CNS tumours (Couldwell et al, 1996; Mastronardi et al, 1998). A number of studies have used tamoxifen to treat progressive, low grade gliomas, myxopapillary ependymoma with promising results (Madden et al, 2001; Walter et al, 2000).

Prognostic Indicators.

Therapy

Tumours with a smaller postoperative volume have a reduced risk of regrowth and extent of resection is critical in prolonged PFS (where the patient remains free of disease) (Smoots et al, 1998).

Although histological grade and radiation dose are important prognostic indicators in ependymoma, the findings of most studies suggest that the extent of resection in the most important. Extent of resection (gross total versus subtotal) was found to be highly statistically

significant in the prediction of 5-year PFS (51% v 21%) in a study of 80 patients with ependymoma. Similar results have been found in other studies (Fouladi et al, 1998; Healey et al, 1991; Mohri et al, 2000; Nitiss and Beck, 1996; Robertson et al, 1998; Sutton et al, 1990-1991).

A number of studies investigating the role of radiation dose on survival in ependymoma have found that survival is improved when the tumour site is irradiated with 45 Gy (Dohrmann et al, 1976; Goldwein et al, 1990; Lyons and Kelly, 1991; Pierre-Khan et al, 1983; Salazar et al, 1983; Wallner et al, 1988). Although the most common cause of failure in ependymoma is recurrence at the primary site, increasing the radiation dose is prohibited by an increase in long-term side effects (McLaughlin et al, 1998; Salazar et al, 1983).

The use of whole brain irradiation for ependymoma and craniospinal irradiation for anaplastic ependymoma has resulted in 10 year survivals of 67% and 47% respectively (Salazar et al, 1983). This form of radiation results in an improved survival compared to using partial brain fields for ependymoma and just cranial irradiation for anaplastic ependymoma, which yielded 10 year survivals of 13% and 8% respectively (Salazar et al, 1983).

Duration of RT was shown to be an important prognostic factor in patients treated with radiation doses less than 45Gy. Survival at 10 years was 78.9% in patients with a treatment duration of less than 50 days compared to 36.4% in patients with a treatment duration of greater than 50 days (Paulino and Wen, 2000).

Patients with astrocytoma located in the cerebellum who underwent complete resection were less likely to have tumour recurrence than those patients who had an incomplete resection (Pencalet et al, 1999). Total removal of tumour offers long term survival even to patients with malignant astrocytoma (Vinchon et al, 2001).

The use of chemotherapy as a standalone treatment does not offer a convincing survival advantage to patients with ependymoma or astrocytoma (Evans et al, 1996; Fouladi et al, 1998; Held-Feindt and Mentlein, 2002; Robertson et al, 1998; Wolff et al, 2000).

Tumour location

Survival differences attributable to tumour location may be explained by variations in the ability to resect the tumour from various sites. The main sites of brain tumours can be seen in Figure 1.9. Supratentorial tumours exhibit infiltrative growth into the brain parenchyma compared with infratentorial tumours which grow exophytically, often filling the ventricle (McLaughlin et al, 1998).

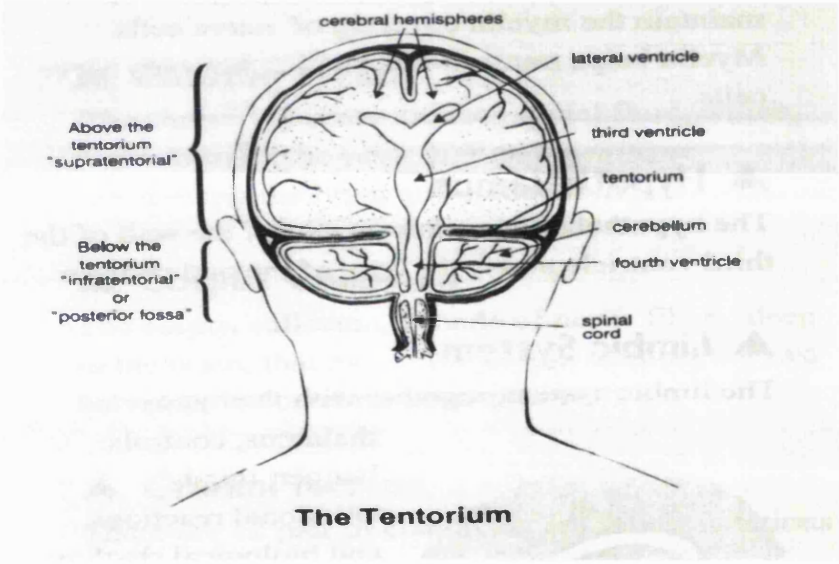


Figure 1.9: Regions of the brain where neoplasms can be located (taken from American Brain tumour Association, A primer of brain tumours).

Surgery is easier in patients with infratentorial tumours compared to those with supratentorial tumours, resulting in a better prognosis for these patients. Five year survival for patients with intracranial ependymoma has been reported at 45-62% compared to 22-38% in patients with supratentorial ependymoma (Lyons and Kelly, 1991). However some studies have found no difference in survival between

these two sites in patients with ependymoma (Salazar et al, 1983). Despite the fact that anaplastic ependymoma are more likely to be supratentorial lesions, a better prognosis has been observed in some studies for these patients than those with infratentorial anaplastic ependymoma (Dohrmann et al, 1976; Goldwein et al, 1990; Kovalic et al, 1993; Pierre-Khan et al, 1983). Several studies have reported a worse prognosis for children with posterior fossa (infratentorial) ependymoma than those with supratentorial tumours (Grill et al, 2001; Rawlings et al, 1988; Timmermann et al, 2000). A study of seventy three patients with ependymoma showed a 4 year PFS of 50% in posterior fossa ependymoma compared to 100% in patients with supratentorial ependymoma (Grill et al, 2001). A second study reported a 3 year PFS of 53% in posterior fossa tumours compared to 72% in supratentorial tumours in a study of 55 patients (Timmermann et al, 2000). A five year overall survival rate for spinal ependymoma has been reported at approximately 100% (Helseth et al, 2001; McCormick et al, 1990; Rawlings et al, 1988).

There is little evidence to support an association between tumour location and survival in patients with low grade astrocytoma (Pollack et al, 1995; Undjian et al, 1989).

The most common reason for incomplete resection is brainstem involvement. Many studies have documented the adverse prognostic effect of brainstem invasion (Pencalet et al, 1999; Schneider et al, 1992). Glial tumours of the brainstem comprise 10% of childhood CNS tumours (Packer et al, 1999) and can be assigned to one of two categories (Fisher et al, 2000). The first, fibrillary astrocytoma, is the most common and has an exceedingly poor prognosis. The second category is pilocytic astrocytoma, which has a more favourable prognosis. The involvement of the brain stem in children with cerebellar astrocytoma has been found to be the sole unifying factor that has both a direct and indirect influence on prognosis with respect to survival, recurrence

and long-term outcome (Pencalet et al, 1999). Patients with brainstem astrocytoma have a 3 year survival rate of only 10%, with 80% of patients dying of disease within 18 months of diagnosis (Albright et al, 1986; Berger et al, 1983; Freeman et al, 1993; Kretschmar et al, 1993; Littman et al, 1982; Packer et al, 1993; Packer et al, 1994; Strange and Wohlert, 1982). The prognosis for patients with brainstem astrocytoma is dependent on the location and growth characteristics of the tumour (Pollack et al, 1993). Brainstem tumours, particularly those located in the pons and medulla oblongata have been shown to have a poorer prognosis than other brain stem locations (Eifel et al, 1987; Littman et al, 1982). A four year survival rate of 100% has been reported for patients with cervicomedullary astrocytoma (Robertson et al, 1994). A nine year survival of 94% has been reported for patients with exophytic astrocytoma (Pollack et al, 1993). There have been reports of children with pilocytic astrocytoma of the brainstem that have long periods of survival (Edwards et al, 1994). Six patients with pilocytic astrocytoma of the pons or fourth ventricle have been followed up for a mean time of 4.6 years after diagnosis, with all patients still alive after this time. Four patients were free of disease, one had stable disease and the remaining patient had a 50% reduction in tumour size after treatment with radiotherapy. Several authors suggest that the adverse risk associated with tumours that infiltrate the brainstem can be explained by the volume of the residual tumour after resection (Schneider et al, 1992; Smoots et al, 1998).

Age

Historically infants with brain tumours have had the worst prognosis of any age group,(Duffner et al, 1986; Evans et al, 1990). The poor outcome may reflect the limitations of treatment: surgery is difficult in young patients and radiation is known to be toxic in young children (Gebhart and Liehr, 2000).

The influence of age on outcome in patients with ependymoma is controversial. Some studies have reported no difference in outcome between young children and older children (McLaughlin et al, 1998; Paulino and Wen, 2000; Robertson et al, 1998; Salazar et al, 1983). In contrast, age has been shown to be a significant prognostic factor in other studies (Chiu et al, 1992; Goldwein et al, 1990; Nazar et al, 1990; Paulino et al, 2002; Pierre-Khan et al, 1983). In one study patients, 24 months or younger had a 5 year survival of 43% compared to 71% for older patients (Healey et al, 1991). Age was shown to be a weak determinant of outcome in childhood intracranial ependymoma (Sutton et al, 1990-1991). The 5 year PFS for 23 patients aged 3 years and under was 26% compared to 51% in children aged 4 years and older. Older children were also seen to be at a smaller risk of disease progression than the younger children in this study (Sutton et al, 1990-1991). Children aged over 3 years have been reported as having 5 year PFS of 67% compared to 31% in children aged less than 3 years (Chiu et al, 1992). In one study, survival in children with ependymoma was analysed according to three age groups, less than 2 years, 2 to 6 years and over 6 years (Nazar et al, 1990). The youngest children had the worse survival, 18% at 5 years. The middle age group had a 5 year survival of 40% and the older age group had a 5 year survival of 60%. The 5 year survival for children aged 3 and under has been reported as 41.7% and for children over 3 years as 74.5% in a recent study (Paulino et al, 2002). The survival at 15 years was very similar for both age groups (42% versus 52%).

The influence of age on the prognosis of paediatric astrocytoma is also controversial. Tumour progression has been found to be associated with infant patients (Vinchon et al, 2001) and some studies have reported earlier recurrences in children younger than 5 years of age (Davis and Joglekar, 1981; Ilgren and Stiller, 1987). Other studies have shown no difference in survival or the risk of progression between

infants and older children (Fisher et al, 2001; Pollack et al, 1995; Smoots et al, 1998).

Tumour histology

In ependymoma there is controversy regarding the prognostic significance of histologic grade. This is due in part to disagreement between pathologists as to how ependymoma should be graded because of heterogeneous histologic patterns within the tumour (Chiu et al, 1992; Goldwein et al, 1990; Healey et al, 1991). Several studies have shown survival differences between patients with ependymoma and anaplastic ependymoma (Kovalic et al, 1993; Liu et al, 1976; Nazar et al, 1990; Salazar et al, 1983). Grade has been shown to independently affect overall survival in children with ependymoma: 76% at 10 years for patients with ependymoma compared to 24% for those with anaplastic ependymoma (McLaughlin et al, 1998). Patients with subependymoma have been seen to have a longer survival than patients with other grades of ependymoma (Schiffer and Giordana, 1998). One study has shown that histologic grade has no prognostic value in infratentorial ependymoma, whereas in supratentorial ependymoma the anaplastic variant is associated with a higher risk of recurrence (Chiu et al, 1992). The rate of survival beyond five years has been found to be significantly different for patients with ependymoma (39%) compared to those with anaplastic ependymoma (26%) (Salazar et al, 1983). A number of other studies have not been able to identify histology as a major prognostic variable in paediatric ependymoma (Goldwein et al, 1990; Healey et al, 1991; Sutton et al, 1990-1991). These discrepancies may be due to the relative rarity of the anaplastic variant of ependymoma.

Histology may also have an important role in the determination of outcome in children with astrocytoma. Juvenile pilocytic astrocytoma have been associated with a better prognosis than other types of low grade astrocytoma (Gjerris and Klinken, 1978). In a study comparing the

survival of children with pilocytic astrocytoma and those with diffuse astrocytoma, 92% of the patients with pilocytic astrocytoma survived 15-40 years postoperatively compared to 55% of patients with diffuse astrocytoma (Gjerris and Klinken, 1978). Pilocytic astrocytoma has an excellent prognosis, with long term survival of almost 100% being reported in several studies (Fisher et al, 2001; Pollack et al, 1995; Vinchon et al, 2001; Wallner et al, 1988). Pilocytic astrocytoma with a small postoperative volume have the best prognosis (Smoots et al, 1998). In a recent study only one of fourteen patients with pilocytic astrocytoma showed any progression of disease 22 years after initial diagnosis (Fisher et al, 2001). Children with anaplastic astrocytoma and GBM have a poorer prognosis than those with low-grade astrocytoma. In one study of paediatric malignant astrocytoma, the 2 year survival was only 33% compared to 100% in pilocytic astrocytoma (Vinchon et al, 2001). In another study the mean time to death in a study of five patients with malignant astrocytoma was 6.8 months (Shinoda et al, 1989). A fibrillary histology has been associated with a poor prognosis in a number of studies (Davis and Joglekar, 1981; Gjerris and Klinken, 1978; Hayostek et al, 1993). One study has shown fibrillary astrocytoma to be associated with an increased risk of progression (Smoots et al, 1998). However a number of other studies have shown no difference in survival between pilocytic and fibrillary astrocytoma (Pencalet et al, 1999; Sutton et al, 1990-1991; Szénásy and Slowik, 1983).

Race

A surprising finding in one study was that Caucasian patients with ependymoma had a better survival rate than non-Caucasian children. Survival at 5 years was 43% and 14% respectively (Goldwein et al, 1990). Another study could find no correlation between patient race and survival (Robertson et al, 1998). There are no other reports of race as a prognostic indicator in paediatric brain tumours.

Genetic markers

Genetic prognostic markers are scarce in children with glial tumours. Gain of 1q has been associated with patient survival, tumour histology and recurrence in some studies of paediatric ependymoma (Carter et al, 2002; Dyer et al, 2002; Sainati et al, 1996).

In a study of low-grade astrocytoma the presence of CNAs was seen to have a positive effect on patient survival (Orr et al, 2002). Gain of 1q has been associated with shorter survival in children with anaplastic astrocytoma (Rickert et al, 2001).

Mutations of *p53* have been found to have no impact on survival in children with astrocytoma. Children with mutations have been found to have a mean survival time of 19 months compared to 23 months in children with no *p53* mutation (Bhattacharjee and Bruner, 1997; Pollack et al, 1997; Sung et al, 2000). Patients with overexpression of p53 have a shorter PFS than those children with low p53 expression levels (Pollack et al, 2002). Deletions of *PTEN* have been associated with poorer survival in children with malignant astrocytoma (Raffel et al, 1999).

Tumour Cell Biology

Cancer occurs as a result of genetic aberrations that disrupt the balance that regulates normal cell growth and development. Such aberrations occur in two classes of interacting genes: those that facilitate cell growth and tumour formation (oncogenes) and those that inhibit these processes (tumour suppressor genes) (Sager, 1989).

Cells that die either *in vitro* or *in situ* do so as a result of the initiation of apoptosis (programmed cell death). Apoptosis can act as a final defense mechanism against the expression of deleterious mutations, and its inhibition may increase the risk of disease in some instances. Apoptosis may be an important factor when considering how cells escape normal controls, resulting in malignant growth. Cancerous cells

are regarded as those that have evaded cellular growth controls and are proliferating at an increased rate. The ability to do this has been associated with altered expression of proto-oncogenes such as *myc* and *ras*, as well as with the loss of tumour suppressor genes (Hunter T, 1991; Klein, 1987; Levine and Momand, 1990).

Oncogenes

Oncogenes are dominantly-acting growth enhancing genes whose transformation by mutation, deletion, amplification or rearrangement leads to uncontrolled cell proliferation (Cogen and McDonald, 1996). They were discovered as a result of studies of oncogenic RNA retroviruses by Rous (1911) and Shope (1932). All oncogenes are derived from normal cellular genes, known as proto-oncogenes. Proto-oncogenes appear to have a role in normal cellular physiology and are often involved in the regulation of normal cell growth and proliferation (Friend et al, 1988). Proto-oncogenes are converted to active oncogenes by aberrations which cause novel or increased activity of the proto-oncogene by altering the structure of a normal protein or by inappropriately increasing the levels of a normal protein in the cell (Friend et al, 1988).

There are four separate mechanisms of proto-oncogene activation which are demonstrated in Figure 1.10 (Land et al, 1983):

- The translocation of an oncogene from an untranscribed site to a position adjacent to an actively transcribed gene. Examples of this mechanism include translocation of the *c-abl* gene from chromosome 9 to chromosome 22 resulting in the formation of the Philadelphia chromosome, expression of abnormally hyperactive *c-abl* gene product in chronic myeloid leukaemia, and translocation of the *c-myc* oncogene from chromosome 8 to chromosome 14, where its expression is assured by juxtaposition with one of the immunoglobulin genes which will be actively transcribed in the B-

cell. This mechanism is the origin of Burkitt's lymphoma (Underwood, 1992).

- The rearrangement of sequences within the genome following chromosomal translocation. The oncogene may undergo a point mutation resulting in a gene product, such as a protein kinase, with increased or inappropriate activity.
- Over-expression due to amplification of the proto-oncogene or oncogene. Examples of this mechanism include *myc* amplification in promyelocytic leukaemia and Ki-*ras* amplification in colon cancer.
- Over-expression of a proto-oncogene following acquisition of a novel transcriptional promotor.

Oncogenes can be classified into five groups according to the function of the gene product (Ruley, 1993; Sherley, 1991; Underwood, 1992):

- Nuclear binding oncoproteins involved in regulating cellular proliferation (e.g. *myc*)
- Tyrosine kinases (e.g. *src*)
- Growth factors (e.g. *sis* coding for PDGF)
- Growth factor receptors (e.g. *erB* coding for EGFR)
- Oncoproteins with cyclic nucleotide binding activity (e.g. *ras* and GTP) that disrupt intracellular signalling

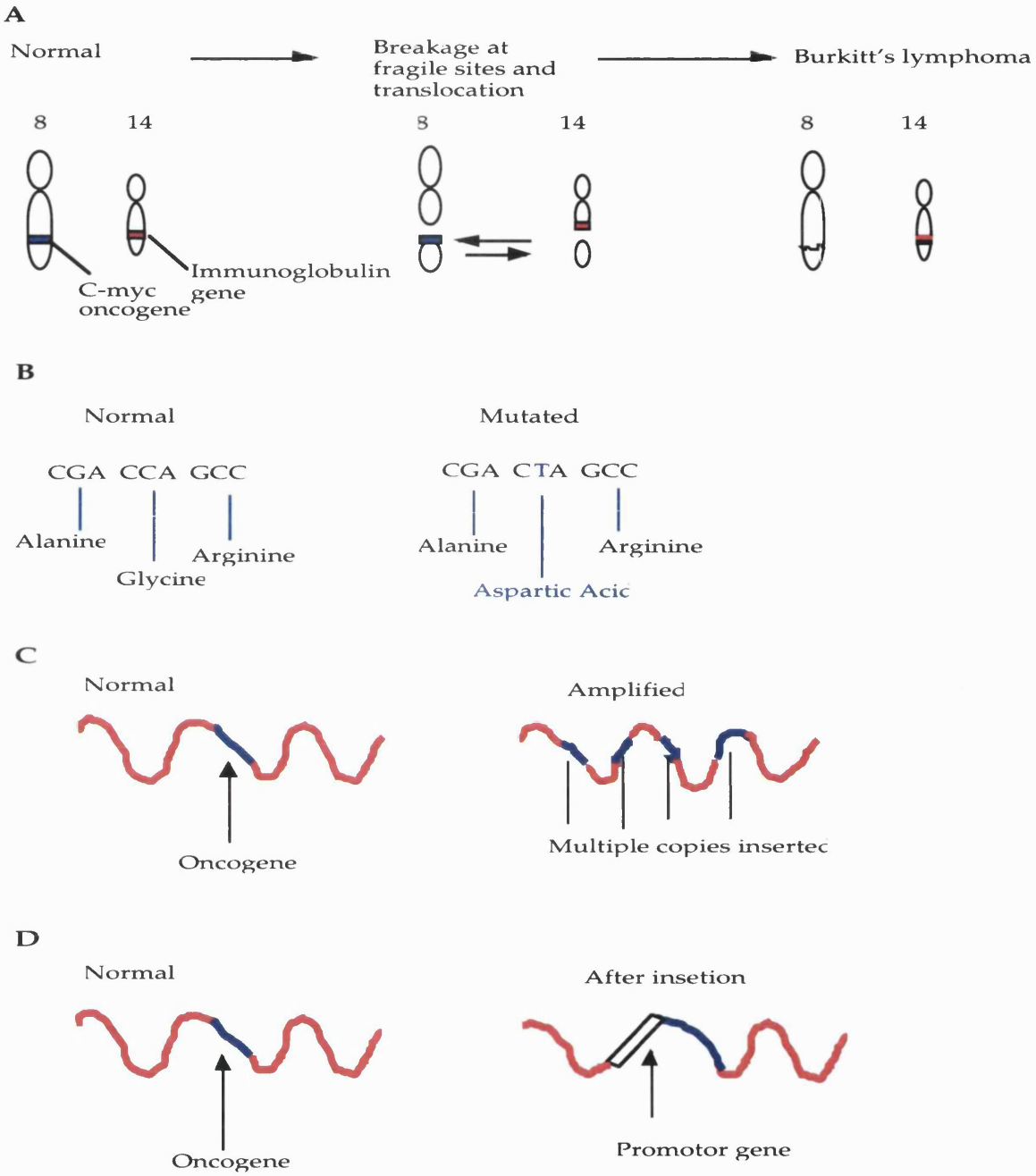


Figure 1.10 :Mechanisms of oncogene activation.

A. Translocation of an oncogene from an untranscribed site to a position adjacent to an actively transcribed gene. **B.** Point mutation in which the substitution of a single base in the oncogene is translated into an amino acid substitution in the oncoprotein causing it to be hyperactive. **C.** Amplification by the insertion of multiple copies of the oncogene resulting in cellular proliferation stimulated by excessive quantities of the oncoprotein. **D.** Increased oncogene expression by gene insertion (insertional mutagenesis) resulting in proximity of an oncogene to a promotor or enhancing gene (Underwood, 1992).

Tumour Suppressor Genes

Tumour suppressor genes have a negative growth regulatory function. They act recessively as both copies of the gene need to be inactivated (Raffel, 1996). The first tumour suppressor gene to be characterised was the *RB1* gene, which is associated with retinoblastoma. Retinoblastoma is a malignant tumour derived from the retina, which occurs almost exclusively in children. In some cases, they are hereditary and occur bilaterally. In other cases, they are sporadic and occur unilaterally with no familial association. Knudsen suggested that the clinical pattern of retinoblastoma could be the result of as few as two genetic events, if each one of these events lead to the inactivation of one copy of a gene whose function is to control cellular proliferation. The loss of both copies of the gene would result on the formation of a tumour (Knudsen, 1971). According to this hypothesis the first inactivation would occur in the germline of patients with the hereditary form of the disease and would therefore be present in all the cells of the individual. The second event would target the somatic cells (in this case the retinoblasts) resulting in the formation of a tumour. Patients with the sporadic form of the disease would also develop a tumour after the two 'hits', but both events would only be present in the somatic tissues (Cogen and McDonald, 1996).

There are four mechanisms of tumour suppressor gene inactivation:

- Loss of one copy of the gene, followed by mutation of the remaining copy of the gene. e.g. *p53*
- Homozygous deletion e.g. *CDKN2A/B*
- Methylation of the CpG island in the 5' region of the gene e.g. *CDKN2A/B*
- Point mutation e.g. *p53* in erythroleukaemia cells

The Pathogenesis of Brain Tumours

The majority of genetic studies have been carried out on adult brain tumours with relatively few studies of paediatric CNS tumours being published. Studies of paediatric brain tumours have been hindered by a number of factors (Bhattacharjee et al, 1997; Karnes et al, 1992). These include:

- Clinical and pathological heterogeneity of paediatric brain tumours
- The low incidence of each histological subtype of tumour and the small number of centres treating large numbers of patients
- Difficulties in generating short term cultures of paediatric biopsy samples. Paediatric brain tumours are more difficult to grow *in vitro* than adult tumours and established tumour cell lines are rare. Paediatric cell cultures have a low mitotic index and metaphases are usually of poor quality.
- Deep seated neurological location of the tumours. A large number of paediatric CNS neoplasms are located in functionally important areas of the brain, making it extremely difficult to remove large pieces of tissue for analysis.

Studies of adult brain tumours have been better documented and have shown consistent cytogenetic abnormalities (Bigner et al, 1990; Leon et al, 1994). In comparison to adult tumours, paediatric brain tumours are more frequently primitive tumours and have different clinical behaviours. These biological differences suggest that the genetic alterations in paediatric tumours might be different from those seen in adult tumours (Bhattacharjee et al, 1997).

Genetic analysis of Ependymoma

The cytogenetic analysis of both paediatric and adult ependymoma has failed to identify consistent patterns of genetic abnormality. In fact, as many as 50% of ependymoma have no detectable cytogenetic aberrations. The most commonly reported abnormality is loss of chromosome 22, which has been observed in 30% of tumours

(Mazewski et al, 1999). Less frequent losses are seen on chromosomes 6, 9, 10, 13,17 and 19 (Bijlsma et al, 1995). Structural rearrangements of chromosomes 2, 4, 10, 11, 16 and 17 and gains of almost all autosomes have also been reported in ependymoma (Bhattacharjee et al, 1997; Debiec-Rychter et al, 1995; Griffin et al, 1988; Hirose et al, 2001; Neumann et al, 1993; Reardon et al, 1999; Scheil et al, 2001; Shlomit et al, 2000; Stratton et al, 1989; Vagner-Capodano et al, 1992; Vagner-Capodano et al, 1999).

Chromosome 22 and the *NF2* gene

To date the most consistent finding in ependymoma has been loss of chromosome 22. Monosomy 22 has been observed in up to 40% of ependymoma (Kramer et al, 1998; Neumann et al, 1993; Ransom et al, 1992; Vagner-Capodano et al, 1992; Vagner-Capodano et al, 1999). The incidence of monosomy 22 in adults is approximately twice that seen in paediatric cases, and has been reported in 56% of adult cases compared to 31% of paediatric cases. (Mazewski et al, 1999). In contrast, several studies have shown loss of 22 to be infrequent in paediatric samples and have suggested this abnormality may be age dependent (Kramer et al, 1998; Von Haken et al, 1996; Zheng et al, 2000).

Neurofibromatosis 2 (NF2) is an autosomal dominant disease characterised by the neoplastic growth of cells derived from the neural crest (Mautner et al, 1993). NF2 patients develop acoustic neuromas, schwannomas and multiple meningiomas and also have a higher incidence of gliomas, particularly ependymoma and to a lesser extent astrocytoma (Rubio et al, 1994). Familial ependymal tumours are rare but the analysis of several afflicted families have eliminated *NF2* as the target of chromosome 22 loss in ependymoma. A five year old boy with an anaplastic ependymoma was found to have a constitutional t(1;22)(p22;q11.2). This breakpoint is between both the DiGeorge locus and *BCR* which are distal to the *NF2* gene and the *EWS* gene (Park et al, 1996). Another family study involved four members of the same family

with anaplastic ependymoma. One affected member of the family showed loss of chromosome 22 in the tumour sample, but not the normal blood control. No mutations of *NF2* were found (Nijssen et al, 1994). A study by Roberts et al, (2001) identified an ependymoma with t(X;22)(p22.1;q11). This region is the site of a putative tumour suppressor gene identified by Hulsebos and colleagues (1999). A segregation analysis of four cousins with ependymoma revealed a susceptibility gene locus proximal to marker *D22S941* in 22pter-22q11.2. The absence of affected individuals in the first two generations of this family and the development of ependymoma in the third generation cannot be explained by simple Mendelian inheritance of a single ependymoma-susceptibility gene. This family data suggests multifactorial inheritance in which ependymoma development is determined by two or more loci. The chromosome 22 loci contributes significantly to the disease phenotype and both copies of the gene have to be inactivated for tumour formation to occur (Hulsebos et al, 1999).

LOH analysis of the region spanning the *NF2* gene has shown loss in 2/15 tumours (Rubio et al, 1994; Slavc et al, 1995). *NF2* mutations may be specific to intramedullary spinal ependymoma as these mutations are most commonly found in tumours at this location (Ebert et al, 1999). *NF2* mutations were detected in 6/14 intramedullary ependymoma but in 0/15 intracranial ependymoma (Ebert et al, 1999). LOH of chromosome 22 was also seen to be associated with intramedullary tumours rather than intracranial tumours. Six out of 20 adult intramedullary ependymoma have been reported with *NF2* mutations compared to 0/25 intracranial ependymoma (Lamszus et al, 2001). Comparative genomic hybridisation (CGH) experiments have also shown loss of 22q to be more frequent in spinal tumours than intracranial ependymoma (Hirose et al, 2001). Lack of *NF2* mutations in intracranial ependymoma suggests the presence of a second tumour suppressor gene on 22q or that mutations of the *NF2* gene may occur in

non-exonic portions of the gene, such as promotor regions, introns or alternatively spliced regions (Rubio et al, 1994).

Chromosome 1

Cytogenetic changes of chromosome 1 are frequent occurrences in ependymoma (Kramer et al, 1998; Neumann et al, 1993). CGH analysis has shown gain of 1q to be a frequent event in paediatric ependymoma, occurring in 21/70 (30%) of tumours (Hirose et al, 2001; Scheil et al, 2001; Shlomit et al, 2000; Zheng et al, 2000). Gain 1p13-22 has been seen in 1/8 ependymoma (Zheng et al, 2000). Loss 1p is a less frequent event, being seen in 5/67 (7%) tumours (Hirose et al, 2001; Reardon et al, 1999; Scheil et al, 2001; Zheng et al, 2000). Structural alterations include add(1)(p12), t(1;20)(q21;q13), t(1;2)(p33;q21), t(1;8)(p31;q22), del(1)(p22), i(1q) and dup(1)(p13p32), (Bigner et al, 1997; Griffin et al, 1988; Neumann et al, 1993; Roberts et al, 2001; Sainati et al, 1996). LOH analysis has shown loss of markers on 1p and 1q in 2/9 ependymoma (Blaeker et al, 1996; Von Deimling et al, 2000).

Chromosome 6

Abnormalities of chromosome 6 occur quite frequently in paediatric ependymoma. Structural abnormalities include i(6p), del(6)(q15), der(6)t(6;16)(q11;p11) (Neumann et al, 1993; Vagner-Capodano et al, 1992). Alterations of 6q as sole abnormalities have been reported in 5 ependymoma (Kramer et al, 1998; Mazewski et al, 1999; McLendon et al, 1996). LOH for alleles on 6p has been observed in 2/8 ependymoma (Blaeker et al, 1996; James et al, 1990).

CGH analysis has revealed a variety of chromosome 6 alterations. Gain in the 6q11-25 region has been seen in 3/19 tumours (Shlomit et al, 2000; Zheng et al, 2000). Gain has also been seen at 6p21.3-p25 in 1/12 ependymoma (Scheil et al, 2001). Losses of chromosome 6 appear to be more frequent than gains. Loss from 6p has been seen in 5/26 tumours (Hirose et al, 2001; Shlomit et al, 2000). Loss of 6q has been observed in

13/67 tumours (Hirose et al, 2001; Reardon et al, 1999; Scheil et al, 2001; Zheng et al, 2000).

Chromosome 7

Alterations of chromosome 7 in ependymoma have only been reported in a relatively small number of cases. Structural alterations of chromosome 7 have been reported including i(7)(q10), t(3;7)(q21;q34) (Bigner et al, 1997; Sainati et al, 1996; Stratton et al, 1989; Vagner-Capodano et al, 1992). Gains of chromosome 7 have been reported in 7/35 ependymoma (Bhattacharjee et al, 1997; Griffin et al, 1992; Hirose et al, 2001) and trisomy 7 has been reported in four cases (Bhattacharjee et al, 1997; Griffin et al, 1992; Vagner-Capodano et al, 1992).

CGH analysis has shown gain of 7q in 6/67 (Hirose et al, 2001; Reardon et al, 1999; Scheil et al, 2001; Zheng et al, 2000).

Chromosome 9

Numerical alterations of chromosome 9 include both gains and losses whereas structural alterations of chromosome 9 have not been reported. Gain of chromosome 9 has been reported in 9/71 ependymoma (Bhattacharjee et al, 1997; Griffin et al, 1992; Hirose et al, 2001; Neumann et al, 1993; Reardon et al, 1999). Loss of 9p has been seen only in paediatric cases of ependymoma (Hirose et al, 2000). Monosomy 9 has been reported as a sole abnormality in one ependymoma and two anaplastic ependymoma (Bigner et al, 1997; Hirose et al, 2001). Monosomy 9 in conjunction with gain of 1q has also been seen in four ependymoma (Hirose et al, 2001).

CGH analysis has shown loss of 9p in 11/36 ependymoma (Hirose et al, 2001; Scheil et al, 2001). More specifically, loss 9p has been seen in 1/5 anaplastic ependymoma (Reardon et al, 1999). Loss of chromosome 9 has been reported in 6/10 recurrent ependymoma (Hirose et al, 2001). Loss of 9q32-qter has been seen in 1/8 ependymoma (Zheng et al, 2000). Gain of 9 has also been seen in 8/59 ependymal tumours (Hirose et al, 2001; Reardon et al, 1999; Scheil et al, 2001).

Chromosome 10 alterations

Loss of chromosome 10 has been reported in 9% of ependymoma (Hirose et al, 2001; Reardon et al, 1999; Vagner-Capodano et al, 1992). LOH analysis of chromosome 10 showed loss for every informative marker in 1/13 ependymoma suggesting loss of the whole chromosome (Blaeker et al, 1996). A second study found LOH for chromosome 10 markers in 2/11 ependymoma (Rasheed et al, 1994). CGH analysis showed loss of chromosome 10 spanning 10p and 10q11-25 in 2/8 paediatric ependymoma as well as gain of 10q26 in an anaplastic ependymoma (Scheil et al, 2001; Zheng et al, 2000). No mutations of *PTEN* were detected in a series of 62 ependymoma, indicating that it is unlikely to play a role in their development (Ebert et al, 1999).

Chromosome 11 abnormalities and the folate receptor

Abnormalities of chromosome 11 have been seen occasionally in glial tumours but may be of more importance in ependymoma than astrocytoma. It has been suggested that the multiple endocrine neoplasia type 1 (*MEN1)* gene, a putative tumour suppressor gene that maps to 11q13, may be involved in the pathogenesis of ependymoma (Lamszus et al, 2001). Spinal ependymoma have been reported as part of the multiple endocrine neoplasia type 1 syndrome, which is characterised by an inherited predisposition to hyperparathyroidism, endocrine pancreatic-duodenal, pituitary, adrenal gland tumours and tumours of the thymus and bronchi (Calender et al, 1998; Giraud et al, 1997; Kato et al, 1996). LOH for 11q13 has been reported in a spinal ependymoma in a patient with MEN1 (Giraud et al, 1997). An ependymoma from a 3 year old female with no family history of MEN1 has been reported with a translocation involving 11q13 and a deletion of 4bp from exon 3 of the *MEN1* gene (Urioste et al, 2002). This mutation leads to truncation of the menin protein and may indicate that inactivation of *MEN1* might contribute to the tumourigenesis of

sporadic ependymoma. Translocations involving 11q have been observed in other ependymoma. A t(11;17)(q13;q21) was reported as a sole abnormality in an anaplastic ependymoma and t(11;18)(q13;q21) has been seen in an ependymoma (Neumann et al, 1993; Sainati et al, 1996). An add(11)(p15) has been reported as a sole abnormality in a grade II ependymoma (Kucerova et al, 2000). Gains of chromosome 11 have been reported in 3/33 cases and five cases of chromosome 11 abnormalities as sole aberrations have been reported (Mazewski et al, 1999; Vagner-Capodano et al, 1999). LOH for markers on 11q was seen in 1/9 ependymoma (Lamszus et al, 2001). CGH analysis has shown loss of 11p in 1/10 recurrent ependymoma (Hirose et al, 2001) and gain of 11 in 2/2 myxopapillary ependymoma (Reardon et al, 1999; Scheil et al, 2001). Gain 11q23-qter has been reported in 1/8 anaplastic ependymoma (Scheil et al, 2001).

The folate receptor is a membrane glycoprotein that initiates the cellular accumulation of 5-methyltetrahydrofolic acid and methotrexate in epithelial cells *in vitro.* The gene for the folate receptor has been localised to 11q13 (Ragoussis et al, 1992). It has been suggested that some malignant cell lines have higher levels of receptor expression than normal epithelial cells or fibroblasts. Using western and northern blot analysis ovarian and renal cell carcinomas have been shown to have overexpression of the folate receptor, suggesting that receptor synthesis or turnover is altered in some malignancies. The cerebrum, cerebellum and spinal cord have all been found to be immunologically nonreactive. It has been suggested that overexpression of the folate receptor may lead to a potential therapeutic advantage in treating patients with cancer. Some of the newer chemotherapeutic agents (such as CB-3717 and ICI-198) bind to the receptor with the same affinity as 5-methyltetrahydrofolic acid. A study several years ago analysed folate receptor expression in biopsies from twenty-three children with primary brain tumours. The most notable expression was observed in

anaplastic ependymoma, with 2/3 tumours showing positive expression (Weitman et al, 1994).

Chromosome 16

Loss of 16 has been reported in ependymoma (Griffin et al, 1988). Structural alterations involving chromosome 16 include t(6;16)(q11;p11), and der(16)t(16;?)(q23;?) (Griffin et al, 1992; Neumann et al, 1993). LOH analysis of chromosome 16 showed one ependymoma to have loss for every informative 16p and 16q marker, indicating loss of the entire chromosome (Blaeker et al, 1996). Loss of chromosome 16q was seen in conjunction with gain of chromosome 1q in 3/3 grade II ependymoma (Shlomit et al, 2000). CGH analysis showed loss on both arms of chromosome 16 in 11/58 ependymoma (Hirose et al, 2001; Reardon et al, 1999; Shlomit et al, 2000; Zheng et al, 2000). Gain of 16 has also been reported in 1/7 ependymoma (Vagner-Capodano et al, 1992).

Chromosome 17 and the *p53* gene

Loss of chromosome 17 has been reported in 8 - 50% of paediatric ependymoma (Bijlsma et al, 1995; Reardon et al, 1999; Stratton et al, 1989; Von Deimling et al, 2000; Von Haken et al, 1996). Structural alterations of chromosome 17 have been reported including i(17q), translocation t(11;17) (q13;q21) and alterations involving 17p11.2 and 17p13 (Kramer et al, 1998; Sainati et al, 1996; Stratton et al, 1989).

Chromosome 17 is the location of both the *p53* gene (17p13.3) and the *NF1* gene (17q). Mutations of the *p53* gene in ependymoma are rare (Fink et al, 1996; Nozaki et al, 1998; Tong et al, 1999) (Orellana et al, 1998), though some mutations have been reported in anaplastic ependymoma. These include a silent mutation in exon 6, a germline mutation resulting in an amino acid substitution in a highly conserved site of exon 7 and a base pair change in exon 5 (Metzger et al, 1991; Ohgaki et al, 1991; Tominaga et al, 1995). One anaplastic ependymoma

showed LOH at all informative loci on both arms of chromosome 17, consistent with monosomy, although no mutations were found in *p53* transcripts from this tumour (Phelan et al, 1995).

MYC genes

The *n-myc* gene is located at 2p23-24. Amplification of *n-myc* is uncommon in paediatric brain tumours but has been reported in some paediatric PNETs (James et al, 1990). Amplification of the region containing *n-myc* was observed in two anaplastic ependymoma in a CGH study (Scheil et al, 2001), and low accumulation of the *n-myc* transcript has been found in 1 ependymoma (Fujimoto et al, 1989). Three other studies did not find any amplification of *n-myc* (James et al, 1990; Kucerova et al, 2000; Wasson et al, 1990). Amplification of *c-myc* has been reported in 3/3 ependymoma (Kucerova et al, 2000). In 30-60% of the cells examined there were five or more copies of this gene. Amplification of *c-myc* occurred without any numerical gains of chromosome 8 being reported in the same tumours.

Resistance to chemotherapy and the *MDR1* gene

Resistance to chemotherapeutic drugs is a major problem in the treatment of human cancer. One type of resistance often seen in tumour cells is multidrug resistance (MDR). MDR is characterised by the simultaneous resistance of cancer cells to a variety of structurally and functionally unrelated cytotoxic drugs (Noonan et al, 1990). These drugs are usually of natural origin or semi-synthetic derivatives of natural product drugs (Lehnert, 1994). Multidrug resistant cells have been shown to overexpress a membrane glycoprotein, P-glycoprotein (P-GP) (Georges et al, 1990). This protein has a molecular mass of 170kDa and functions as an energy-dependent drug efflux pump, decreasing intracellular cytotoxic drug concentrations to sublethal levels (Horio et al, 1988; Thiebaut et al, 1989). P-GP is the product of the *MDR1* gene, which is located on chromosome 7q21.1. Expression of P-GP is not restricted to transformed cells, but is also expressed in many human cells such as the epithelial cells of the adrenal glands, liver,

colon and kidney (Becker et al, 1991). P-GP is localised in the luminal membrane of the endothelial cells that form the blood vessels of the brain (Thiebaut et al, 1989) and may contribute to drug resistance by expelling drugs from the endothelial cells into the circulation in addition to extruding them from tumour cells (Beaulieu et al, 1997). The expression of P-GP in capillary endothelial cells of the blood brain barrier may also act to prevent the influx of cytotoxic drugs into the brain parenchyma (Becker, 1997; Geddes et al, 1994). *MDR1* overexpression has been observed in a variety of tumour types including sarcoma, retinoblastoma, neuroblastoma, lymphoma and leukaemia (Billson et al, 1994). Recently, the expression of *MDR1* has been investigated in human brain tumours. As many as 40% of PNET have *MDR1* mRNA expression (Tishler et al, 1992), and the presence of *MDR1* mRNA has also been shown in meningioma, lymphoma, metastatic adenocarcinoma and meningeal sarcoma (Henson et al, 1992; Nabors et al, 1991). P-GP expression has been shown in all grades of astrocytoma (Abe et al, 1998; Becker et al, 1991; Henson et al, 1992; Kirches et al, 1997; Nagane et al, 1999; Toth et al, 1996; von Bossanyi et al, 1997). Oda et al. (1997) have shown that 76% of paediatric solid tumours express *MDR1*.

Normal ependymal cells do not express P-GP but the finding that as many as 95% of ependymoma show expression of P-GP suggests that expression occurs along with cellular transformation (see Table 1.5). An increase in P-GP expression in one ependymoma has been demonstrated, suggesting *MDR1* expression may be induced by chemotherapy (Billson et al, 1994).

Number of Tumours	% with P-GP expression	Reference
10	70	(Geddes et al, 1994)
34	100	(Korshunov et al, 1999)
35	94	(Chou et al, 1996)
8	75	(Billson et al, 1994)

Table 1.5: Reported expression of P-GP in paediatric ependymoma

Adult Astrocytoma

An extensive body of research has identified some of the genetic alterations in adult astrocytoma. There is strong evidence that the progression from low-grade to anaplastic astrocytoma and GBM is associated with the cumulative acquisition of multiple genetic alterations (Kleihues and Ohgaki, 1999). There is little overlap between the two pathways, suggesting that genetically, primary and secondary GBM may be two separate diseases (Kleihues et al, 2000).

EGFR

The epidermal growth factor gene is located at 7p12 and is involved in the control of cell proliferation. *EGFR* has been associated with cancer for three reasons. Firstly, it is the cellular homolog of the *v-erbB* oncogene found in the transforming avian erythroblastosis virus. Secondly, when is expressed in cells it is capable of setting up a transforming autocrine loop so that cellular transformation is ligand-dependent, and thirdly, it has been shown to be amplified in several tumour types, with an increased copy number that can be directly correlated to an increase in the number of cell surface receptors (Cavenee et al, 2000). This gene has been found to be amplified and overexpressed in more than a third of GBM (Bigner et al, 1988; Hurtt et al, 1992; Wong et al, 1992). In about 50% of GBM with *EGFR* amplification, the event is coupled with gene rearrangement, resulting in a variant form of *EGFR*, known as mutant *EGFR*. Mutant *EGFR*

bestows enhanced tumourigenecity through increased cellular proliferation and reduced apoptosis in GBM (Nagane et al, 1996). Amplification and overexpression of *EGFR* are a hallmark of primary GBM, with more than 60% of tumours showing upregulated expression GBM (Watanabe et al, 1996). Overexpression of EGFR occurs independently of mutations of the *p53* gene suggesting these are mutually exclusive events in the formation of primary and secondary GBM (Watanabe et al, 1996). Amplification of *EGFR* often occurs in association with a loss of chromosome 10 (Von Deimling et al, 1992). *EGFR* amplification is also associated with deletions of *CDKN2A* (Hayashi et al, 1997).

LOH on chromosome 17p and mutations of the *p53* Gene

Cytogenetic evidence for loss of chromosome 17 has been documented in a variety of human neoplasms and is frequently associated with the presence of point mutations in the *p53* gene (Frankel et al, 1992). The *p53* gene has been localised to 17p13.1 and encodes a nuclear phosphoprotein involved in the regulation of cell proliferation. The wild-type *p53* gene acts as a tumour suppressor gene and some *p53* mutations not only cause loss of tumour suppressor function but also may activate *p53* to an oncogenic state in a dominant-negative fashion (Ohgaki et al, 1993). The *p53* gene is altered in many human cancers and in cell cultures expression of wild-type *p53* can suppress both the transformation of primary cells by other oncogenes and the growth of transformed and tumour derived cell lines (Sherley, 1991). *p53* is involved in at least four critical cellular pathways: arrest of the cell cycle in G_1 phase, initiation of DNA repair, induction of apoptosis and promotion of cellular differentiation (Louis, 1994).

Wild-type *p53* accumulates in the nucleus in response to either DNA damage or deregulated proliferation (Kastan et al, 1991; Lowe and Ruley, 1993). Accumulation of *p53* results in G_1 arrest and deregulated proliferation and replication of damaged DNA. Once a cell has been

arrested in G_1 it must either repair its DNA before entering S-phase or, if the damage is irreparable, instigate a suicidal, apoptotic response. *p53* is necessary for the induction of the growth arrest and DNA damage-inducible (GADD) DNA repair enzymes, which stimulate a DNA repair cascade (Kastan et al, 1992). (Wang et al, 2002). Induction of wild-type p53 expression in exponentially growing cells inhibits cell cycle progression, suggesting a negative role for p53 in growth regulation (Mercer et al, 1990). *p53* has also been implicated as a crucial element in programmed cell death (apoptosis) (Lane, 1993). Apoptosis in response to radiation and chemotherapy does not occur in the absence of wild-type *p53* (Lowe et al, 1993).

p53 plays a central role in cellular responses to aberrant growth signals and cytotoxic stresses such as DNA damage, by enhancing the transcription of genes that regulate a variety of cellular processes including cell cycle progression, apoptosis, genetic stability and angiogenesis (Irwin and Kaelin, 2001). Under the conditions of normal cell growth, p53 is a short lived protein, which is very tightly regulated. p53 turnover is regulated by ubiquitin and ubiquitin ligase MDM2. *MDM2* itself is a p53-inducible gene, and therefore activation of p53 establishes a negative feedback loop where MDM2 limits p53 accumulation (Barak et al, 1993). The stabilization and accumulation of p53 can be induced by a number of cellular oncoproteins including c-myc, E1A, Ras and E2F1. This can be attributed to the induction of the protein ARF, which binds directly to MDM2 inhibiting the E3-ligase activity of MDM2 and interfering with the nucleocytoplasmic shuttling of MDM2 that is necessary for p53 degradation (Tao and Levine, 1999; Zhang et al, 1998). p53 can also repress the activity of certain promoters, although the mechanism for this action is not understood (Ginsberg et al, 1991).

The most common genetic abnormality in astrocytoma is LOH on chromosome 17p, reported in 65% of low grade astrocytoma and in a similar frequency of anaplastic astrocytoma (Kleihues et al, 2000).

Positive *p53* immunoreactivity has been reported in 44% of anaplastic astrocytoma and 46% of GBM (Kirla et al, 2000).

Allelic losses of genes on chromosome 17 and mutations of the *p53* gene have been reported to be associated with all grades of astrocytoma and inactivation of *p53*, usually with mutation of one copy and chromosomal loss of the remaining allele, occurs in approximately a third of astrocytoma. Mutations of *p53* have been seen in approximately 45% of diffuse astrocytoma (Kraus et al, 1994; Ohgaki et al, 1993). The *p53* pathway has been seen to be disrupted in 82% of malignant gliomas, either by mutation of the *p53* gene (32%) or by $p14^{ARF}$ deletion (54.5%) (Fulci et al, 2000). In high-grade astrocytoma, mutations of *p53* have been found in 62% of anaplastic astrocytoma and GBM that exhibit LOH for alleles on 17p (Fults et al, 1992). Mutations usually occur in the form of mis-sense mutations and target the conserved domains in exons 5, 7 and 8. These mutations affect the *p53* residues that are essential for DNA binding and result in the loss of *p53*-mediated transcriptional activity (Louis, 1997). Mutations outside of these exons are rare in astrocytoma, but have been reported in exons 4, 9 and 10, and in introns 4 and 6 (Chung et al, 1991; Frankel et al, 1992; Fults et al, 1992; Mashiyama et al, 1991). No mutations have been described in exons 2, 3 or 11 or in the 5′ promotor region. Mutations that occur in exons 5 – 8 will most often be in the conserved domains II, III, IV and V, and 95% of the mutations will occur in codons that have been highly conserved. The hot spots of mutation in adult astrocytoma appear to be codons 130-142, 151-164, 171-181, 193-200, 213-223, 234-258 and 276-286 (Cavenee et al, 2000).

Overexpression and amplification of *MDM2*

The *MDM2* (murine double minute 2) gene has been localised to chromosome 12q14.3-q15. The MDM2 protein binds to both mutant and wild type p53, inhibiting the ability of wild type p53 to activate transcription (Zauberman et al, 1995). The transcription of the *MDM2* gene is induced by wild type p53. Amplification or over expression of

MDM2 is an alternative mechanism for escaping the *p53* regulated control of cell growth (Kleihues et al, 2000). *MDM2* has been shown to be amplified and/or overexpressed in primary GBM that lack *p53* mutations (Biernat et al, 1997; Reifenberger et al, 1993; Waber et al, 1993). Amplification of *MDM2* has been found in 10% of anaplastic astrocytoma and GBM, although overexpression of MDM2 has been found in 50% of primary GBM and only 10% of secondary GBM (Biernat et al, 1997; Reifenberger et al, 1993). A splice variant of *MDM2* has been identified in 70% of GBM without correlation with *p53* status, suggesting *MDM2* may have a role in the development of malignant astrocytoma independent of the *p53* regulatory pathway (Matsumoto et al, 1998).

The *p16/p15/CDK4/CDK6/RB* pathway

The p16 and p16 tumour suppressor genes are encoded by *CDKN2A/B* respectively and exerts growth control by the inhibition of the cyclin-dependent kinases CDK4 and CDK6. This reduces the ability of the cyclins to phosphorylate the RB protein in conjunction with cyclin D and allows G_1/S phase transition of the cell cycle to occur. Loss of cell cycle control may therefore be a result of altered expression of any of these genes, i.e. loss of *p16/p15* expression, overexpression/amplification of CDKs or loss of RB function (Kleihues et al, 2000).

CDKN2A/B maps to 9p21, a site associated with interstitial and homozygous deletions in high-grade astrocytoma and 60% of glioma cell lines (Olopade et al, 1992). Deletions involving *p16* have been found to occur more frequently in primary GBM than secondary GBM (Biernat et al, 1997). Homozygous deletions of *p16* occur in a third of primary GBM but rarely occur in secondary GBM, and a third of GBM have normal p16 expression and accumulate MDM2 protein (Biernat et al, 1997; Newcomb et al, 2000). A proportion of malignant astrocytoma with intact *CDKN2A/B* genes do not express the protein and have been shown to have hypermethylation of the CpG island in the 5′ region of

the gene. This causes structural changes in the chromatin and silences gene transcription (Costello et al, 1996).

p14 ^{ARF} is another gene encoded by *CDKN2A* and increases *p53* stability by sequestering *MDM2* in the nucleolus and preventing *MDM2*-mediated ubiquitin ligase activity of *p53* (Fulci et al, 2000). A recent study has shown *p53* mutations and deletions of *p14* ^{ARF} to be mutually exclusive in GBM (Fulci et al, 2000). Recent data suggests that the loss of the *Ink4a-Arf* locus (which encodes the proteins p16 ^{INK4a} and p14 ^{ARF}) might have a role in the sensitising astrocytes to transformation thorough dedifferentiation in response to the appropriate oncogenic stimuli (Uhrbom et al, 2002).

The gene encoding CDK4 maps to chromosome 12q13-14 and the gene encoding CDK6 to 7q21-22. Both CDK4 and CDK6 are proteins with catalytic kinase activity that can form complexes with members of the cyclin D family, and are both inhibited by p16 and p15. Overexpression of either protein can mimic mutation of the p16/p15 inhibitors and over-ride their function. *CDK4* is amplified in approximately 15% of high-grade astrocytoma, especially those with no *CDKN2A/B* alterations (Nishikawa et al, 1995; Reifenberger et al, 1994). A few tumours without *CDKN2A/B* mutations or *CDK4* amplification have been shown to have amplification of *CDK6* (Costello et al, 1997). This suggests the two proteins can functionally compensate for one another.

Phosphorylation of RB allows the release of the transcription factor E2F, which in turn activates the genes necessary for cell proliferation. The *RB1* gene is located at 13q14, a region which is altered in about a third of high-grade astrocytoma (Henson et al, 1994; James et al, 1988). LOH of *RB1* is not found in grade II astrocytoma and is present in approximately 20% of anaplastic astrocytoma and 30% of GBM. Mutations of *RB1* were seen in 3/23 anaplastic astrocytoma and 17/100 GBM and in no diffuse astrocytoma (Henson et al, 1994; Ueki et al, 1996). Mutations of the *RB1* gene have the same functional

consequences as amplification of *CDK4/CDK6* or loss of *CDKN2A /CDKN2B*. These events occur exclusively of one another in astrocytoma and a large proportion of anaplastic astrocytoma and GBM have alterations in one or other component of the pathway (see Figure 1.11).

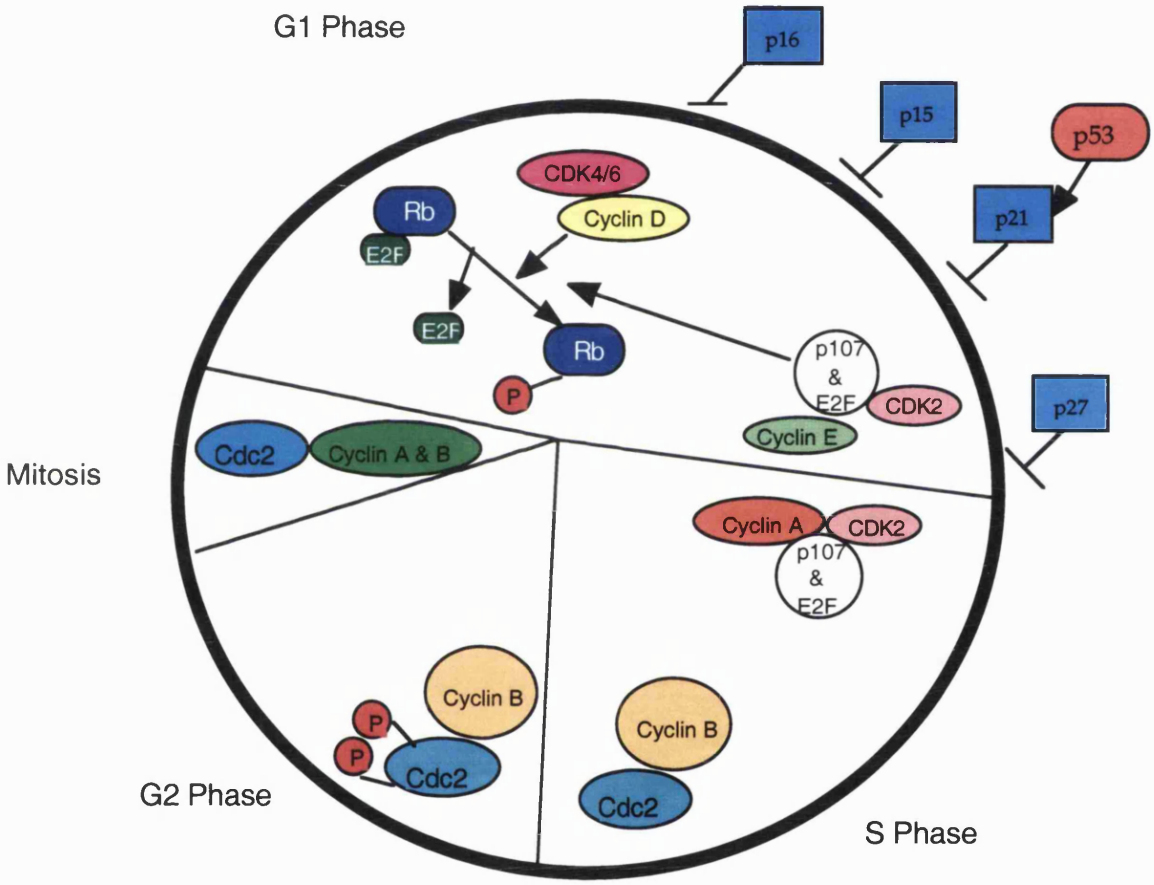


Figure 1.11: The G1 phase of the cell cycle as a target for mutations in glioma. The proteins required for progression through the mitotic cycle are shown in the region where they exert their effects. The proteins in circles are checkpoint accelerators while those in squares are inhibitors (taken from (van Dekken et al, 2001).

Loss on Chromosome 10, *PTEN* and *DMBT1*

Deletions of chromosome 10 are the most frequent genetic abnormality in GBM (Albarosa et al, 1996; James et al, 1988; Von Deimling et al, 1992). Deletion mapping has identified three distinct regions of loss, implying the presence of multiple loci involved in glial tumourigenesis (Steck et al, 1999). A putative tumour suppressor gene (*PTEN*) was cloned from 10q23 in 1997 and suggested as a possible tumour suppressor gene for gliomas (Ichimura et al, 1998). *PTEN* encodes an enzyme with phosphatase activity towards both acidic protein substrates and the lipid second messenger, PIP3. *PTEN* also regulates cell motility and invasion by the dephosphorylation of the focal adhesion kinase (FAK) and the adapter protein Shc (Torres et al, 2001). Loss of an entire copy of chromosome 10 has been reported in GBM as well as extensive loss of the PTEN region (Steck et al, 1999; Zhou et al, 1999). Loss of alleles for chromosome 10 have been found in 93% GBM, 66% anaplastic astrocytoma and 35% of grade II astrocytoma (Steck et al, 1999). Mutations in exons 1-8 of the *PTEN* gene have been reported in 1/14 anaplastic astrocytoma and 21/78 GBM. These mutations consisted of silent mutations, frameshift mutations and deletions (Teng et al, 1997; Zhou et al, 1999).

DMBTI has been mapped to the 10q25 region and has been proposed as candidate tumour suppressor gene for medulloblastoma and gliomas (Mollenhauer et al, 1997). This region was found to be frequently lost in GBM (8/10) and in anaplastic astrocytoma (15/18) (Steck et al, 1999).

The *MXI1* gene has been mapped to 10q24-25 (Wechsler et al, 1994). This gene encodes a 228-amino acid protein that shares homology with *c-Myc*, *Max* and members of the *Mad* family (Wechsler et al, 1997). *MXI1* is thought to function in a regulatory network with *Max* and members of the myc family, in which *myc* activates transcription and stimulates cell proliferation and *MXI1* negatively regulates these actions. Polymorphic microsatellite repeat analysis has shown 7/11 informative GBMs to have *MXI1* loss suggesting it may have a role in GBM development (Wechsler et al, 1997).

Paediatric Astrocytoma

Pilocytic astrocytoma accounts for around 68% of all paediatric astrocytoma (Roberts et al, 2001). Anaplastic astrocytoma and GBM are

less common and comprise about 25% of astrocytic tumours (Roberts et al, 2001). Cytogenetic studies have shown that as many as 70% of pilocytic astrocytoma have normal karyotypes (Agamanolis and Malone, 1995; Bhattacharjee et al, 1997; Bigner et al, 1997; Griffin et al, 1992; Karnes et al, 1992; Kucerova et al, 2000; Roberts et al, 2001; Sanoudou et al, 2000; Shlomit et al, 2000). Normal karyotypes have also been reported in 55% of diffuse astrocytoma, 38% of anaplastic astrocytoma and 25% GBM (Agamanolis and Malone, 1995; Bhattacharjee et al, 1997; Bigner et al, 1997; Fujii et al, 1994; Griffin et al, 1988; Karnes et al, 1992; Kucerova et al, 2000; Neumann et al, 1993; Rickert et al, 2001; Roberts et al, 2001; Sainati et al, 1996; Schröck et al, 1996; Vagner-Capodano et al, 1992; Warr et al, 2001). A number of numerical and structural chromosomal abnormalities have been observed in paediatric astrocytic tumours but definitive non-random aberrations have not been identified. Some tumours have abnormalities that are present in adult astrocytoma, such as gains of 7 and loss of chromosome 10, but the pattern of alterations in paediatric tumours appears to be vastly different to those seen in adult tumours. (Agamanolis and Malone, 1995; Blaeker et al, 1996; Cheng et al, 1999; Griffin et al, 1992; Karnes et al, 1992; Neumann et al, 1993; Schröck et al, 1994; Vagner-Capodano et al, 1992). Structural changes of chromosomes 1, 7, 9, 17 and 22 have been observed but no consistent breakpoints have been identified. (Agamanolis and Malone, 1995; Bhattacharjee et al, 1997; Fujii et al, 1994; Griffin et al, 1988; Griffin et al, 1992; Karnes et al, 1992; Neumann et al, 1993; Sainati et al, 1996; Sawyer et al, 1992; Vagner-Capodano et al, 1992).

Comparison of adult and paediatric astrocytoma

EGFR

Gains of chromosome 7 have been reported in all grades of paediatric astrocytoma at low frequencies (Agamanolis and Malone, 1995; Bigner et al, 1997; Griffin et al, 1988; Karnes et al, 1992; Roberts et al, 2001;

Schröck et al, 1994; Vagner-Capodano et al, 1992). The frequency of chromosome 7 gains increases with tumour grade: 9.7% in pilocytic astrocytoma, 18% in diffuse astrocytoma, 31.5% in anaplastic astrocytoma and 47% in GBM (Agamanolis and Malone, 1995; Bhattacharjee et al, 1997; Bigner et al, 1997; Karnes et al, 1992; Kucerova et al, 2000; Orr et al, 2002; Roberts et al, 2001; Sainati et al, 1996; Vagner-Capodano et al, 1992). Gain of 7q31 has been reported in 1/8 pilocytic astrocytoma (Shlomit et al, 2000). Gain of chromosome 7 was also the most frequent alteration in a genetic study of pleomorphic xanthoastrocytoma using CGH (Yin et al, 2002). CGH analysis has identified a region of amplification in high grade astrocytoma that is localised to 7q21-qter (Rickert et al, 2001; Warr et al, 2001). The target of this amplification is as yet unknown. Structural aberrations of chromosome include t(7;22)(q21;q13.2), der 7 t(7;7), der 7 t(7;?)(p11;?), t(7;17)(q22;q21) (Agamanolis and Malone, 1995; Griffin et al, 1992; Roberts et al, 2001).

In contrast to adult astrocytoma *EGFR* alterations are rare in paediatric astrocytoma. Three studies have found no evidence for alterations of *EGFR* in anaplastic astrocytoma and GBM (Cheng et al, 1999; Raffel et al, 1999; Sung et al, 2000). Other studies have found some alterations of *EGFR* in paediatric astrocytoma. *EGFR* amplification has been reported in 31/105 (29.5%) paediatric GBM (Bredel et al, 1999; Rasheed et al, 1994; Von Deimling et al, 2000). Positive immunoreactivity has been observed in approximately 80% of paediatric high-grade astrocytoma (Bredel et al, 1999; Maruno et al, 2000) suggesting that expression of *EGFR* may be enhanced by a mechanism other than a simple alteration in gene copy number.

LOH on chromosome 17p and mutation of the *p53* gene

There is some evidence that loci on chromosome 17 may contribute to the pathogenesis of paediatric astrocytoma. Loss of 17p has been detected in all grades of paediatric astrocytoma, suggesting the presence

of a tumour suppressor gene (Cheng et al, 1999; Orellana et al, 1998; Von Deimling et al, 2000; Warr et al, 2001; Willert et al, 1995). CGH analysis has also confirmed loss of 17p in high grade astrocytoma (Schröck et al, 1994; Warr et al, 2001). Loss of 17p has been reported through i(17q) or unbalanced translocations in paediatric malignant astrocytoma (Griffin et al, 1988; Rasheed et al, 1994). LOH analysis has revealed regions of loss at 17p13.1 and 17p13.3 (Cheng et al, 1999; Orellana et al, 1998; Von Deimling et al, 2000; Willert et al, 1995). The only structural alteration of 17p reported is dic(12;17)(p11;p11) (Roberts et al, 2001).

Although there have been many studies of p53 immunoreactivity and *p53* gene mutations in astrocytic tumours in adults, there have been few studies in childhood astrocytoma. p53 immunoreactivity has been reported in 5/7 pilocytic astrocytoma, 6/18 diffuse astrocytoma, 19/35 anaplastic astrocytoma and 7/13 GBM (Bhattacharjee and Bruner, 1997; Drach et al, 1996; Lang et al, 1994). This incidence of p53 immunoreactivity is similar to that reported in adult high-grade astrocytoma. However, there has been a wide variation in *p53* mutation rates reported in paediatric astrocytoma. Mutation rates of 0% to 80 % have been reported in pilocytic astrocytoma and 0% to 20% in diffuse astrocytoma (Chozick et al, 1994; Hayes et al, 1999; Lang et al, 1994; Phelan et al, 1995). In malignant astrocytoma, *p53* mutations have been reported in between 0% and 50% of cases (Bhattacharjee and Bruner, 1997; Cheng et al, 1999; Frankel et al, 1992; Phelan et al, 1995; Pollack et al, 2001; Pollack et al, 2002; Pollack et al, 1997). A summary of *p53* mutations in paediatric astrocytoma can be seen in Table 1.6. There appear to be five hot spots of mutation, compared to seven in adult astrocytoma: codons 151-164, 171-181, 213-223, 234-258 and 276-286. *p53* mutations in paediatric high grade astrocytoma appear to be age related, with mutations being less common in tumours from children diagnosed before 3 years of age than in older children (Pollack et al, 1997). The data reports a *p53* mutation rate of 7% in children aged

younger than three years and 31% in children aged 4 –18 years (Bhattacharjee and Bruner, 1997; Cheng et al, 1999; Chozick et al, 1994; Dam et al, 2000; Frankel et al, 1992; Lang et al, 1994; Pollack et al, 2001; Pollack et al, 1997; Rasheed et al, 1994; Sung et al, 2000). This suggests that infant high grade astrocytoma may differ on a biological and molecular basis from similar tumours in older children (Pollack et al, 2001). Two mis-sense mutations of the *p53* gene have been reported in pleomorphic xanthoastrocytoma (Brunner et al, 2000). These mutations were unusual in that they were located outside the conserved domain of the *p53* gene. Mutations of codons 220 and 292 have not been reported previously in brain tumours, but have been observed in carcinoma and lymphoma. One of the mutations was present in a benign recurrence of a PXA but not in the primary tumour. This data suggests *p53* mutations may occur after establishment of PXA but are not associated with malignant progression.

Grade	No. cases	No with mutation (%)	Reference
PA	28	1 (3.5)	(Rasheed et al, 1994)
PA	7	1 (14)	(Lang et al, 1994)
PA	10	4 (40)	(Orellana et al, 1998)
PA	6	1 (16)	(Willert et al, 1995)
PA	1	1 (100)	(Dam et al, 2000)
PA	20	17 (85)	(Hayes et al, 1999)
DA	5	1 (20)	(Lang et al, 1994)
DA	20	12 (60)	(Von Deimling et al, 2000)
AA	2	1 (50)	(Rasheed et al, 1994)
AA	30	14 (47)	(Von Deimling et al, 2000)
AA	8	2 (25)	(Pollack et al, 1997)
AA	35	9 (25.7)	(Pollack et al, 2001)
AA	6	1 (16)	(Willert et al, 1995)
AA	7	2 (28.5)	(Sung et al, 2000)
AA	17	4 (23.5)	(Raffel et al, 1999)
AA GBM	24	9 (38)	(Cheng et al, 1999)
GBM	4	0 (0)	(Rasheed et al, 1994)
GBM	76	15 (20)	(Von Deimling et al, 2000)
GBM	19	9 (50)	(Pollack et al, 1997)
GBM	42	17 (40)	(Pollack et al, 2001)
GBM	15	7 (45)	(Raffel et al, 1999)
GBM	22	8 (36)	(Sung et al, 2000)
GBM	13	3 (23)	(Bhattacharjee and Bruner, 1997)

Table 1.6: Summary of *p53* mutations reported in paediatric astrocytoma.

In adults only 10% of primary GBM have *p53* mutations, whereas the data in paediatric GBM suggest that mutations may occur in as many as 50% of tumours, a rate approaching that seen in adult secondary GBM. The types of mutation are also similar to those reported in adult tumours. The finding that tumours from children under 3 years of age have a lower frequency of *p53* mutations than older children suggests there might by different pathways leading to the formation of GBM in children in the same way as in adult astrocytoma. The fact that mutations appear to be frequent in the higher grade tumours in children suggests that *p53* mutation is a late event in the formation of these tumours.

Overexpression and amplification of *MDM2*

No mutations of *MDM2* have been found in twenty-nine paediatric high-grade astrocytoma. However, around 60% of the tumours showed positive immunoreactivity for MDM2 (Sung et al, 2000). Southern blot analysis detected no amplifications in six high-grade astrocytoma and fourteen pilocytic astrocytoma (Willert et al, 1995). Two anaplastic astrocytoma (11%) have been reported with amplification of *MDM2* (Raffel et al, 1999). The low frequency of *MDM2* amplification in paediatric high grade astrocytoma does not account for the overexpression of MDM2 protein that is observed in these tumours. The $p14^{ARF}$ protein encoded by *the INK4a-ARF* locus may regulate MDM2 protein expression. Homozygous deletions of this locus have been reported in 10% of paediatric high-grade astrocytoma (Sung et al, 2000).

The *p16/p15/CDK4/CDK6/RB* pathway

LOH for loci on chromosome 9p have been reported in 2/24 diffuse astrocytoma, 5/30 anaplastic astrocytoma and 31/89 GBM (Blaeker et al, 1996; Von Deimling et al, 2000). In a recent study, 10% of paediatric astrocytoma were shown to have homozygous deletions of $p16^{INK4a}$ (Newcomb et al, 2000). Deletions of *CDKN2A* have been reported in 2/25 pilocytic astrocytoma, 2/18 diffuse astrocytoma, 6/26 anaplastic astrocytoma and 40/81 GBM (Raffel et al, 1999; Von Deimling et al, 2000).

Amplification of *CDK4* has been reported 4/43 anaplastic astrocytoma and 7/66 GBM.

Loss of chromosome 13 has been reported in all grades of astrocytoma (Agamanolis and Malone, 1995; Bhattacharjee et al, 1997; Bigner et al, 1997; Kucerova et al, 2000; Rickert et al, 2001; Roberts et al, 2001; Shlomit et al, 2000). The highest frequency of loss was seen in GBM (22.5%) (Agamanolis and Malone, 1995; Bhattacharjee et al, 1997; Bigner et al, 1997; Rickert et al, 2001; Roberts et al, 2001), with the other grades of tumour having a similar frequency of approximately 10% (Bigner et

al, 1997; Kucerova et al, 2000; Rickert et al, 2001; Roberts et al, 2001; Shlomit et al, 2000). LOH for loci on 13q have been reported in 7/28 anaplastic astrocytoma and 22/81 GBM (Von Deimling et al, 2000). Twenty-two out of twenty-nine paediatric high-grade astrocytoma have shown positive immunoreactivity for the *RB1* gene (Sung et al, 2000).

Loss on chromosome 10, *PTEN* and *DMBT1*

Loss of chromosome 10 has been reported in all grades of paediatric astrocytoma at similar frequencies: 11% in pilocytic astrocytoma, 17% in anaplastic astrocytoma and 16% in GBM (Agamanolis and Malone, 1995; Bigner et al, 1997; Griffin et al, 1992; Karnes et al, 1992; Neumann et al, 1993; Roberts et al, 2001; Shlomit et al, 2000; Wernicke et al, 1997). Some studies have suggested deletions of chromosome 10 are restricted to GBM (Agamanolis and Malone, 1995; Roberts et al, 2001; Vagner-Capodano et al, 1992). Monosomy 10 has been observed in a third of both anaplastic astrocytoma and GBM (Agamanolis and Malone, 1995; Bhattacharjee et al, 1997; Bigner et al, 1997; Roberts et al, 2001; Vagner-Capodano et al, 1992). There has only been one report of monosomy 10 in diffuse astrocytoma (Kucerova et al, 2000) and no reports of chromosome 10 loss in pilocytic astrocytoma. CGH analysis has shown loss of chromosome 10 to be confined to the 10q22-25 region in paediatric malignant astrocytoma (Rickert et al, 2001; Schröck et al, 1994; Warr et al, 2001), and has also shown loss from 10p in two cases of malignant astrocytoma (Warr et al, 2001). LOH analysis has shown loss of markers from the 10q23-25 region in 4/5 anaplastic astrocytoma and 3/10 GBM (Blaeker et al, 1996; Cheng et al, 1999). LOH of markers for 10p have been reported in 1/5 GBM (Blaeker et al, 1996). There is only one report of a structural alteration involving chromosome 10 which is der(10) t(10:?:1)(qter→p15::?::p11-qter) in a case of anaplastic astrocytoma (Karnes et al, 1992).

Deletions of *PTEN* have been observed in approximately 10% of paediatric GBM but not in other grades of astrocytoma (Cheng et al, 1999; Sung et al, 2000). *PTEN* mutations have been reported in 1/28 anaplastic astrocytoma and 7/33 GBM (Cheng et al, 1999; Raffel et al, 1999; Teng et al, 1997). These mutations included deletions, insertions, nonsense mutations, two splicing variants and base changes (Cheng et al, 1999; Raffel et al, 1999; Raffel et al, 1999; Von Deimling et al, 2000).

One GBM had a double mutation: a mis-sense mutation at exon 6 and a nonsense mutation at exon 8 that was expected to lead to the formation of a truncated protein (Cheng et al, 1999). This data is consistent with results of *PTEN* alterations in adult high-grade astrocytoma that indicate that *PTEN* mutations are associated with increasing malignancy. There have been no reports of mutations of *DMBT1* or *MXI1* in paediatric astrocytoma.

Other non-random genetic alterations

Chromosome 1

Alterations of chromosome 1 have been reported in all grades of astrocytoma (Agamanolis and Malone, 1995; Bigner et al, 1997; Karnes et al, 1992; Schröck et al, 1994; Vagner-Capodano et al, 1992). Simple numerical gain of chromosome 1 has only been reported in anaplastic astrocytoma (9%) and GBM (25%) (Agamanolis and Malone, 1995; Bigner et al, 1997; Bobola et al, 2001; Karnes et al, 1992; Vagner-Capodano et al, 1992). CGH has also identified gain of chromosome 1 in malignant astrocytoma (Rickert et al, 2001; Schröck et al, 1994; Warr et al, 2001). A region of amplification at 1p21-22 has been reported in 1/10 GBM (Warr et al, 2001). High level gains of 1q have also been reported in 3/10 anaplastic astrocytoma and 3/13 GBM (Rickert et al, 2001; Warr et al, 2001).

Monosomy of chromosome 1 has been reported in one malignant astrocytoma (Bhattacharjee et al, 1997), one recurrent diffuse astrocytoma (Wernicke et al, 1997) and one recurrent malignant astrocytoma (Bigner et al, 1997). CGH analyses has also shown loss of 1p32-pter and 1q23-32 in pilocytic astrocytoma and malignant astrocytoma (Shlomit et al, 2000; Warr et al, 2001). LOH for loci on 1p have been reported in 6/25 anaplastic astrocytoma and 4/70 GBM (Von Deimling et al, 2000). LOH for loci on 1q have been reported in 2/76 GBM (Von Deimling et al, 2000).

A wide variety of chromosome 1 structural abnormalities have been reported in paediatric astrocytoma (see Table 1.7).

Chromosome 8 and *c-myc*

The *c-myc* gene has been is localised to 8q24.1 and gains of 8q have been reported in 3/10 paediatric malignant astrocytoma (Warr et al, 2001). Elevated expression of *c-myc* has been shown to be a common feature of paediatric anaplastic astrocytoma and GBM (Orian et al, 1992). In comparison two studies have shown *c-myc* and *n-myc* amplifications to be infrequent events in all types of paediatric astrocytoma, including pleomorphic xanthoastrocytoma, cerebellar astrocytoma, anaplastic astrocytoma and GBM (Hurtt et al, 1992; Wasson et al, 1990).

Chromosome 17

There have also been reports of loss of 17q in paediatric anaplastic astrocytoma and GBM (Agamanolis and Malone, 1995; Bigner et al, 1997; Roberts et al, 2001). Structural alterations of chromosome 17q include i(17q), t(9;17)(q34;q21), der(17) and t(7;17)(q22;q21), t(1;17)(q21;q25), and t(17;20)(q11;p13) (Agamanolis and Malone, 1995; Bigner et al, 1997; Fujii et al, 1994; Griffin et al, 1988; Roberts et al, 2001). LOH on 17q has been reported in low grade astrocytoma and anaplastic astrocytoma (Blaeker et al, 1996; Phelan et al, 1995; Von Deimling et al, 2000).

Gains of chromosome 17 have also been reported in all grades of astrocytoma (Agamanolis and Malone, 1995; Bhattacharjee et al, 1997; Karnes et al, 1992; Roberts et al, 2001; Schröck et al, 1994; White et al, 1995). Agamanolis et al, (1995) observed dup 17(q21qter) in a cerebral astrocytoma. Gain of this region was also observed using CGH in a GBM (Schröck et al, 1994).

Structural alteration	Grade	Reference
add(1)(p11)	PA	(Roberts et al, 2001)
del (1)(p11)	rec AA	(Wernicke et al, 1997)
inv(1)(p11p36)	GBM	(Fujii et al, 1994)
del(1)(p13.3)	LGA	(Bhattacharjee et al, 1997)
dic(1;3)(p13;q29)	MA	(Bhattacharjee et al, 1997)
del (1)(p21)	GBM	(Sainati et al, 1996)
del (1)(p22)	LGA	(Orr et al, 2002)
del (1)(p32)	PA	(Bigner et al, 1997)
t(1;22)(p12;q13)	MA	(Bhattacharjee et al, 1997)
t(1;14)(p36;q24)	DA, GBM	(Griffin et al, 1988)
r(1)(?p36?q42)	PA	(Neumann et al, 1993)
del(1)(q10)	GBM	(Roberts et al, 2001)
del(1)(q41)	AA	(Vagner-Capodano et al, 1992)
der(1)t(1;3)(q21→p36::q21→qter)	AA	(Karnes et al, 1992)
der(1;13)(q10;q10)	DA	(Roberts et al, 2001)
i(1)(q10)	AA	(Kucerova et al, 2000)
add (1)(q21)	rec GBM	(Wernicke et al, 1997) (Dam et al, 2000)
der(1)t(1;19)(q11;q13)	GBM	(Bigner et al, 1997)
del(1)(q11)	GBM	(Bigner et al, 1997)
der(1)t(1;21)(q21;p11.2)	GBM	(Agamanolis and Malone, 1995)

Table 1.7: Reported chromosome 1 structural alterations in paediatric astrocytoma. PA = pilocytic astrocytoma, DA = diffuse astrocytoma, AA = anaplastic astrocytoma, GBM = GBM, LGA = low grade astrocytoma, MA = malignant astrocytoma, rec = recurrent tumour.

Chromosome 22

Both deletions and gains of chromosome 22 have been reported in paediatric astrocytoma. Gains of chromosome 22 have been reported in pilocytic astrocytoma (Agamanolis and Malone, 1995; Roberts et al, 2001), anaplastic astrocytoma (Karnes et al, 1992) and GBM (Agamanolis and Malone, 1995). Gain of 22 has also been reported in a low grade spinal astrocytoma (Neumann et al, 1993). A region of amplification at 22q12 has been found in a GBM using CGH (Schröck et al, 1994). Deletions of chromosome 22 are more common than gains and have been reported in all grades of tumour (Agamanolis and Malone, 1995; Griffin et al, 1988; Karnes et al, 1992; Neumann et al, 1993). Deletions of 22q have been detected by CGH in pilocytic astrocytoma and high grade astrocytoma (Schröck et al, 1996; Shlomit et al, 2000; Warr et al, 2001). Structural alterations of chromosome 22 include add(22)(p13), add(22)(q13), t(18;22)(q11;p11), t(1;22)(p12;q11), t(15;22)(q22;q13), del(22)(q11-qter) (Agamanolis and Malone, 1995; Bhattacharjee et al, 1997; Griffin et al, 1988; Karnes et al, 1992; Kucerova et al, 2000; Neumann et al, 1993). LOH for loci on 22q have been reported in 2/22 grade II astrocytoma, 9/30 anaplastic astrocytoma and 16/67 GBM (Von Deimling et al, 2000).

Oncogenic viruses

Human polyomaviruses JC, BK and the simian polyomavirus (SV40) have been shown to induce brain tumours in experimental animals (Cheng et al, 1999; Weggen et al, 2000). SV40 and BK DNA have been found in a range of human tumours including lung, bone and brain tumour, and the JC virus has been isolated from human glial tumours (Caldarelli-Stefano et al, 2000). There is evidence that different JCV strains have different pathogenic potential, due to rearrangements in their transcriptional control regions (TCR) generating mutated strains.

Mad-4 is one mutated strain that has been shown to have oncogenic potential in experimental animals. Caldarelli-Stefano et al. 2000 have observed 1 astrocytoma and 1 ependymoma with rearranged *Mad-4* in the viral TCR suggesting that JCV could have played a role in the pathogenesis of these tumours. SV40 has the ability to transform normal cells into cells with a neoplastic phenotype. The transforming ability is dependent on the expression of the early region gene product known as large tumour antigen (Tag), which has multiple biological and biochemical properties. SV40 Tag forms complexes with p53, pRb, p107, p130, p300 and p400 inactivating their functions. When these proteins are inactivated, cells can enter the cell cycle unrestricted. This leads to an alteration in the integrity and stability of the host cell genome, resulting in numerical and structural chromosomal aberrations (Zhen et al, 1999). The complex formation of Tag-p53 and Tag-pRb may be another method to inactivate *p53* and *pRb* at the protein level in addition to gene mutation.

Studies involving SV40 large T antigen gene sequences showed 10/11 ependymoma to have amplification products that hybridised specifically to SV40 probes. SV40 Tag has been shown to be expressed in most histologic types of brain tumours, including ependymoma (8/8), choroid plexus papilloma (2/2), pituitary adenoma (9/10), astrocytoma (9/11), anaplastic astrocytoma (2/4), GBM multiforme (4/8), meningioma (7/10) and medulloblastoma (2/6), but not in normal brain (Zhen et al, 1999). This suggests that SV40 or a related virus may play a role in the development of ependymoma and that inactivation of *p53*, $p110^{rb}$, or both is important in the genesis of these tumours (Raffel, 1996). Although SV40 does not naturally infect humans, it was a major contaminant in a polio vaccine given to children between 1955 and 1963. The finding of the SV40 early region gene in peripheral blood samples and sperm fluids from healthy individuals indicates a possible manner of transmission of SV40 from person to person in the normal population (Zhen et al, 1999).

Comparative Genomic Hybridisation

For many years analysis of metaphase chromosomes has been the cornerstone of cancer genetics. G-banding analysis has proved to be useful in identifying chromosome abnormalities in leukaemias and lymphomas but has been less successful in studying solid tumours because of the difficulty in obtaining good quality metaphases preparations (Gray and Collins, 2000). CGH was first developed by Kallioniemi and colleagues in 1992 and has allowed the entire genome to be studied in a single experiment from just a few nanograms of starting DNA, without the need for cell culture (Forozan et al, 1997). It enables the screening of tumours for DNA copy-number changes and provides a map of chromosomal regions that are lost or gained, which can help in locating specific chromosomal regions that might play a role in the pathogenesis or progression of tumours. Once results from CGH have been obtained, more specific molecular genetic techniques, such as FISH, LOH analysis and sequencing can be used in order to identify oncogenes and/or tumour suppressor genes in these regions (Weiss et al, 1999).

CGH is based on a modified in situ hybridisation method where differentially labelled test (tumour) and reference (normal) genomic DNA are co-hybridised in the presence of human Cot-1 DNA to normal human metaphase chromosome spreads. The relative amounts of test and reference DNA that bind at any given chromosome locus are dependent on the abundance of those sequences in the test and reference DNA samples. Copy number differences between the two samples can be seen as green:red fluorescence intensity differences on metaphase chromosomes. Ratios of the signal intensities can be quantitated using a digital image analysis system which calculates intensity profiles for both colours and the green:red ratio along each chromosome (Kallioniemi et al, 1992). Gains and amplifications in the test DNA are seen as chromosomal regions with an increased fluorescence ratio (>1) whereas losses and deletions show a reduced

fluorescence ratio (<1) (Forozan et al, 1997; Weiss et al, 1999). The main limitations of CGH are its resolution (10-20Mb), it does not provide quantitative information about gene dosage and it is insensitive to structural aberrations that do not result in a DNA sequence copy number change (Gray and Collins, 2000). However it is sensitive enough to detect amplification events (e.g. 5-10 fold amplifications of 1mb regions). The technique cannot be used to detect balanced rearrangements such as inversions and translocations, and deletions within a chromosomal band (Biegal, 1999; Forozan et al, 1997).

CGH has helped identify the androgen receptor (*AR*) gene located in a region of increased copy number at Xq12 in hormone refractory prostate cancers (Koivisto et al, 1997) and the *PIK3CA* gene located at 3q26 in ovarian cancer (Shayesteh et al, 1999). Several genes have been identified in regions of amplification at 20q, including *AIB1* a steroid receptor co-activator (Anzick et al, 1997), *ZNF217* a putative Zn-finger transcription factor associated with instability and immortalisation (Collins et al, 1998) and *STK15* a centrosome-associated serine/threonine protein kinase (Zhou et al, 1998).

CGH has been used to study brain tumours in both children and adults and has identified six new regions of amplification at 1q32, 4q12, 7q21.1, 7q21.2-3, 12p and 22q12, as well as amplification of the *EGFR* gene at 7p12 in malignant gliomas. Gain of chromosome 7 and loss of chromosome 10 were the most common events in this group of tumours (Schröck et al, 1994). The work of Brunner et al. (2000) also showed gain of 7 and loss of 10 to be the most common aberrations in adult GBM. They also observed a novel amplification at 20p11-12 in an oligoastrocytoma. CGH has identified two major sub-groups of high-grade oligodendroglial tumours, 1 group with –1p/-19q and the other group with +7/-10 (Jeuken et al, 1999).

The first group to publish work on CGH analysis of ependymoma identified losses of 6q and 22q as the most common aberration in primary ependymoma in paediatric patients (Reardon et al, 1999). Loss of 22q was the most frequent abnormality in a mixed group of adult and paediatric ependymoma studied by Zheng et al, (2000). Gains of 1q have been seen to occur exclusively in paediatric ependymoma (Scheil et al, 2001). Loss of 13q was a frequent observation especially in myxopapillary ependymoma. Shlomit et al, (2000) showed gain of 1q and loss of 16q to be the most frequent alterations in paediatric low grade ependymoma. Gain of 1q and loss of 9 were the most frequent alterations in intracranial ependymoma in a study by Hirose et al, (2001), whilst gain of 7 was seen exclusively in spinal ependymoma. A recent study showed isochromosome 1q to be an early genetic event in a case of primary and recurrent ependymoma from a child with an intracranial ependymoma (Vinchon et al, 2001).

Only a handful of studies have investigated the genetic alterations in paediatric astrocytoma using CGH. Only a small number of juvenile pilocytic astrocytoma have been found to have detectable alterations. The tumours that did show alterations had gains of a single chromosome, the most common being gain of 7 (Sanoudou et al, 2000). These findings are in agreement with an earlier study that found no consistent changes in paediatric low grade astrocytoma (Schröck et al, 1996). Another study also showed juvenile pilocytic astrocytoma to have a low number of copy number aberrations. The most common alterations were gain of 6q and loss of 9q (Shlomit et al, 2000). In paediatric high grade astrocytoma the most common imbalance was loss of 16p and high copy number amplifications were seen at 1p, 2q22, 7q22-23, 8q21-22, 12q and 13q11-14 (Warr et al, 2001). A recent CGH study identified loss of 8p as a sole abnormality in a case of paediatric pleomorphic xanthoastrocytoma (Yin et al, 2002).

The application of CGH has been expanded through combination with tissue microdissection and PCR amplification using a universal primer. This allows test DNA to be obtained from minute subregions and even from individual cells (Forozan et al, 1997). The combination of microdissection and CGH allows the detection of cytogenetic aberrations from clones that may be missed when analysing DNA extracted from large cell populations (Aubele et al, 1999). Degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) has been reported to be a reliable method to produce a representation of small amounts of DNA (Cheung, 1996; Huang et al, 2000; Kuukasjarvi et al, 1997; Speicher et al, 1993). DOP-PCR allows the analysis of less than 1ng of DNA and is useful for amplifying DNA from microdissected tissue.

Aims of this Research

Tumors of the central nervous system are significant cause of neurological morbidity and mortality in children, yet few genetic investigations have focused on the events that lead to the formation of these tumours. A large proportion of ependymoma and astrocytoma have been found to have normal karyotypes. A number of chromosomes appear to be non-randomly associated with glial tumour development, including deletions or rearrangements of 6q, 17 and 22 in ependymoma and gain of 7, loss of 10 and structural abnormalities of 9p in a small subset of astrocytoma. At present, the only significant prognostic factors are age at diagnosis and extent of tumour resection. With these facts in mind, this study aimed to:

- Identify consistent chromosome abnormalities in paediatric ependymoma and astrocytoma using comparative genomic hybridisation in order to determine candidate genes that might be involved in the development/or progression of these tumours. By defining minimum regions of gain/loss it might be possible to identify target genes in these regions that are involved in the

genesis of these tumours and have been reported to have a role in other malignancies.

- From the characterization of genetic alterations that occur in paediatric astrocytoma, identify the main differences or similarities between paediatric and adult astrocytoma.

- Identify chromosome abnormalities that could be used as potential prognostic markers. CGH analysis would identify copy number aberrations (CNAs) that are associated with clinicopathological features such as tumour histology, location and recurrence as well as patient age and sex that might prove useful as diagnostic markers. From the clinical data available for the patients in this study it should be possible to identify clinicopathological features as well as genetic aberrations (identified by CGH) that influence patient survival.

- Ependymoma are known to respond poorly to treatment with chemotherapeutic agents and this is often due to the development of multidrug resistance in some tumour cells. Recent research has suggested a role for the multidrug resistance gene, *MDR1*, in the chemoresistance of paediatric ependymoma. This study will investigate the expression of *MDR1* in a panel of ependymoma in order to determine whether overexpression of this gene may be a factor in the failure of chemotherapy in these tumours.

- The number of samples available for analysis is quite small and it would therefore be advantageous to utilize archival material in the form of paraffin embedded tissue sections. Such material often results in small quantities of DNA that are of a poor quality. One technical aim of this study is to analyse different techniques available for the extraction of DNA from archival material and

methods that can be used to amplify and label this DNA for use in CGH experiments.

CHAPTER 2
MATERIALS & METHODS

Origin of samples

Paired blood and biopsy samples were kindly provided by W. Harkness, R. Haywood and B. Harding (Great Ormond Street Hospital), DGT Thomas (Queen Square) and N. Kitchen and P. Wilkins (Atkinson Morley Hospital) between 1981 and March 2002. Tumour samples were removed at the time of surgery and were obtained from material not required for diagnostic purposes, which would otherwise have been discarded. Also taken at the time of surgery was a 10ml venous blood sample from each patient. These procedures and the present study were approved by the Joint Medical Ethics Committee of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology. The samples were used fresh (received straight from the hospital) or from older samples stored in liquid nitrogen (LN_2). Ependymoma patient information can be seen in Table 2.1 and astrocytoma patient information in Table 2.2. A total of 54 ependymoma and 64 astrocytoma samples were obtained from 110 patients. The age range of the ependymoma patients was 7 months to 17 years with a mean age of 5.2 years. The male to female ratio was 16:11. There were 9 anaplastic ependymoma, 41 ependymoma and 4 subependymoma. The age range of the astrocytoma patients was 8 months to 33 years with a mean age of 10.36 years. The male to female ratio was 1:1. The histological breakdown of astrocytoma samples was 11 glioblastoma multiforme, 6 anaplastic astrocytoma, 18 diffuse astrocytoma and 29 pilocytic astrocytoma.

Details of reagents and buffers

All instructions for the preparation of the buffers and reagents used in this study, along with the names and addresses of the companies which supplied them, can be found in Appendix II. All primers were synthesised by Amersham Pharmacia Biotech.

IN[1]	Age[2]	Sex[3]	Grade[4]	P/R[5]	Location	Treatment[6]	Survival[7]
Primary Samples							
1932	2	M	SE	P	Posterior Fossa	Macroscopic removal,	4 (D)
113	Unknown	F	E	P	Unknown	Unknown	Unknown
959	Unknown	M	E	P	Unknown	Unknown	Unknown
1594	1.67	M	E	P	Posterior Fossa	Partial removal, radiotherapy, chemotherapy	18 (D)
1638	1.4	M	E	P	Posterior Fossa	Macroscopic removal, chemotherapy	49 (D)
2186	12.5	F	E	P	Posterior Fossa	Macroscopic removal, radiotherapy	118 (A)
2242	2	F	E	P	Posterior Fossa	Macroscopic removal,	17 (D)
2511	13	F	E	P	Posterior Fossa	Partial excision, radiotherapy	35 (A)
2536	7	F	E	P	Supratentorial	Macroscopic removal, radiotherapy	51 (A)
2628	17	M	E	P	Unknown	Unknown	9 (D)
2699	11.5	F	E	P	Posterior Fossa	Macroscopic removal, radiotherapy	2 (A)
2752	2.25	M	E	P	Posterior Fossa	Partial removal, chemotherapy (BB)	62 (A)
2776	6	F	E	P	Posterior Fossa	Macroscopic removal, radiotherapy	71 (A)
2871	1.3	F	E	P	Posterior Fossa	Macroscopic removal, chemotherapy (BB)	63 (A)
2891	1.9	F	E	P	Posterior Fossa	Sub-total removal, chemotherapy (BB)	20 (A)
2922	2.75	M	E	P	Posterior Fossa	Macroscopic removal, radiotherapy	47 (A)
2931	0.58	F	E	P	Posterior Fossa	Macroscopic removal, chemotherapy (BB)	18 (D)
2935	2.58	M	E	P	Posterior Fossa	Macroscopic removal, radiotherapy	39 (A)
2939	11.5	F	E	P	Posterior Fossa	Macroscopic removal, radiotherapy	38 (A)
2941	5.5	M	E	P	Posterior Fossa	Macroscopic removal, radiotherapy	24 (A)
2944	10.5	M	E	P	Supratentorial	Partial removal, radiotherapy	37 (A)
3008	2.25	M	E	P	Posterior Fossa	Macroscopic removal, chemotherapy (BB)	27 (A)
3014	6.75	M	E	P	Supratentorial	Macroscopic removal, radiotherapy	23 (A)
3022	1.75	F	E	P	Posterior Fossa	Macroscopic removal, chemotherapy (BB)	18 (A)
3029	1.75	M	E	P	Posterior Fossa	Macroscopic removal, chemotherapy (BB)	23 (A)
3071	4	M	E	P	Posterior Fossa	Macroscopic removal, radiotherapy	4 (A)
3087	3.5	M	E	P	Posterior Fossa	Macroscopic removal, local field radiotherapy	5 (A)
U36	3	M	E	P	Posterior Fossa	Unknown	Unknown
U38	1.5	F	E	P	Posterior Fossa	Unknown	Unknown
U40	3	M	E	P	Posterior Fossa	Unknown	Unknown
2443	4.5	M	AE	P	Posterior Fossa	Partial removal, radiotherapy, chemotherapy	64 (D)
2767	1.8	F	AE	P	Posterior Fossa	Macroscopic removal, chemotherapy (BB)	72 (A)
2827	9.25	M	AE	P	Supratentorial	Macroscopic removal, radiotherapy	69 (A)
Recurrent Samples							
2798	15	F	SE	R	Posterior Fossa	Unknown	Unknown
1258	2.5	F	E	R	Posterior Fossa	Partial removal, radiotherapy	36 (A)
1497	1.25	M	E	R	Posterior Fossa	Macroscopic removal, radiotherapy, chemotherapy	45 (D)
2923	1.3	M	E	R	Posterior Fossa	Surgery (extent unknown)	20 (D)
2855	12	F	AE	R	Posterior Fossa	Debulking surgery, chemotherapy	26 (A)
2886	5.25	M	AE	R	Posterior Fossa	Partial removal, radiotherapy	21 (D)
2929	9.33	F	AE	R	Supratentorial	Surgery (extent unknown), palliative radiotherapy	14 (D)
Paired samples							
772	4	M	SE	P	Supratentorial	Partial removal, radiotherapy, chemotherapy	72 (D)
1134	5.5	M	SE	R	Supratentorial	Partial removal, chemotherapy	72 (D)
1231	7	F	E	P	Supratentorial	Macroscopic removal, radiotherapy	64 (D)
U37	7	F	AE	R	Supratentorial	Macroscopic removal	64 (D)
1759	10	M	E	P	Posterior Fossa	Partial excision, radiotherapy	98 (A)
2376	15	M	E	R	Posterior Fossa	Excision (extent unknown), chemotherapy	98 (A)
2638	1.9	M	E	P	Posterior Fossa	Partial removal, chemotherapy (BB)	91 (A)
2887	4.5	M	E	R	Posterior Fossa	Excision (extent unknown), radiotherapy	91 (A)
2682	0.66	F	E	P	Posterior Fossa	Macroscopic removal, chemotherapy (BB)	11 (D)
2766	2	F	E	R	Posterior Fossa	Excision (extent unknown)	11 (D)
2904	7	M	E	R	Posterior Fossa	Surgery (extent unknown), radiotherapy	97 (D)
2970	8	M	E	R	Posterior Fossa	Surgery (extent unknown), palliative chemotherapy	97 (D)
3037	2	M	AE	P	Posterior Fossa	Macroscopic removal, chemotherapy (BB)	21 (A)
3108	4	M	AE	R	Posterior Fossa	Surgery (extent unknown)	21 (A)

TABLE 2.1: Clinical and pathological data for ependymoma patients

Key to table:
IN[1] : Institute of Neurology assigned number
Age[2] : Age in years at diagnosis
Sex[3]: M = male; F = female
Grade[4]: SE = subependymoma; E = ependymoma; AE = anaplastic ependymoma
P/R[5]: P = primary sample; R = recurrent sample
Treatment[6]:BB = baby brain protocol (see Appendix I)
Survival [7]: survival in months from date of diagnosis A = alive; D = deceased

IN[1]	Age[2]	Sex[3]	Grade[4]	P/R[5]	Location[6]	Treatment[7]	Survival[8]
17/81	14	F	PA	P	L.temporal	Unknown	Unknown
168	15	M	PA	P	Unknown	Unknown	Unknown
324	15	M	PA	P	Supratentorial	Unknown	190 (D)
1133	5.5	M	PA	P	Posterior Fossa	Macroscopic removal	1 (A)
1533	1.75	F	PA	P	Posterior Fossa	Macroscopic removal	143 (A)
1950	4	M	PA	P	Posterior Fossa	Macroscopic removal	26 (A)
1953	2.5	F	PA	P	Posterior Fossa	Partial excision	159 (A)
2017	7	M	PA	P	Posterior Fossa	Macroscopic removal	24 (A)
2110	2.5	M	PA	P	Posterior Fossa	Macroscopic removal	124 (A)
2356	4	M	PA	P	Posterior Fossa	Macroscopic removal	106 (A)
2368	8	F	PA	P	Posterior Fossa	Macroscopic removal	87 (A)
2466	3.5	F	PA	P	Posterior Fossa	Partial excision	103 (A)
2576	4.8	M	PA	P	Posterior Fossa	Macroscopic removal, radiotherapy	68 (A)
2596	4.5	F	GCA	P	III Ventricle	Partial excision	45 (A)
2775	9.5	F	PA	P	Posterior Fossa	Macroscopic removal	75 (A)
2788	9	M	PA	P	Posterior Fossa	Macroscopic removal	39 (A)
2797	6.5	M	PA	P	L.temporal	Macroscopic removal	59 (A)
2825	2.75	F	PA	P	Posterior Fossa	Incomplete removal	67 (A)
2826	7	M	PA	P	Posterior Fossa	Macroscopic removal	54 (A)
2893	11.6	M	PA	P	Posterior Fossa	Macroscopic removal, LGG (wait & see)	47 (A)
2921	3	M	PA	P	Posterior Fossa	Macroscopic removal, LGG (wait & see)	2 (A)
2940	3	M	PA	P	Posterior Fossa	Macroscopic removal, LGG (wait & see)	39 (A)
2946	9	F	PA	P	Posterior Fossa	Macroscopic removal, LGG (wait & see)	28 (A)
2969	2.5	F	PA	P	Posterior Fossa	Macroscopic removal, LGG (wait & see)	34 (A)
2977	5	F	PA	P	Posterior Fossa	Macroscopic removal	1 (A)
3002	6	F	PA	P	Posterior Fossa	Sub total removal	13 (A)
3017	8.25	F	PA	P	Posterior Fossa	Sub total removal, LGG (wait & see)	25 (A)
3066	0.75	M	PA	P	Posterior Fossa	Sub total removal, chemotherapy (LGG)	12 (A)
3085	9	F	PA	P	Posterior Fossa	Macroscopic removal, LGG (wait & see)	8 (A)
18	33	M	DA	P	Unknown	Unknown	12 (D)
276	4.5	F	DA	P	Thalamic	Macroscopic removal	120 (A)
380	1.8	F	DA	P	Posterior Fossa	Macroscopic removal, radiotherapy	92 (D)
699	15	M	DA	P	R.Thalamus	Stereotactic biopsy	Unknown
1145	11	M	DA	P	Midline Cerebellar	Macroscopic removal	60 (A)
1382	10	F	DA	P	Posterior Fossa	Partial excision, radiotherapy	Unknown
1520	7	M	DA	P	Supratentorial	Partial excision, radiotherapy	110 (A)
1524	13	M	DA	P	Posterior Fossa	Macroscopic removal	13 (A)
1805	3	F	DA	P	Thalamus	Stereotactic biopsy, radiotherapy	2 (A)
1859	3	F	DA	P	Brainstem	Partial excision, radiotherapy	139 (A)
1930	13	F	DA	P	R. Temporal	Temporal lobectomy	60 (A)
2003*	9	F	DA	P	R. Temporal	Macroscopic removal	117 (A)
2591*	12	F	DA	R	Supratentorial	Radiotherapy	117 (A)
2102	8	M	DA	P	Brainstem	Partial excision, radiotherapy	67 (A)
2122	10	F	DA	P	Optic Chiasm	Partial excision, radiotherapy	78 (A)
2355	19	M	DA	P	Unknown	Unknown	Unknown
2587	13	F	PXA	P	L.temporal	Macroscopic removal	44 (A)
2698	9.33	M	DA	P	Brainstem	Partial excision	80 (A)
2988	26	F	DA	P	L.Frontal	Unknown	14 (A)
3032	9.5	F	GA	P	L.Thalamic	Partial excision, radiotherapy	12 (D)
1651	2.5	M	AA	P	Midbrain	CT biopsy	Unknown
2538	21	M	AA	P	L.Frontal	Stereotactic biopsy	3 (A)
2563	24	F	AA	P	L.Parietal	Stereotatic biopsy, radiotherapy	45 (A)
2758	31	F	AA	P	R.Frontal	Stereotatic biopsy, radiotherapy	132 (A)
2774	30	M	AA	P	L.Frontal	Stereotatic biopsy, radiotherapy	Unknown
146/81	27	M	GBM	P	Unknown	Unknown	100 (D)
178	13	M	GBM	P	Unknown	Unknown	3 (D)
1180	8	M	GBM	P	R.Parietal-occipital	Macroscopic removal, chemotherapy (BB)	4 (D)
1262	14	M	GBM	P	L.Parietal	Removal (extent unknown), radiotherapy	12 (A)
1419	8.5	F	GBM	P	Supratentorial	Partial excision, radiotherapy	12 (D)
1495	6	F	GBM	P	Supratentorial	Removal (extent unknown)	80 (A)
1566	6.4	F	GBM	P	Posterior Fossa	Partial removal	7 (A)
1786	27	M	GBM	P	L.temporal	Stereotatic biopsy, radiotherapy	120 (A)
2240	14	F	GBM	P	Unknown	Unknown	4 (D)
3046	15.9	M	GBM	P	L.Frontal	Partial excision, radiotherapy	14 (D)

TABLE 2.2: Clinical and pathological data for astrocytoma patients

Key to table:
IN[1] : Institute of Neurology assigned number
Age[2] : Age in years at diagnosis
Sex[3]: M = male; F = female
Grade[4]: PA = pilocytic astrocytoma; GCA = giant cell astrocytoma; DA = diffuse astrocytoma
PXA = pleomorphic xanthoastrocytoma; GA = gemistocytic astrocytoma
AA = anaplastic astrocytoma; GBM = glioblastoma multiforme
P/R[5]: P = primary sample; R = recurrent sample
Location [6]: L = left; R = right
Treatment[7]: LGG = low grade glioma protocol, BB = baby brain protocol (see Appendix I)
Survival [8]: survival in months from date of diagnosis A = alive; D = deceased

Tissue Culture Techniques

Preparation of Biopsy Material

In the operating theatre, tumour specimens were immediately placed in sterile 30ml universals (Scientific Laboratory Supplies, Nottingham) containing Hams F10 media (Invitrogen Ltd., Inchinan, Renfrewshire) supplemented with kanamycin (50μg/ml), penicillin/streptomycin (100IU/ml/100μg/ml) and amphotericin B (2.5μg/ml). Samples were processed immediately upon arrival in the laboratory, although they remained viable for up to 5 days in the transport media. Venous blood samples were collected in sterile 30ml universals containing EDTA and stored at –70°C.

All handling of the biopsy and blood samples was carried out in a Class II laminar air flow cabinet (ICN Pharmaceuticals, Costa Mesa, CA). The biopsy was removed from the universal containing transport media and placed in a sterile petri dish (Helena Biosciences, Sunderland, UK).

A sample of the biopsy weighing approximately 25mg was removed for DNA extraction. As much tissue as possible was frozen down in LN_2 to allow for subsequent extractions. The remaining tissue (approximately 10mg) was sliced using crossed sterile scalpels (size 10 blades) until it was fine enough to be pipetted. The minced tissue was then pipetted into a sterile universal and 2ml Hams F10 culture media, containing 10% foetal calf serum (FCS) (Labtech International, Sussex), and 1ml collagenase solution (Sigma Aldrich, Gillingham, Dorset) were added. The tissue was incubated at 37°C to allow the tissue structure to be broken down by the action of the collagenase. After 30 minutes incubation, 7ml culture media (Hams F10 + 10% FCS) was added to the universal and the sample centrifuged for 5 minutes at 1000rpm. The supernatant was removed and the sample resuspended in 10ml culture media and transferred to a $25cm^2$ tissue culture flask (Triple Red, Thame, Oxfordshire). The sample was incubated at 37°C and checked daily for growth.

Thawing Frozen Cell Stocks

A small beaker was filled with water at approximately 37°C. A vial of cells was removed from LN_2 and placed into the beaker to thaw. The cells were pipetted out of the vial and slowly allowed to drip down the side of a 25cm^2 flask containing 9ml media. The cells were then incubated at 37°C and the media changed after 24 hours to allow the cells time to attach to the flask.

Feeding Cells.

Cells were fed once a week or when the media had changed from dark pink to yellow in colour, depending on which was sooner. The absorption of nutrients from the media by the cells results in the presence of waste products which turn the media acidic and cause the media to become yellow in colour. The media was aspirated from the flasks using a sterile glass pipette and replaced with new media: 10ml for 25cm^2 flasks; 20ml for 75cm^2 flasks and 35ml for 150cm^2 flasks.

Passaging Cells

Cells were passaged when they had reached confluence, in the following way:

1 x 25cm^2 flask transferred into 1 x 75cm^2 flask

1 x 75cm^2 flask split into 3 x 75cm^2 flask

1 x 75cm^2 flask transferred into 1 x 150cm^2 flask

1 x 150cm^2 flask split into 3 x 150cm^2 flask

The old media was aspirated using a sterile glass pipette. The cells were washed twice with sterile Hanks Buffered Saline Solution (HBSS) (Invitrogen). In order to detach the cells from the bottom of the flask, 3ml of trypsin solution (Sigma Aldrich) were added to the flask and the cells incubated at 37°C for 15-30 minutes. To inactivate the trypsin, 7ml of media were added to the cells and the flask contents pipetted into a sterile

universal. The cells were then centrifuged at 1000rpm for 5 minutes. The supernatant was removed and the cells resuspended in 10ml of media.
Each time the cells were treated with trypsin solution the passage number was increased by 1.

Freezing Cells

Any cells that were not required for experimentation were frozen. A 0.4ml sample of the cell suspension was added to 19.6ml of Isoton II (Counter (Beckman Coulter UK Ltd., High Wycombe, Bucks) and the cells counted using a Coulter Counter (Beckman Coulter). The remaining cells were centrifuged at 1000rpm for 5 minutes and the supernatant removed before the cell pellet was resuspended in FCS containing 10% dimethyl sulphoxide (Sigma Aldrich) to a final concentration of 1 million cells per 1ml. The cell suspension was pipetted into a cryovial (1ml suspension per vial) and stored at –70°C for 24 hours. The frozen cells were then transferred to LN_2 storage tanks.

DNA Extraction Techniques

Extraction of genomic DNA from short term cell cultures

DNA was extracted from short term cell cultures using the Qiagen 100/G tips (Qiagen, Hilden, Germany). Once the cells in 3 x 150cm^2 flasks were confluent, they were trypsinised as previously described, using 4ml of trypsin solution. The cells were resuspended in PBS to a final concentration of 1 x 10^7 cells per ml in a 50ml polypropylene tube (Helena Biosciences, Sunderland, Tyne & Wear). One volume of ice-cold buffer C1 and 3 volumes of ice cold dH_2O were added to the sample and mixed by inverting the tube several times. The cells were then incubated on ice for 10 minutes to allow the cells to lyse. The lysed cells were centrifuged for 15 minutes at 1300 x g and the supernatant discarded. The cell pellet was resuspended in 1ml of ice-cold buffer C1 and 3ml ice-cold dH_2O before being centrifuged at 1300 x g for 15 minutes. The supernatant was discarded

and 5ml of buffer G2 added to the cell pellet. The nuclei were completely resuspended by vortexing. The nuclei were incubated at 50°C for 60 minutes in the presence of 95μl of proteinase K stock (20mg/ml) (ICN Biochemicals, Basingstoke, Hampshire) until the lysate became clear. A Genomic 100/G tip was equilibrated with 4ml buffer QBT and allowed to empty into a 50ml Falcon tube by gravity flow. The sample was vortexed before being applied to the genomic tip and allowed to enter the resin by gravity flow. The genomic tip was washed twice with 7.5ml of buffer QC in order to remove contaminants from the sample. The DNA was eluted into a clean 50ml polypropylene tube with 5ml of buffer QF, prewarmed to 50°C. Isopropanol (0.7 volumes) (Sigma Aldrich) was added in order to precipitate the DNA. The sample was then centrifuged at 3000rpm for 15 minutes to pellet the DNA. The supernatant was removed and 1ml of 70% ethanol added to the tube. The tube was vortexed and the contents transferred to a 1.5ml eppendorf tube (Alpha Laboratories, Eastleigh, Hants). The sample was centrifuged for 15 minutes at 13000rpm before the supernatant was removed and the DNA pellet dried in a Speed Vac (Savant Instruments Inc, Holbrook, NY) for 10 minutes. The DNA was resuspended in 200μl TE buffer (pH 8.0) and left overnight to dissolve before being sheared with a 19 gauge needle. The DNA concentration was determined fluorimetrically using a Hoefer Dynaquant (Amersham Pharmacia Biotech, Little Chalfont, Bucks).

Extraction of genomic DNA from blood

Normal blood DNA was required as a control for CGH experiments. Venous blood samples were taken from 10 healthy female volunteers. A small amount of EDTA (Sigma Aldrich) was added to each blood sample to prevent it clotting. To lyse the cells, 3ml of each blood sample were pipetted into separate 50ml Falcon tubes along with 3ml ice-cold C1 and 9ml of ice-cold water and incubated on ice for 10 minutes. The lysed cells were centrifuged at 3000 rpm for 15 minutes and the supernatant removed. The protocol for extraction of DNA from short term cell cultures was then followed to the point of the precipitation of the DNA with

isopropanol. The tubes were gently shaken to allow the DNA to spool. The liquid was stirred gently with a glass rod to allow the DNA to collect on the rod. The DNA from all the samples was placed into a single eppendorf tube and resuspended in 1ml TE buffer (pH 8.0). The DNA was dissolved at room temperature overnight before being sheared with a 19 gauge needle. The DNA concentration was determined fluorimetrically.

Extraction of genomic DNA from biopsy material

Genomic DNA was extracted from fresh or frozen biopsy samples using a Qiagen QiAmp Kit (Qiagen Ltd) which contained pre-prepared buffers and extraction columns.

The tissue was allowed to equilibrate at room temperature before a 25mg piece was removed and placed in a sterile petri dish. The tissue was then cut up into small pieces using crossed scalpel blades (size 10) and transferred to a 1.5ml Eppendorf tube, where 180μl buffer ATL and 20μl proteinase K stock solution (20mg/ml) were added and mixed by vortexing. The tissue was incubated at 55°C until it had completely lysed, usually around 2-3 hours. Some samples were incubated overnight at 55°C to allow complete lysis to occur. When lysis was complete, 200μl buffer AL was added to the sample and mixed thoroughly by vortexing. The sample was incubated at 70°C for 10 minutes and 200μl absolute alcohol was added to the sample and mixed thoroughly by vortexing. A QIAmp spin column was placed in a 2ml collection tube and the sample mixture carefully applied to the column without moistening the rim. The cap was closed and the sample centrifuged at 8000rpm for 1 minute. The filtrate was discarded and the column placed in a clean collection tube. The column was washed by the addition of 500μl AW1 and centrifugation at 8000rpm for 1 minute. The filtrate was discarded and the column placed in a clean collection tube prior to a second wash with 500μl AW2. The column was centrifuged at 1500rpm for 3 minutes to remove all traces of AW from the column before elution. The collection tube containing the filtrate was discarded and the column placed in a clean collection tube. To elute the DNA, 200μl buffer AE was added to the column and the column incubated

at room temperature for 1 minute before being centrifuged at 8000rpm for 1 minute. The eluted DNA was pipetted into a sterile 1.5ml Eppendorf tube. The elution step was repeated twice. The eluted DNA was quantified fluorimetrically.

Extraction of DNA from paraffin embedded tissues

Method 1: QIAamp DNA mini kit

Six 5μm mounted sections of each tumour sample were used. Each section was carefully scraped off the microscope slide into a 1.5ml eppendorf tube using a sterile size 10 scalpel blade and 1200μl of xylene (Fisher Chemicals, Loughborough, Leicestershire) was added to the tube. The sample was vortexed vigorously and centrifuged at 1500rpm for 5 minutes at room temperature. The superanatant was removed by pipetting and 1200μl of ethanol added to the sample to remove any remaining xylene. The sample was gently vortexed before being centrifuged at 1500rpm for 5 minutes. The ethanol was then removed by pipetting and the alcohol step repeated. After the ethanol had been removed, the open tube was incubated at 37°C for 15 minutes to allow any remaining ethanol to evaporate. The tissue pellet was resuspended in 180μl of buffer ATL (Qiagen) and the procedure for extracting DNA from biopsy samples followed from this point. After the extraction procedure had been completed a 10μl sample for each tumour was electrophoresed at 100v for 1 hour on a 1% agarose gel in order to determine the fragment size.

Method 2: Personal communication from Jeuken, University Hospital Nijmegen, The Netherlands (2001)

Six 20μm unmounted sections of each tumour were used. The sections were placed in a 50ml polypropylene centrifuge tube and 30ml of xylene was added. The samples were incubated at room temperature for 10 minutes and then centrifuged at 3000rpm for 15 minutes. The supernatant was removed and the xylene step repeated at least twice until all the paraffin had been removed. The pellet was washed twice in ethanol, air dried and then incubated in 10ml 1M sodium thiocynate (Sigma Aldrich)

at 80°C for 1 hour. The sample was centrifuged at 3000rpm for 15 minutes and the supernatant removed. After washing in dH_2O, the pellet was resuspended in 3ml lysis buffer, 300μl 10% SDS and 25μl 20mg/ml proteinase K and then incubated at 37°C overnight. In order to remove any contaminating RNA, 50μl 5mg/ml RNase (Sigma Aldrich) was added to the sample and incubated at 37°C for 1 hour. The sample was cooled to room temperature and 1ml 6M NaCl added. The sample was shaken vigorously by hand in order to precipitate the proteins and then centrifuged at 4000rpm for 15 minutes. The supernatant was transferred to a 15ml polypropylene centrifuge tube and the shaking step repeated until the supernatant was clear. When the supernatant was clear it was transferred to a 50ml polypropylene centrifuge tube and 2 volumes of ethanol were added. The supernatant was stirred gently with a glass rod and the spooled DNA transferred to a sterile 1.5ml eppendorf tube. The DNA was then air dried and dissolved in 200μl dH_2O. If the DNA could not be removed by spooling the tubes were incubated at –20°C overnight and then centrifuged at 3000rpm for 15 minutes. The pellet was washed in 70% ethanol to remove any co-precipitated salt and air dried before being dissolved in 150μl dH_2O. A 10μl sample from each tumour was electrophoresed at 100v for 1 hour on a 1% agarose gel in order to determine the fragment size. The concentration of the DNA was measured fluorimetrically.

Fluorimetric quantification of DNA

The DyNAQuant Fluorimeter (Amersham Pharmacia Biotech) accurately quantitates DNA concentrations using Hoechst 33258 dye (Bisbenzimide). The Hoechst 33258 dye binds to DNA and the light from the mercury lamp excites the DNA-dye complex causing light at 458nm to be emitted. The measured fluorescence is a direct indicator of the DNA concentration. All DNA concentrations were measured using the Capillary Cuvette method, which allows the detection of DNA concentrations ranging from 0 to 100ng/μl depending on the capillary assay solution used.

A blank was prepared by mixing 100μl 1X TNE with 100μl Capillary Assay solution (CAS). The standard was made to give a final concentration of 100ng/μl by mixing 10μl CAS with 10μl 200ng/μl Calf Thymus DNA (Sigma Aldrich). To measure the sample 2μl of the test DNA was mixed with 2μl CAS. The DNA was measured twice and an average of the two readings taken to give the final concentration in ng/μl.

Comparative Genomic Hybridisation

DNA Labelling

SpectrumGreen dUTP (Vysis, Downers Grove, IL) was incorporated into between 500ng and 1000ng of each tumour sample or normal control DNA by nick translation.

500ng-1000ng genomic DNA, 2.5μl 0.2mM SpectrumGreen dUTP, 5μl 0.1mM dTTP, 10μl 0.1mM dNTP mix and 5μl nick translation buffer (Vysis) were added to a 0.5ml eppendorf and cooled on ice. 5μl or 10μl nick translation enzyme (Vysis) were added and the volume made up to 50μl with nuclease free water (Vysis). The tubes were vortexed briefly before being incubated on a Techne PHC-3 Thermal Cycler for 2 hours at 15°C. The reaction was stopped by incubating at 70°C for 10 minutes. The samples were then chilled on ice.

Determining the probe size

The probe size was estimated by running 10μl of each SpectrumGreen labelled sample on a 1% agarose gel immersed in 1 x TAE buffer containing 30μl 10mg/ml ethidium bromide at 100V for 1 hour with 1μl Lambda DNA/*Eco*911 (MBI Fermentas, Vilnius, Lithuania) as a size marker. Probes were visualized on a UV transilluminator.

Samples were used for hybridisation if the majority of the DNA smear was in the range of 300-3000bp. For samples in which the probe size was too

large, DNA was re-labelled and incubated for 4 hours at 15°C with 10μl nick translation enzyme. A 10μl aliquot was electrophoresed on a 1% agarose gel for 1 hour to check the probe size. If the fragment size was still too large the labelling procedure was repeated with a 6 hour incubation. None of the samples in this study required longer than 6 hours.

CGH procedure

In order to produce a hybridisation signal with equivalent intensities, a ratio of 2:1 SpectrumGreen to SpectrumRed labelled DNA was used.

The hybridisation mix consisted of 400ng SpectrumGreen labelled test DNA, 200ng SpectrumRed labelled total genomic reference DNA (Vysis) and 10μg Human Cot-1 DNA (Vysis). After precipitation with 3M sodium acetate (Sigma Aldrich) and ethanol, the pellet was resuspended in 10μl hybridization buffer and denatured at 73°C for 5 minutes.

Normal metaphase target slides were purchased from Vysis. These slides had been prepared using phytohaemaglutin (PHA) stimulated lymphocytes derived from a karyotypically normal male donor. The lymphocytes were cultured to produce chromosome lengths of 400-550 bands. Slides were incubated in denaturation solution at 73°C for 5 minutes and dehydrated through an alcohol series prior to hybridisation. The denatured probe mix was applied to the slide and a coverslip was immediately applied and sealed with cow gum. The slides were then incubated in a sealed, humidified chamber at 37°C for 72 hours.

Following stringency washes of 0.4x SSC/0.3% NP-40 at 73°C for 2 minutes and 2x SSC/0.1% NP-40 at room temperature for 1 minute, the slides were air dried and mounted in 4,6-diamidinophenylindole (DAPI II) counterstain (Vysis).

Digital Imaging and Analysis

Metaphase images with uniform hybridisation were acquired using a Zeiss Axioscope epifluorescent microscope equipped with a triple bandpass filter (Vysis) designed to simultaneously excite and emit light specific for DAPI, SpectrumGreen and SpectrumRed, and a cooled charge-coupled device camera (Photometrics, Tuscon AZ). The hybridisations were evaluated by eye prior to digital image analysis as a high quality hybridisation ensures an accurate interpretation of data both within and across experiments. Metaphases used for analysis had low and uniform background fluorescence around each chromosome, minimal surrounding cytoplasm and balanced red and green fluorescence. Mean ratios of SpectrumGreen to SpectrumRed signal intensities were calculated using Quips karyotype software (Vysis). Between 8-20 metaphases were analysed and averaged in order to obtain CGH profiles for each tumour. Upper and lower threshold values of 1±0.2 were used to interpret chromosomal gains and losses. High level amplifications were determined using an upper threshold of >1.4. For normal blood controls, the mean green to red rations remained between 1.2 and 0.8 along the whole length of all the chromosomes. The CGH procedure is illustrated in Figure 2.1.

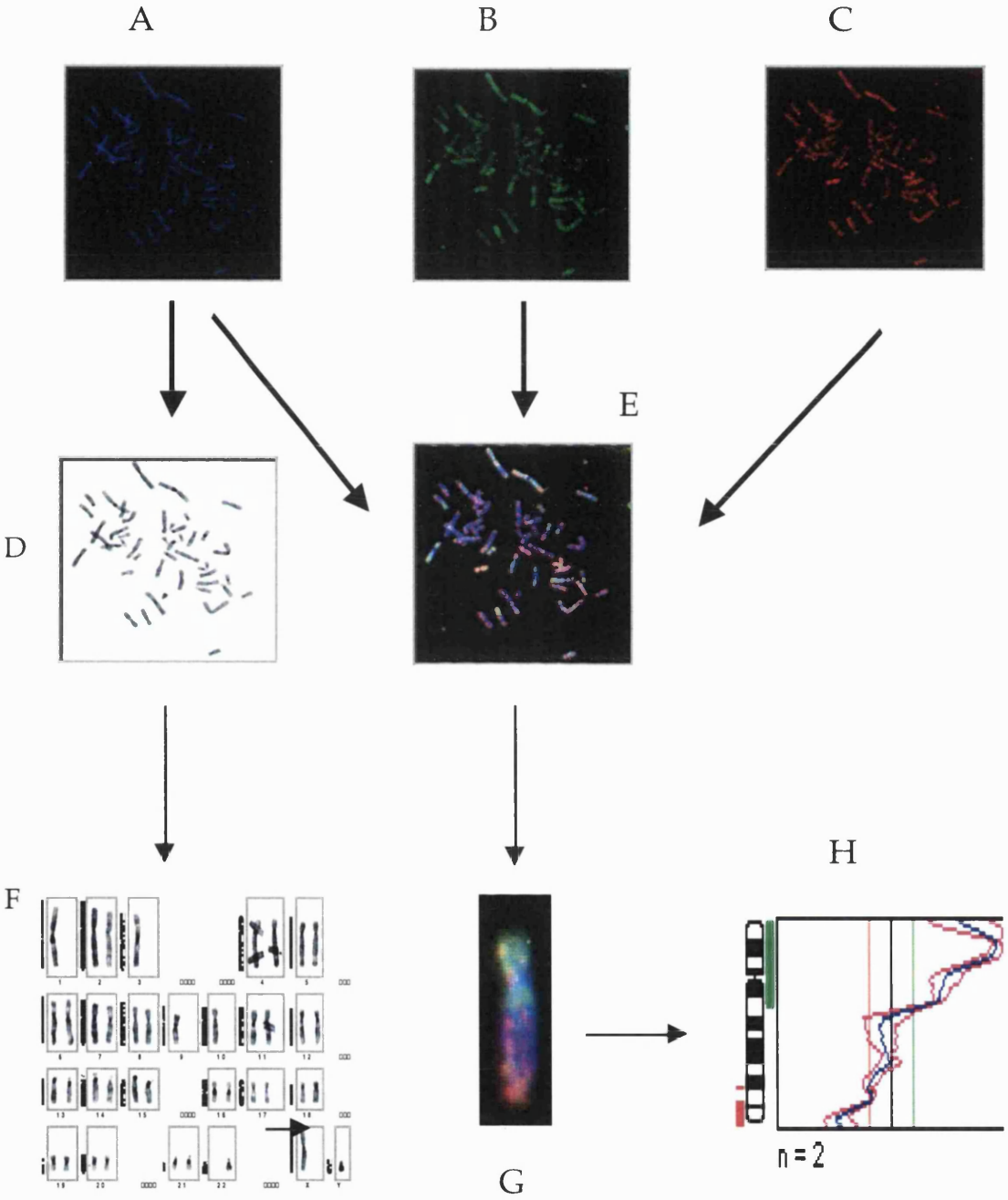


Figure 2.1: Schematic diagram of comparative genomic hybridisation.

Digital images of DAPI counterstain (A), SpectrumGreen tumour (B) and SpectrumRed normal (C) are combined to produce a composite image (E). The DAPI counterstain also produces a pseudo G-banding pattern to enable identification of each individual chromosome (D,F). A region of amplification (green) can be seen in the composite image of chromosome 12 (G) and in the fluorescence ratio profile (H).

CGH Control experiments

A control hybridisation was included in each set of experiments. This was either a normal control or an MPE 600 control. The hybridisation mix contained 200ng SpectrumGreen labelled MPE 600 DNA (Vysis) or 200ng SpectrumGreen labelled normal blood DNA with 100ng SpectrumRed reference DNA and 10μg Cot-1 DNA. The controls were hybridised under the same conditions as the tumour samples.

MPE 600 DNA has been extracted from an immortalized female breast cancer cell line with pre-defined genetic abnormalities, which are:

- a small deletion near 1pter; gain of 1q
- loss of 9p
- distal deletion of 11q
- loss of 16q
- normal chromosomes 2 and X

In order to establish that any alterations observed are a result of true genetic change and not labelling or hybridisation artifacts, hybridisation of each tumour was repeated at least twice. An example of the reproducibility of the technique can be seen in Figure 2.2.

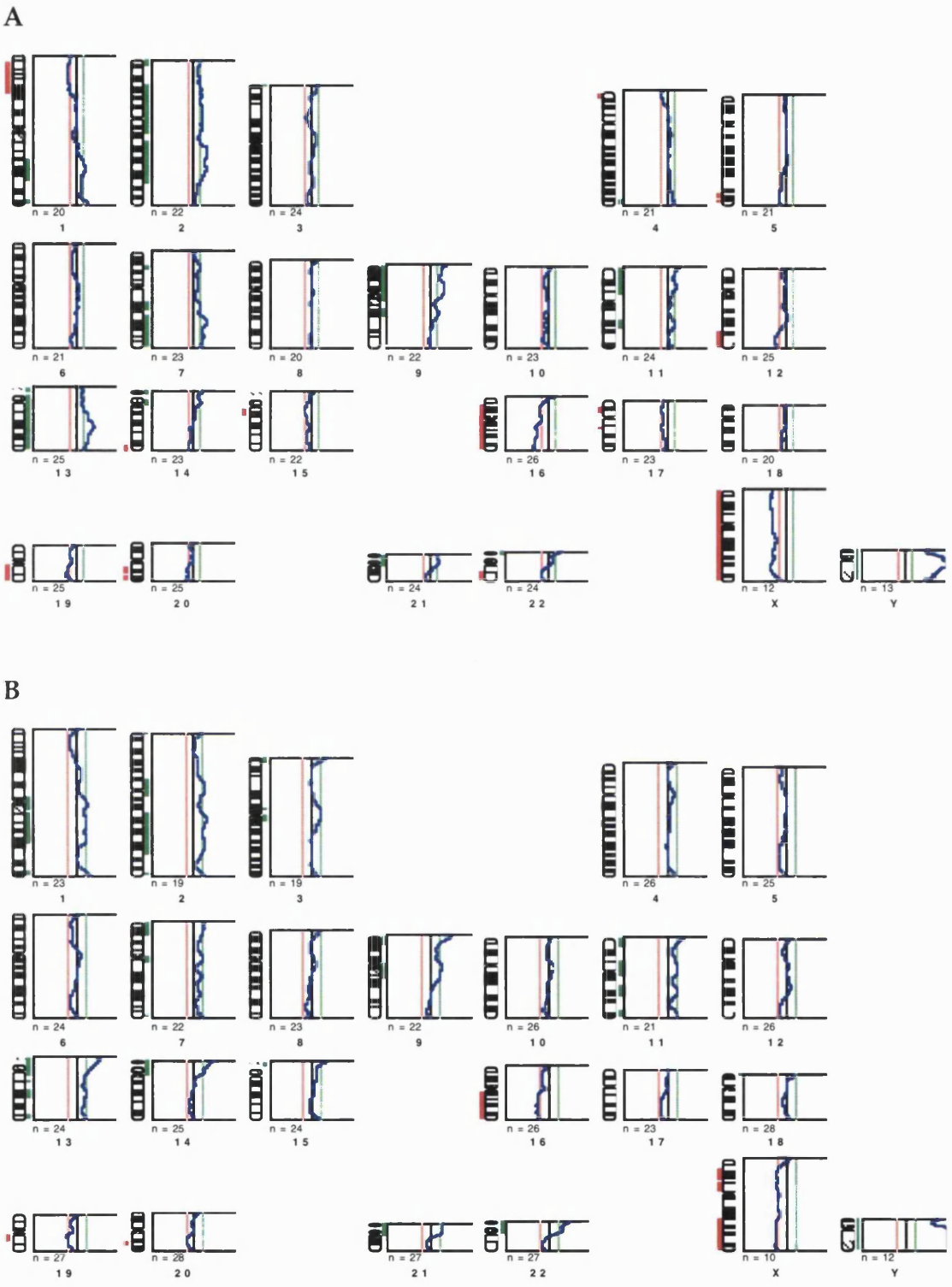


Figure 2.2: Paediatric ependymoma IN2376, hybridised in two separate experiments (A and B). The same alterations can be seen in the CGH profiles of both hybridisations, and include gains of 1q, 2q,7q, 9p, 11 and 13, and loss of 1p, 12q, 16q, 19q, 20q and 22.

Verification of patient sex using Y-PCR

PCR amplification using Y chromosome specific primers was used to verify the sex of the patient samples used for CGH. Two sets of primers were used. Y1.7 and Y1.8 were designed to amplify a single-copy sequence (DYS14) and produced a 198bp product (Isaka, Takei et al, 2000), and SRY primers were designed to amplify the sex determining region of the Y chromosome and produced a product 470-472bp in size (http://www.ncbi.nlm.nih.gov). The primer sequences were as follows:

Y1.7	5′	CAT CCA GAG CGT CCC TGG CTT	3′ (forward)
Y1.8	5′	CTT TCC ACA GCC ACA TTT GTC	3′ (reverse)
SRYF	5′	GAA TAT TCC CGC TCT CCG GA	3′ (forward)
SRYR	5′	GCT GGT GCT CCA TTC TTG AG	3′ (reverse)

Normal male blood DNA was used as a positive control in all reactions.

PCR amplifications were carried out in a 20μl reaction volume containing 200ng template DNA, 1.25μmol of each dNTP, 1pmol each of forward and reverse primer, 2mM $MgCl_2$, 10x PCR buffer and 1.5U *Taq*-polymerase. An initial denaturation step at 94°C for 1 min was followed by 40 cycles of denaturation at 94°C for 1 min, annealing at either 60°C (Y1.7/1.8) or 57°C (SRY) for 30 sec and extension at 72°C for 30 sec, followed by a final extension step at 72°C for 7 min.

The presence of the amplified sequences was confirmed by running 10μl of each product on a 3% agarose gel immersed in 1x TAE buffer containing 30μl 10mg/ml ethidium bromide for 1 hour at 100V. Products were visualized using a UV transilluminator. The presence of a band on the gel showed the sample came from a male patient, the absence of a band indicated the sample was from a female patient.

DNA from biopsies or short term cell cultures were used for analyses, along with the matched normal blood control for each patient where available.

RNA Techniques

Isolation of total RNA from biopsy samples and cell cultures

The Qiagen RNA/DNA buffer kit and Qiagen-tip 100 was used to extract RNA from biopsy samples and short term cell cultures.

Preparation of cell lysate

Three confluent 150cm^2 tissue culture flasks of cells ($\leq$ 1 x 10^7) were required. The media was aspirated from the flasks and the cells washed with 10ml HBSS before 4ml of trypsin was added. The cells were incubated at 37°C until they had detached from the bottom of the flasks at which point 6ml of PBS was added to the cells and the total volume transferred to a 30ml universal. The cells were pelleted by centrifugation 1000rpm for 5 minutes. The supernatant was aspirated and 2ml of buffer QRL1 added to the cell pellet. The lysate was then passed 3 or 4 times through an 18 gauge needle to homogenise the cells which were then transferred to a 50ml polypropylene tube.

Preparation of tissue lysate

Fresh tissue or thawed biopsy tissue ($\leq$25mg) was finally chopped with crossed scalpel blades (size 10) before being transferred to a 50ml polypropylene tube containing 2ml of buffer QRL1. The tissue was homogenised by drawing it through an 18 gauge needle 3 or 4 times.

RNA isolation

The lysate was mixed with 2ml of buffer QRV1 and centrifuged for 20 minutes at 3000rpm. The supernatant was carefully decanted into a sterile 15ml polypropylene centrifuge tube (Helena Biosciences) and 3.2ml of ice-cold isopropanol added. The tube was then incubated on ice for 5 minutes before being centrifuged at 3000rpm for 30 minutes. Meanwhile 3ml of buffer QRE was pipetted onto a Qiagen tip 100 to equilibrate it. The column was allowed to empty by gravity flow. The supernatant was discarded and

the pellet resuspended in 1ml of buffer QRL1. The pellet was vortexed until it had dissolved. The sample was mixed with 9ml of buffer QRV2 and pipetted on to the Qiagen tip. The sample was allowed to enter the resin by gravity flow before 12ml of buffer QRW was added to the tip. When the buffer had passed though the tip was placed over a clean 15ml polypropylene centrifuge tube and 6ml of preheated (75°C) buffer QRU was added to the tip. The RNA was eluted by gravity flow. One volume of ice-cold isopropanol was added to the elute and incubated for 10 minutes on ice before being centrifuged for 30 minutes at 15,000 x g in order to precipitate the RNA. The supernatant was discarded and 5ml of 70% ethanol added to the pellet. The sample was vortexed and centrifuged at 3000rpm for 15 minutes. The supernatant was removed and this step repeated. The RNA pellet was air dried for approximately 10 minutes before being resuspended in 200μl of DEPC water and pipetted into a 1.5ml polypropylene tube for storage at -70°C. Each RNA extract was electrophoresed on a sodium phosphate gel to determine its quality.

Agarose gel analysis of RNA

All electrophoresis equipment was cleaned with detergent solution, rinsed in water, dried with ethanol and then filled/covered with a 3% solution of hydrogen peroxide. After 10 minutes at room temperature the equipment was then rinsed thoroughly with DEPC treated water.

A 1% agarose gel was prepared using 1X sodium phosphate buffer. Ten microlitres of each RNA sample was electrophoresed for 1 hour at 100V in 1X sodium phosphate buffer containing 30μl 10mg/ml ethidium bromide. A 0.24-9.5Kb RNA ladder (Invitrogen Ltd) was used to determine the size of the RNA sample. Products were visualized using a UV transilluminator.

Expression of *MDR1* gene in ependymomas using RT-PCR

The Qiagen OneStep RT-PCR kit was used to investigate the expression of the *MDR1* gene in RNA samples from paediatric ependymomas. The

protocol for the Qiagen OneStep RT-PCR kit allows reverse transcription and PCR amplification to be carried out sequentially in the same tube. The primer sequences for each primer used are detailed in Table 2.3. RNA was extracted from a sample of normal human brain tissue, as previously described, taken during routine operation and used as a control in all RT-PCR amplifications. A negative control was included with all reactions, which consisted of a reaction containing no template RNA. No product should be detected after amplification unless there has been cross contamination during the set-up of the reaction.

Primer Name		Primer Sequence	Size	Reference
MDR1F	F	5' GGG ACC GCA ATG GAG GAG 3'	308bp	(Sugawara, Watanabe et al, 1992)
	R	5' CAG ATT CAT GAA GAA CCC TG 3'		
MDR1E	F	5' GGG ACC GCA ATG GAG GAG 3'	179bp	(Sugawara, Watanabe et al, 1992)
	R	5' TCC AGC CCC ATG GAT GA 3'		
β2 MG	F	5' ACC CCC ACT GAA AAA GAT GA 3'	120bp	(Noonan, Beck et al, 1990)
	R	5' ATC TTC AAA CCT CCA TGA TG 3'		

Table 2.3: MDR1 primer information

PCR amplifications were carried out in a total volume of 50μl containing 5μl of RNA solution. The reaction mix consisted of 400μm of each dNTP, 0.6μm of each forward and reverse primer, 10μl of 5 X RT-PCR buffer containing 12.5mM $MgCl_2$ and 10μl 5 X Q-solution. For reverse transcription the samples were incubated at 50°C for 30 minutes. For PCR amplification the samples were heated at 95°C for 15 minutes on a thermal cycler followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. A final extension step consisted of incubation at 72°C for 10 minutes.

After amplification 10μl of each sample was electrophoresed on a 2% agarose gel containing 5μl 10mg/ml ethidium bromide for 1 hour at 100v in 1 x TAE buffer. A 100bp DNA ladder (MBI Fermentas) was used to size

the PCR products. Images of the gel were captured using the GeneGenius Bio-imaging system and Gene snap software (Syngene, Cambridge, UK). Photographs of each gel were also taken using a polaroid direct screen instant camera fitted with polaroid black and white film. *MDR1* mRNA was judged to be present in the tumour sample if a band could be clearly seen on the gel image. If no band was present for *MDR1* but a band could be seen for *β2MG,* that tumour was judged not to express *MDR1*. Expression was graded according to band intensity: +++ to samples with a strong band, ++ to samples with an obvious band and + to samples with a faint positive band.

Modification of labelling technique

Degenerate oligonucleotide primed polymerase chain reaction

The standard CGH protocol requires 1μg of genomic tumour DNA for labelling by nick translation. However in some cases in this study smaller amounts of tumour DNA were available for analysis. One way of overcoming a low DNA concentration is to use universal or whole genome amplification using the degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR). This technique allows the amplification of small DNA samples resulting in the production of severalfold more DNA for genetic analysis (Sanchez-Cespedes, Cairns et al, 1998). In this study six different DOP-PCR protocols were investigated to determine the most efficient way of amplifying and labelling small quantities of DNA for use in CGH. All DOP-PCR protocols were performed on a PTC 200 DNA engine (MJ Research, Inc) in 0.5ml thin walled PCR tubes using the UN1 primer sequence:

5′ - CCG ACT CGA GNN NNN NAT GTG G-3′ (where N= A, C, G or T in approximately equal proportions).

Following DOP-PCR amplification, 10μl of product was electrophoresed through a 1% TAE agarose gel containing 5μl of 10mg/ml ethidium bromide for 1 hour at 100v. The molecular weight marker Lambda DNA/*Eco*911 was used to size the amplification products. Products were visualized using a UV transilluminator.

Method (1) was taken from (Cheung, 1996) and was a one step protocol. The reaction was carried out in a 50μl reaction volume consisting of 2μm UNI Primer, 200μm dNTP, 10mM Tris.Cl (pH 9.0), 50mM KCl, 1.5mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin and 50ng template DNA. After an initial denaturation step of 8 min at 96°C, 2.5U Amplitaq DNA polymerase (Perkin Elmer, Warrington, Cheshire) was added. This was followed by 8 cycles of denaturation at 93°C for 1 min, 1 minute annealing at 30°C and 3 minute extension at 72°C and 28 cycles of 1 minute denaturation at 93°C, 1 minute annealing at 60°C and 3 minute extension at 72°C.

Method (2) was taken from (Speicher, du Manoir et al, 1993) and was also a one step reaction. The 50μl reaction volume consisted of 2mM $MgCl_2$, 50mM KCl, 10mM TrisHCl pH 8.4, 10μg/ml gelatine, 200μm each dNTP, 1.7μm UN1 primer, 2.5U *Taq*-polymerase and 50ng template DNA. An initial denaturation step for 10 minutes at 93°C was followed by five cycles of 1 min at 94°C, 1 min 30 sec at 30°C, 3 min transition 30-72°C and 3 min extension at 72°C and 35 cycles of 1 minute at 94°C, 1 min at 62°C and 3 min at 72°C, with an addition of 1 sec/cycle to the extension step and a final extension of 10 min.

Method (3) was taken from (Kuukasjarvi, Tanner et al, 1997). This protocol was a two step reaction, with the option to incorporate a fluorescent label in the second step. Four cycles of the preamplification step were carried out in a 5μl reaction volume containing 10x high salt buffer, 2mM dNTP, 10μM UN1 primer, 2U ThermoSequenase (Amersham Pharmacia Biotech) and 50ng sample DNA. The reaction consisted of 1 minute denaturation at 94°C, 1 minute annealing at 25°C, 3 minute ramp from 25 to 74°C and 2

minute extension at 74°C. The preamplification step was followed by 30 cycles of PCR in degenerate conditions. The reaction was carried out in a 30μl reaction volume containing 10x low salt buffer, 2mM each dATP, dCTP and dGTP, 0.5mM dTTP, 3nmol 12-dUTP FITC (Amersham Pharmacia Biotech), 100μM UN1 primer, 2.5U *Taq*-polymerase and 5μl preamplification product.

Method (4) was a personal communication from James, University of Manchester, UK (1998) and was a two step reaction incorporating a labelling step. The first step 50μl reaction mixture consisted of 10x PCR buffer, 0.5μM each dNTP, 13.5ng UN1 primer and 50ng template DNA. An initial denaturation step of 5 min at 96°C was followed by the addition of 2.5U *Taq*-polymerase and nine cycles of denaturation at 92°C for 1min, annealing at 30°C for 1 min, 30-72°C ramp for 3 min and extension of 72°C for 3 min. This was followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 3 min.
The labelling reaction was carried out in a final volume of 100μl containing 4μl of product from the first round PCR, 10x PCR buffer, 0.5μM dATP, dCTP and dGTP, 0.25μM dTTP, 13.5ng UN1 primer and 1.5mM SpectrumGreen dUTP. After an initial denaturation step of 5 min at 96°C the samples were cooled to 92°C for 5 min and 2.5U of *Taq*-polymerase added. This was followed by 40 cycles of denaturation at 92°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 3 min.

Method (5) was taken from (Huang, Schantz et al, 2000) and consisted of two steps with the option to incorporate a fluorescent label in the second step. The first step reaction was carried out in a final volume of 10μl containing ThermoSequenase buffer, 2mM each dNTP, 1μm UN1 primer, 4U Thermosequenase and 50ng template DNA. An initial denaturation step of 95°C for 3 min was followed by 4 cycles of 1 min at 94°C, 1 min at 25°C, 3 min transition at 25-74°C, 2 min extension at 74°C and a final extension at 74°C for 10 min.

The second reaction was carried out in a final volume of 50μl containing 10μl step 1 product, 10x PCR buffer, 2mM each dATP, dCTP and dGTP, 0.5mM dTTP, 1.5mM SpectrumGreen dUTP, 1.2μm UN1 primer and 5U *Taq*-polymerase. An initial denaturation step of 95°C for 3 min was followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, 2 min extension at 72°C and a final extension of 10 min.

Method (6) was a personal communication from Feuerstein, University of California, San Francisco, USA (2000). This was a three step protocol that enabled the incorporation of a fluorescent label in the third step. The first step used the Thermosequenase enzyme and was carried out in a 5μl reaction volume. The reaction mix consisted of 300μm each dNTP, 1μm UN1 primer, 5x Sequenase reaction buffer and 50ng template DNA. An initial denaturation step of 96°C for 10 min was followed by five cycles of 30°C for 5 min, 37°C for 2 min and 96°C for 2 min. The reaction was paused at the start of the 30°C incubation in each cycle for the addition of 1.6U Thermosequenase. After the low stringency reaction 200μm each dNTP, 1.4μm UN1 primer, 2.5U *Taq*-polymerase, 10x PCR buffer and 14.9μl dH_2O were added to the first step product. The amplification consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 2 min with auto-extension of 1 sec/cycle. A final extension of 72°C for 10 min completed this reaction step. A 5μl aliquot of each reaction product was run on a 1.5% TAE agarose gel for 1 hour at 100v to check the efficiency off the reaction. For the labelling reaction, 100μm of each dNTP, 1.4μm UN1 primer, 1.5mM $MgCl_2$, 5x Sequenase reaction buffer, 1.5U *Taq*-polymerase, 1.5nmol 12-dUTP FITC and 10.4μl dH_2O were added to 5μl of step 2 reaction product to give a total reaction volume of 50μl. The labelling reaction consisted of an initial denaturation of 95°C for 10 min, followed by 25 cycles of denaturation at 94°C for 1 min 10 sec, annealing at 56°C for 1 min 10 sec, extension at 72°C for 3 min with auto-extension of 1 sec/cycle and a final extension step of 72°C for 10 min completed the reaction.

In order to test the efficiency of the DOP-PCR protocols, DNA from samples that had been analysed previously by conventional CGH were used as controls. IN859 and IN1265 had been analysed by Andrew Ellsmore (Institute of Neurology), IN2809 and IN2675 had been analysed by Dr Tracy Warr (Institute of Neurology) and IN2376 is included in the analysis of ependymoma in this study. The protocol that resulted in similar CGH profiles being produced for these samples as were obtained from conventional CGH were then used to amplify and label tumour samples.

Universal linkage system

The second problem encountered in this study was that the DNA isolated from formalin-fixed, paraffin-embedded material was often too degraded to be labelled enzymatically by standard nick translation. The size of archival DNA after nick translation is often smaller than 300bp, which would produce a CGH with very low signal intensity. The universal linkage system (ULS) uses a platinum compound that has two free binding sites, one of which is used to bind to a marker group, such as biotin or digoxigenin. The other site is used to link the platinum/marker complex to the DNA molecule where it will bind preferentially to guanine groups. As there is no enzymatic action there should be no reduction in size of the DNA and a successful CGH should be achievable. The ULS kit and reagents were obtained from Kreatech Diagnostics, Amsterdam, The Netherlands.

Labelling of paraffin section derived DNA with d-Green ULS

500ng of tumour DNA was aliquoted into a 0.5ml eppendorf tube and the volume adjusted to 15μl with ULS labelling buffer before 5μl (0.5U) of d-Green ULS was added. The samples were incubated at 65°C for 15 minutes on a Techne Progene thermal cycler. The eppendorf was then centrifuged briefly at 13000rpm to collect the sample at the bottom of the tube and 200μl buffer PN (Qiagen) added to the tube before the DNA was purified according to the QIAquick nucleotide removal protocol.

Labelling of normal blood DNA with Rhodamine-ULS

In order for successful labelling with ULS, the DNA fragments must be smaller than 2kb, preferably between 500 and 1000bp. Genomic DNA from normal blood has a much larger fragment size (> 8Kb). A 300μl sample of normal blood DNA was sonicated for 3 x 20 seconds. A 10μl sample was electrophoresed at 100v for 1 hour on a 1% TAE agarose gel containing 5μl 10mg/ml ethidium bromide to check the size range and if it was in the range of 500 – 1500bp it was used. 1μg of DNA was pipetted into a 0.5ml eppendorf and 1μl 5mg/ml RNase (Sigma Aldrich) was added for each 5μl of sample. The sample was then incubated in a water bath at 37°C for 1 hour in order to destroy any residual RNA in the sample.

500ng of DNA was pipetted into a 0.5ml eppendorf and the volume adjusted to 15μl with ULS labelling buffer before 5.0μl (0.5U) of Rhodamine ULS was added to the tube. The samples were labelled according to the protocol described for d-Green ULS.

QIAquick Nucleotide Removal Protocol.

This protocol is designed to remove primers <10 bases, enzymes, salts, and unincorporated nucleotides from labelled DNA.

After the addition of 10 volumes of buffer PN the sample was pipetted onto a QIAquick spin column placed on a 2ml collection tube and centrifuged for 1 minute at 6000rpm to bind the DNA. The flow through was discarded and the column placed back in the collection tube. The DNA was washed by the addition of 750μl PE buffer to the column and centrifugation for 1 minute at 6000rpm. To remove residual ethanol from the column it was centrifuged for 1 minute at 13000 rpm. The column was placed in a sterile 1.5ml eppendorf and the DNA eluted by the addition of 100μl buffer EB and centrifugation for 1 min at 13000 rpm.

CGH of ULS Labelled DNA

The hybridisation mix consisted of 500ng d-Green labelled tumour DNA, 500ng Rhodamine labelled reference DNA and 20μg human Cot-1 DNA in

a 0.5ml eppendorf tube. The DNA was precipitated and denatured as previously described.

Statistical Analysis

Fishers exact test (Rees, 1994), using two-way contingency tables was used to compare the incidence of chromosomal gains and losses between different histologic and clinical subgroups of tumours. Associations between different CNAs were analysed using Fishers exact test.

The software package GraphPad Prism® (GraphPad Software Inc, San Diego, CA) was used to produce Kaplin-Meiyer survival curves in order to analyse survival rates amongst the different tumour groups.

The Mantel-Haenszel test (Kirkwood, 1998) was used to identify large groups of CNAs that occurred together.

A p value of <0.05 was considered to be statistically significant at the 95% confidence level.

CHAPTER 3
RESULTS OF ALTERNATIVE LABELLING METHODS

A number of the samples available for analysis in this study had DNA concentrations that were too low to enable the sample to be labelled using the conventional nick translation method. In order to use this material for CGH analysis the DNA had to be labelled using non-enzymatic methods. Several different protocols have been published utilising the degenerate oligonucleotide primed PCR method. A number of these protocols were evaluated in this study in order to identify a method that produced DNA fragments of the optimum size for efficient labelling and subsequent CGH analysis. There were also a number of tumour samples that had been fixed in formalin and then embedded in paraffin. The DNA from these samples is too degraded to be labelled by nick translation. The Universal linkage system provides a means of labelling small DNA fragments non-enzymatically and this method was analysed to determine its suitability for the labelling of degraded DNA samples.

DOP-PCR Methods

Amplification of normal DNA

Six different methods were used to amplify genomic DNA. In the first instance DNA from pooled normal reference DNA was used to optimise each method before any tumour DNA was used. Concentrations ranging from 1ng, 10ng, 50ng and 100ng were used as a representation of the low concentrations of DNA available in some of the tumour samples. Two methods, Cheung (1996) and Kuukasjarvi et al. (1997) did not successfully amplify normal DNA and a decision was made not to continue the evaluation of these methods. The methods of Speicher et al. (1993), James (personal communication), Feuerstein (personal communication) and Huang et al. (2000) were all successful in the amplification of normal DNA. A comparison of the fragment sizes produced can be seen in Figure 3.1. and Table 3.1.

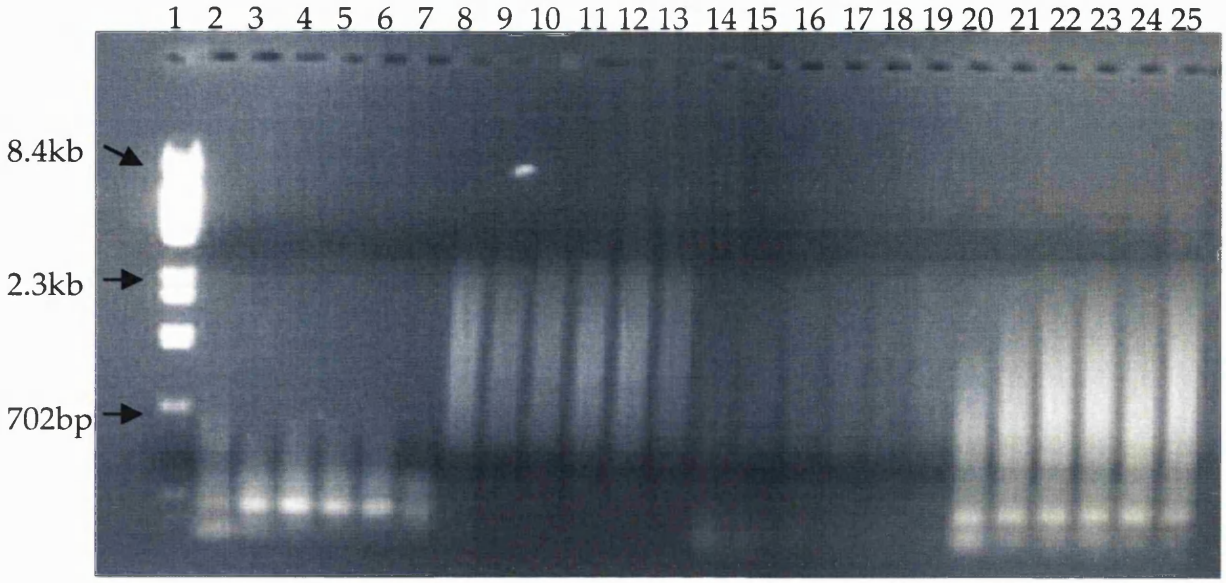


Figure 3.1: DOP-PCR amplification of normal blood DNA.
For each method analysed 1ng, 10ng, 25ng, 50ng, 75ng and 100ng starting DNA were used. Lane 1 is Lambda DNA/*Eco*911 marker; Lanes 2 – 7 DNA amplified using Feuerstein's method; Lanes 8 – 13 DNA amplified using James' method; Lanes 14 -19 DNA amplified using Huang's method; Lanes 20-25 DNA amplified using Speicher's method.

	Feuerstein	James	Huang	Speicher
1ng	<702bp	<702bp – 3.6Kb	<702bp – 2.3Kb	<702bp – 1.2Kb
10ng	<702bp	<702bp – 3.6Kb	<702bp – 2.3Kb	<702bp – 1.3Kb
25ng	<702bp	<702bp – 3.6Kb	<702bp – 3.6Kb	<702bp – 1.9Kb
50ng	<702bp	<702bp – 4.3Kb	<702bp – 6.3Kb	<702bp – 2.3Kb
75ng	<702bp	<702bp – 8.4Kb	<702bp – 6.3Kb	<702bp – 8.4Kb
100ng	<702bp	<702bp – 8.4Kb	<702bp – 8.4Kb	<702bp – 8.4Kb

Table 3.1: Comparison of fragment sized produced by four DOP-PCR methods. The amount of starting DNA is given in the left-hand column.

Feuerstein's method resulted in the smallest fragments of DNA being produced. Regardless of the amount of starting DNA used, the fragments produced were always less than 702bp in size. With all the other methods the fragment size increased when the amount of starting DNA was increased. The methods of

James and Huang both resulted in a large volume of DNA in the 702bp – 2.3Kb size range being produced. This was seen with all starting concentrations using Huang's method, but only with starting concentrations of 50 – 100ng using James' method. The DNA in this size range is optimal for use in CGH. It was decided to use 50ng of DNA in all subsequent DOP-PCR experiments, as this was a fair representation of the samples available for analysis and produced DOP-PCR fragments within the size range required for CGH.

Amplification of tumour DNA

Tumour samples that had been analysed previously by Tracy Warr and Andrew Ellsmore using standard CGH protocols were used as controls to determine the sensitivity of each DOP-PCR protocol for amplifying tumour DNA. Tumours IN2809 and IN2675, were used as controls in experiments using James', Feuerstein's and Speicher's protocols, and IN859, IN1265 and IN2376 were used as controls in experiments using Huang's protocol. A summary of the aberrations found in these tumours can be seen in Table 3.2. Each protocol resulted in the successful amplification of each of the control DNA samples (data not shown).

IN	Source	Amplicons	Gains	Losses
859	CC		1p 7p 20p	14 16 17p 22q
1265	CC		3q 6q 7p 9p	4
2376	FF		1q 2 7q 9p 11p 13	12q 16q
2675	FF		2q 4q 5q 6q 12q 13q 18q	16p 17 20 22q
2809	FF	1p 7q 12p	1p 2p 2q 7 8q 12p X	15 22

Table 3.2: Summary of copy number aberrations in the tumours used as controls for DOP-PCR experiments. CC = short term cell culture, FF = fresh frozen biopsy material

Labelling of normal DNA for use in CGH experiments

After the successful optimisation of the DOP-PCR for amplification of normal DNA, a labelling step was incorporated into the reaction in order to assess the

effects of fluorochrome incorporation on the DNA fragment size. In each instance 50ng DNA was amplified and 10mM SpectrumGreen dUTP incorporated into the DNA. Normal DNA was labelled using Feuerstein's, James' and Huang's methods. DNA amplified using Speicher's method was labelled using the labelling steps from the other three methods. In each instance an identical reaction was performed with no fluorochrome added. Figure 3.2 shows the fragment sizes produced using each method, and Table 3.3 gives a summary of the fragment sizes produced using each method.

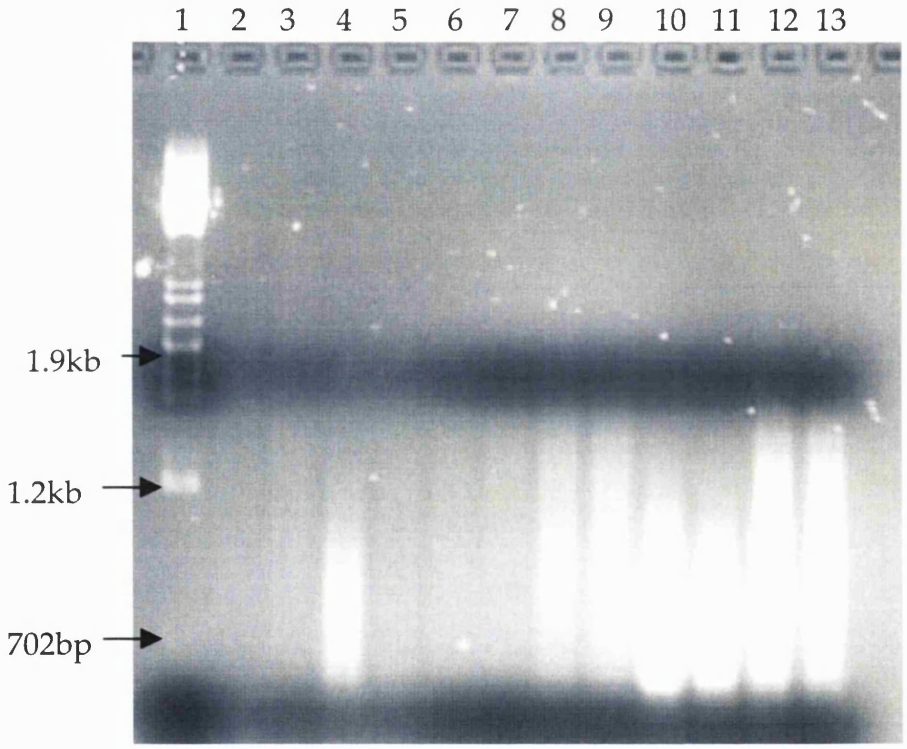


Figure 3.2: Comparison of product sizes from different DOP-PCR labelling methods. Lane 1 shows the Lambda DNA/*Eco*91I marker. Lanes 2 & 3 show DNA amplified and labelled using James' method. Lanes 4 & 5 show DNA amplified by Speicher's method and labelled using James' method. Lanes 6 & 7 show DNA amplified and labelled using Huang's method. Lanes 8 & 9 show DNA amplified using Speicher's method and labelled using Huang's method. Lanes 10 & 11 show DNA amplified and labelled using Feuerstein's method and Lanes 12 & 13 show DNA amplified by Speicher's method and labelled using Feuerstein's method.

	Labelling method		
Amplification method	James	Huang	Feuerstein
James	702bp – 1.9Kb		
Huang		702bp – 1.3 Kb	
Feuerstein			702bp – 1.1Kb
Speicher	702bp – 1.3 Kb	702bp – 1.3 Kb	702bp – 1.9kb

Table 3.3: Summary of fragment sizes produced during DOP-PCR labelling of 50ng normal DNA. Speicher's protocol did not include a labelling step therefore DNA amplified by this method was labelled using the other three methods.

The labelling step resulted in reduction in DNA fragment size to between 702bp and 1.9Kb. Fragments of this size are suitable for use in CGH experiments. The addition of the fluorochrome resulted in fragments of equal size to the reactions where no fluorochrome had been added.

CGH experiments

In the first experiments using SpectrumGreen DOP-PCR labelled tumour DNA, the DNA was co-hybridised with normal reference DNA that had been labelled with SpectrumRed dUTP by the nick translation method. The resulting CGH profiles were not always smooth and it was decided to label the normal DNA in the same manner as the tumour DNA. These "homo-hybridisations" resulted in smoother CGH profiles. Figures 3.3 – 3.10 show examples of the CGH profiles produced from the various DOP-PCR protocols. After evaluating these methods, the method described by Huang et al. (2000) was used for the amplification and labelling of tumour DNA for use in CGH experiments as this method resulted in the smoothest profiles and produced profiles consistent with those obtained by conventional CGH methods.

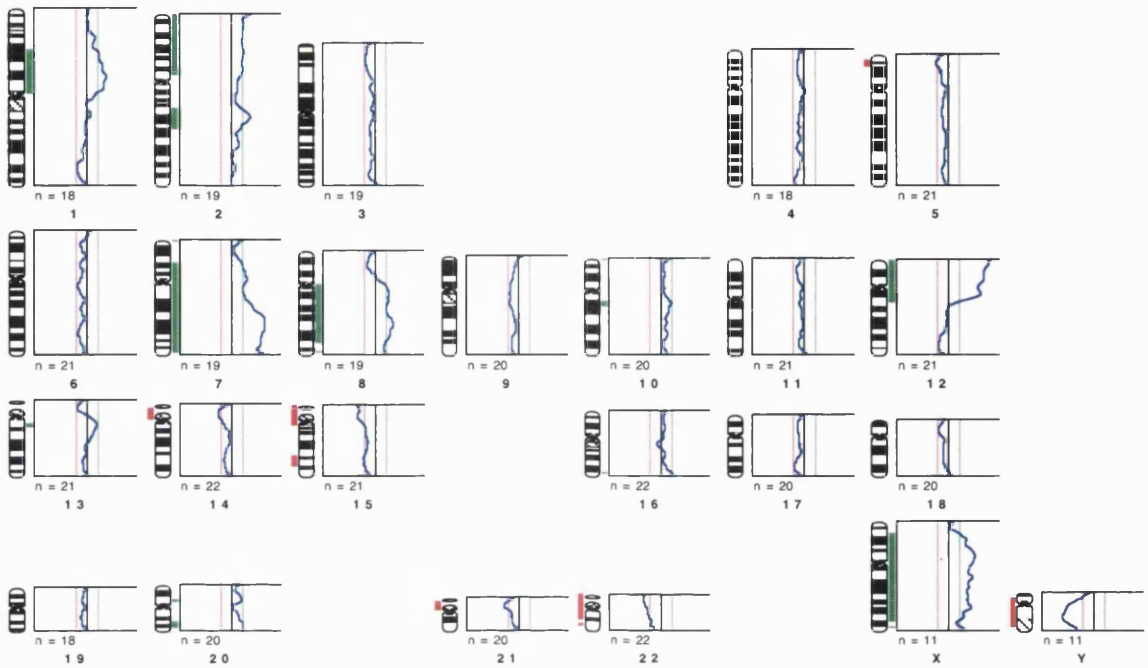


Figure 3.3a: IN2809 labelled by nick translation. There are nine CNAs. There are two regions of high copy number amplification on 7q and 12p, as well as regions of gain on 1p, 2p, 7q, 8q and X. There are regions of loss on chromosome 15 and 22.

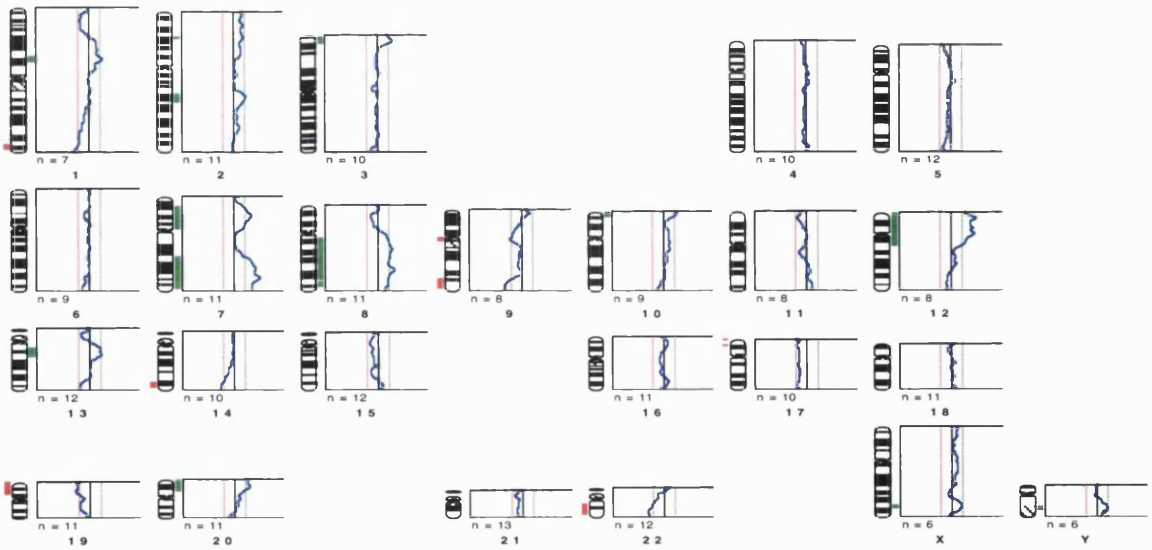


Figure 3.3b: IN2809 amplified and labelled using Feuerstein's method. This composite shows four CNAs. The regions of high copy number amplification on 7q and 12p can be observed as well as the region of gain on 8q and region of loss on 22q. The regions of gain on1p and 2p are much smaller than seen with nick translation

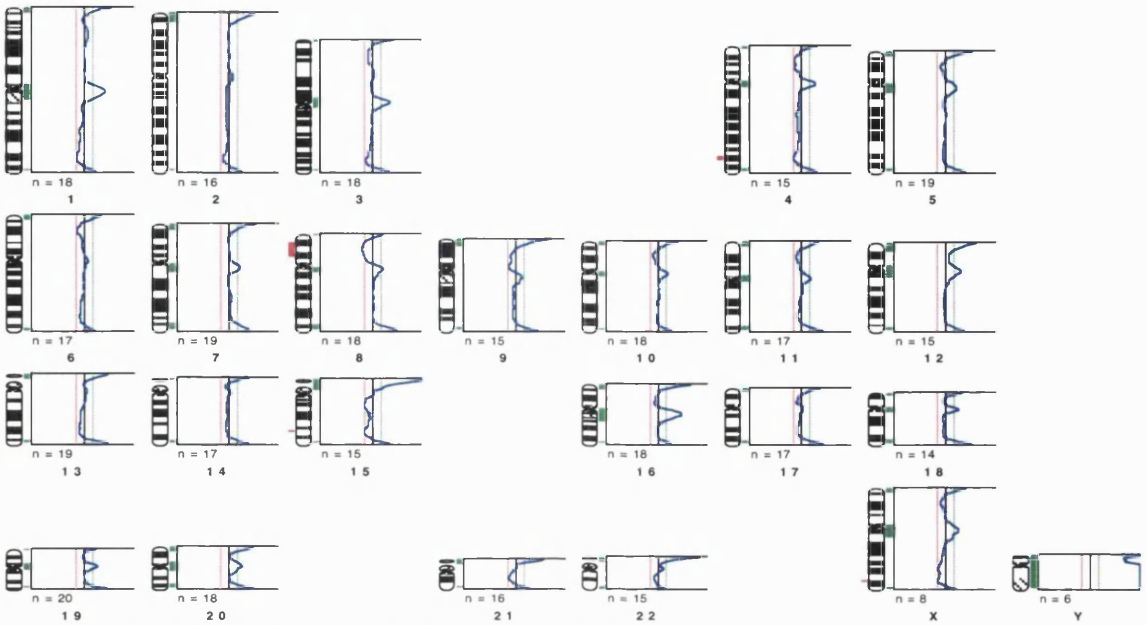


Figure 3.3c: IN2809 amplified and labelled using James' method. This method did not identify any of the alterations observed using nick translation or Feuersteins method.

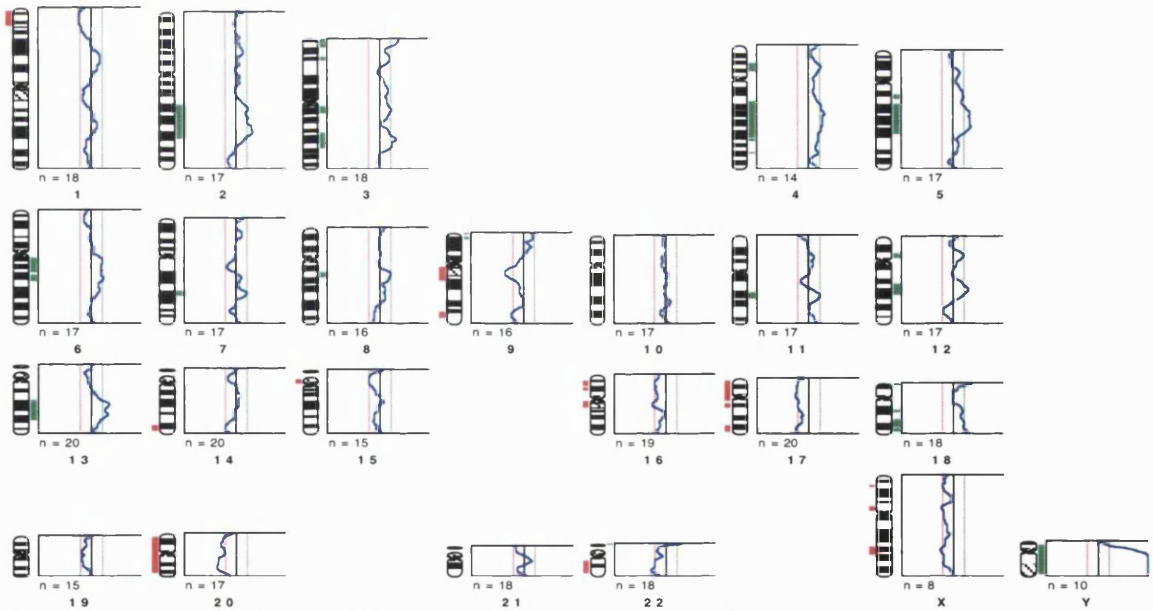


Figure 3.4a: IN2675 labelled by nick translation. This tumour has eleven CNAs. There are gains on chromosomes 2q, 4q, 5q, 6q, 12q, 13q and 18q, and regions of loss on chromosomes 16p, 17, 20 and 22q.

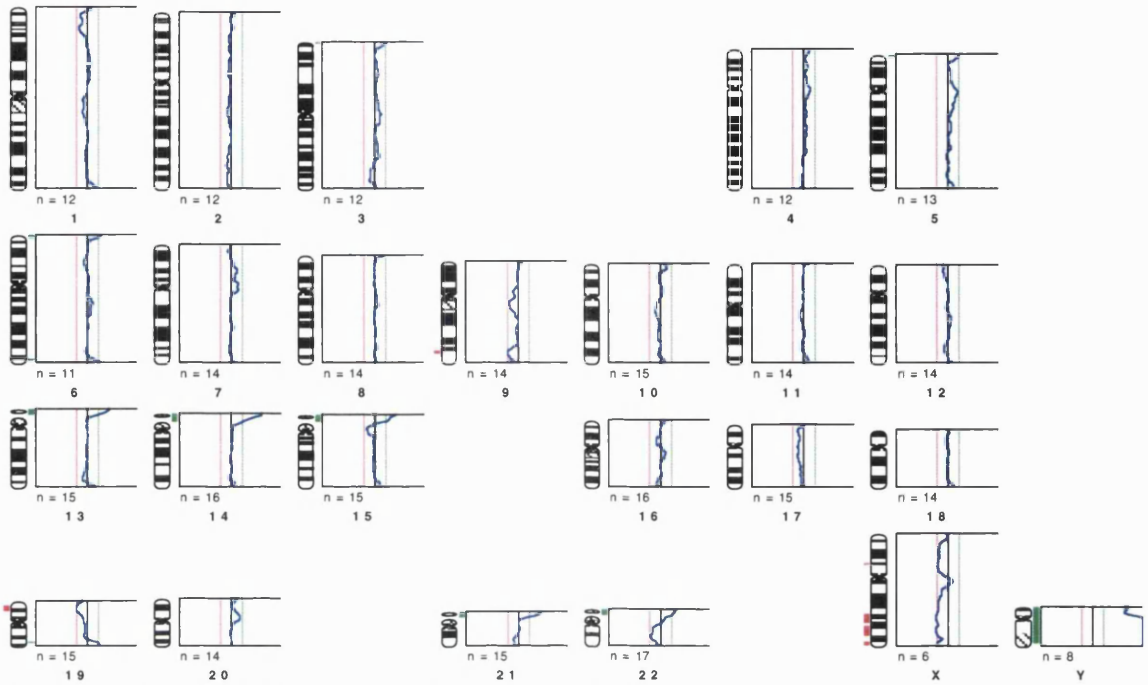


Figure 3.4b: IN2675 amplified and labelled using Feuerstein's technique. The composite shows none of the alterations seen with nick translation.

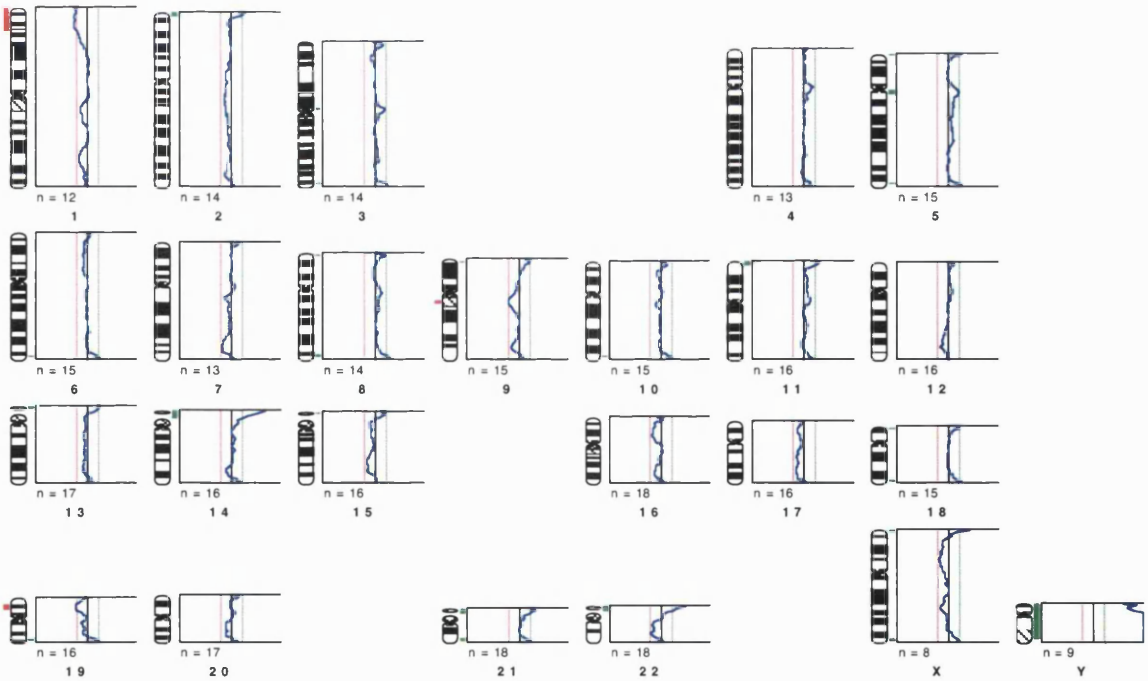


Figure 3.4c: IN2675 amplified and labelled using James' technique. The composite shows none of the alterations seen with nick translation.

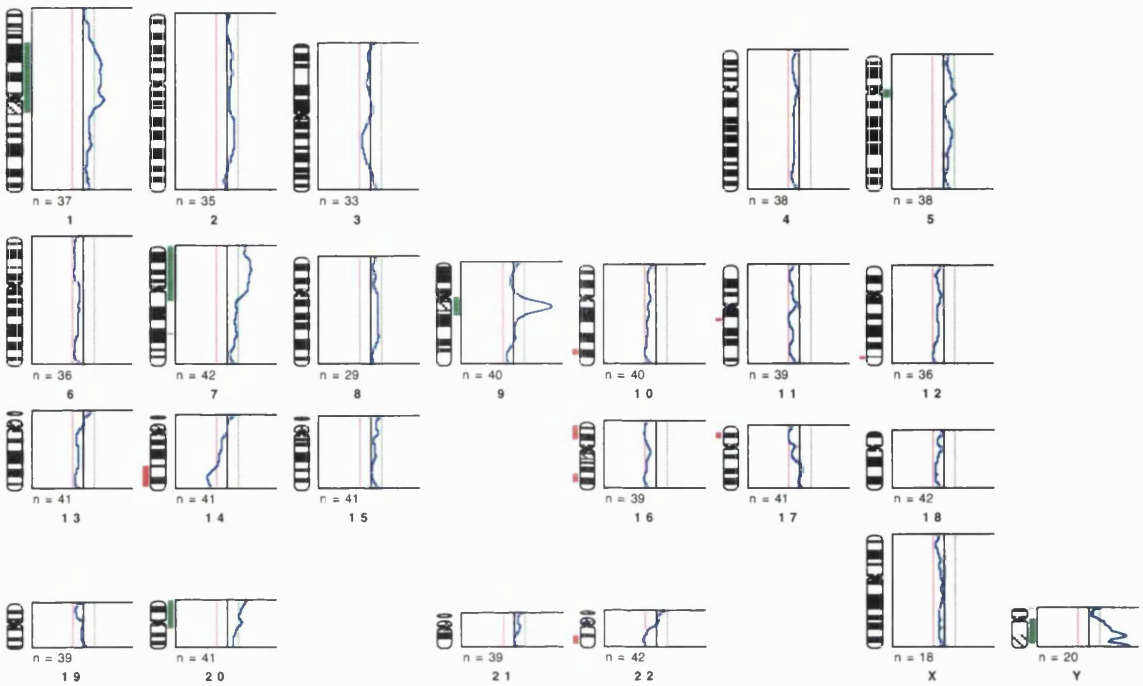


Figure 3.5a: IN859 labelled by nick translation. This tumour has seven CNAs. There are gains on 1p, 7p and 20p and regions of loss on chromosomes 14, 16, 17p and 22q.

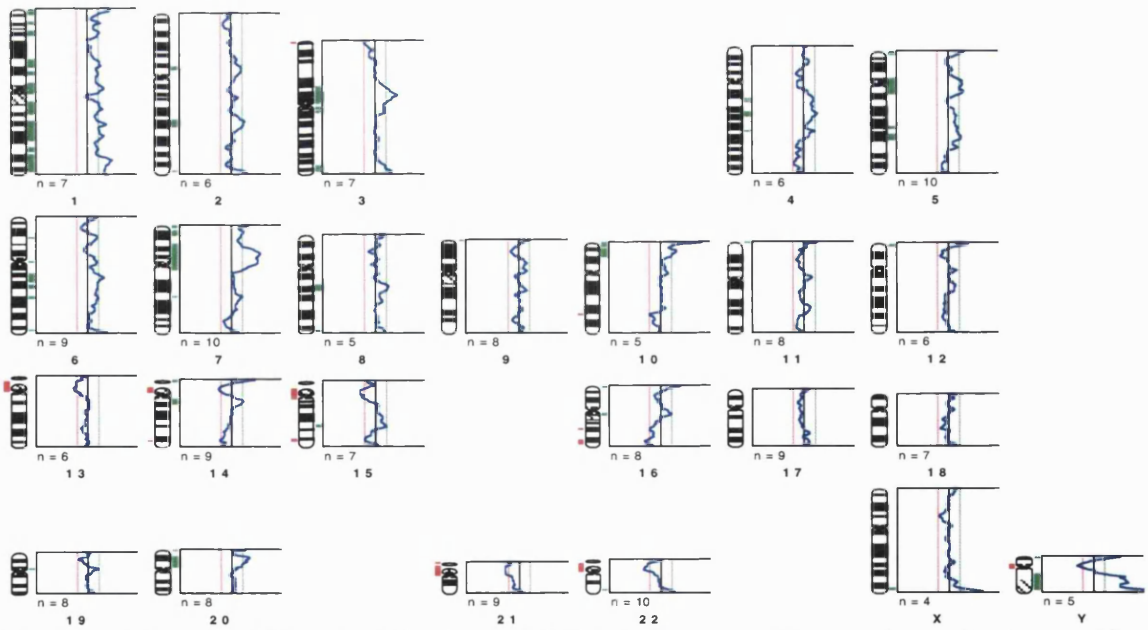


Figure 3.5b: IN859 amplified and labelled using Huang's technique. The composite shows three CNAs, which are gain of chromosome 1, gain of 7p and gain 20p. The regions of loss were not seen in this composite.

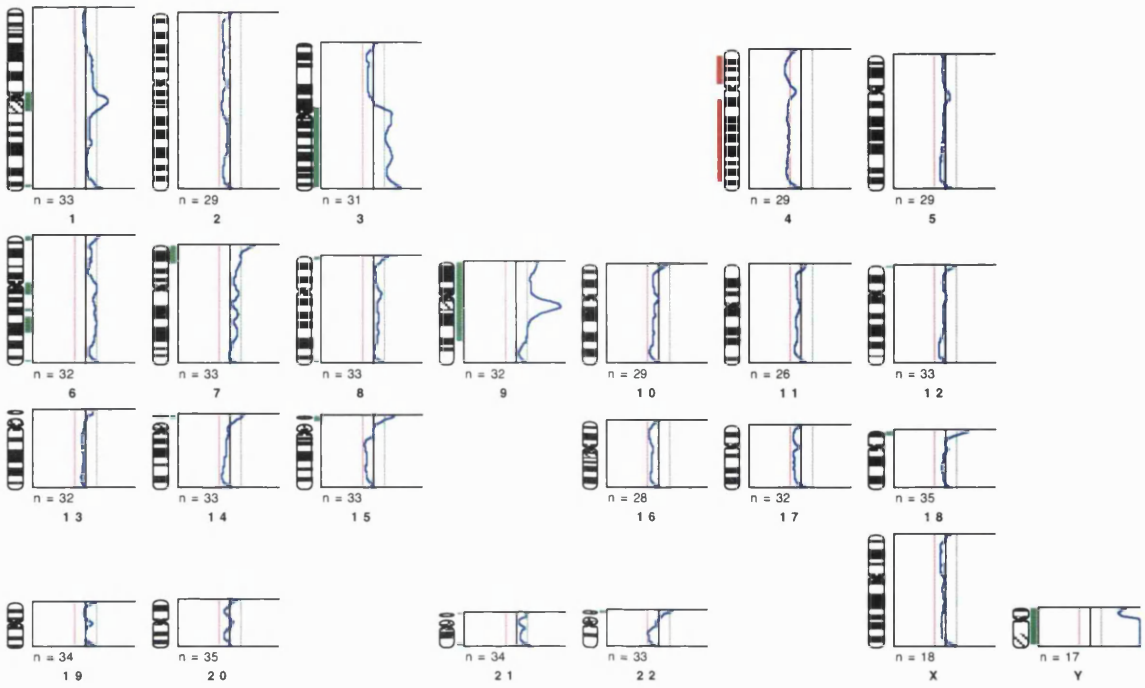


Figure 3.6a: IN1265 labelled by nick translation. This tumour has five CNAs. There are regions of gain on chromosomes 3q, 6q, 7p and 9p and monosomy of chromosome 4.

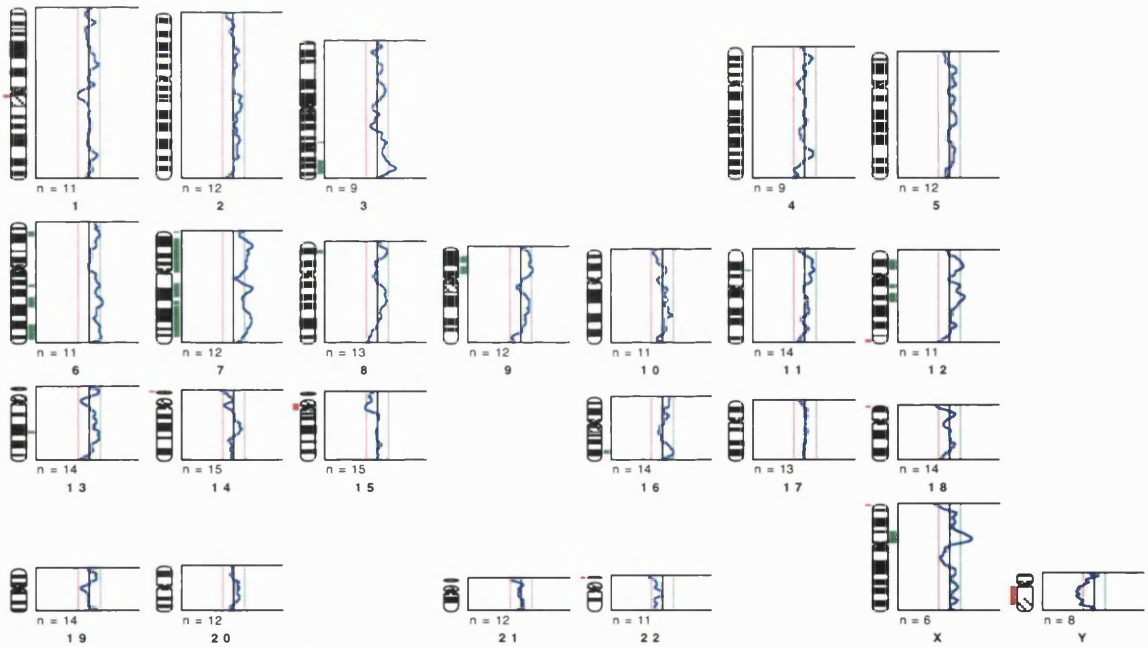


Figure 3.6b: IN1265 amplified and labelled using Huang's technique. The composite shows regions of gain on chromosome 6q, 7 and 9p. The alterations on chromosomes 3 and 4 could not be detected.

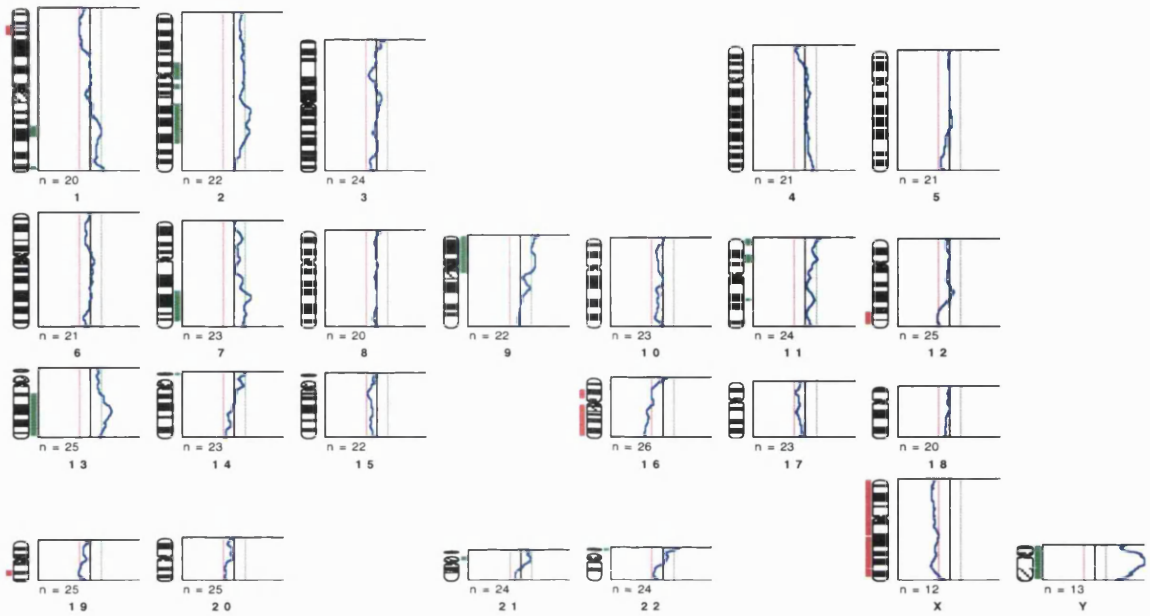


Figure 3.7a: IN2376 labelled by nick translation. This tumour has seven CNAs. There are regions of gain on 2q, 7q, 9p, 11p and 13q, a region of loss on 12q and monosomy of chromosome 16.

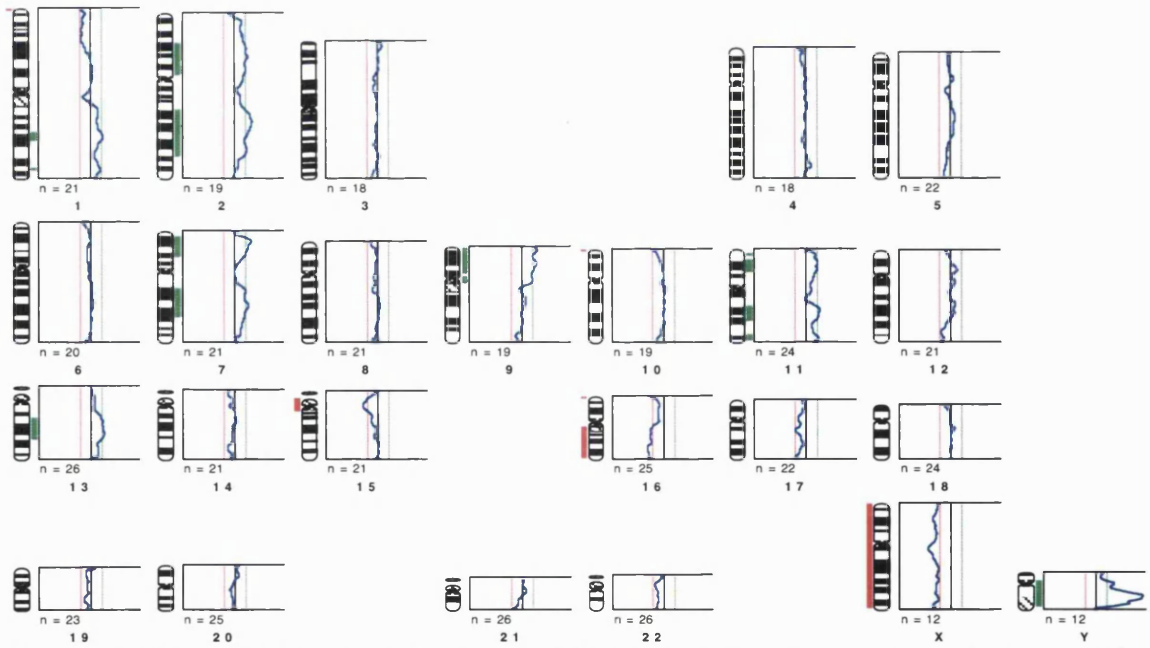


Figure 3.7b: IN2376 amplified and labelled using Huang's technique. The composite shows six CNAs. The regions of gain on chromosomes 2, 7, 9p, 11 and 13q as well as the loss from 16q can be observed, but not the region of loss on 12q or loss from 16p.

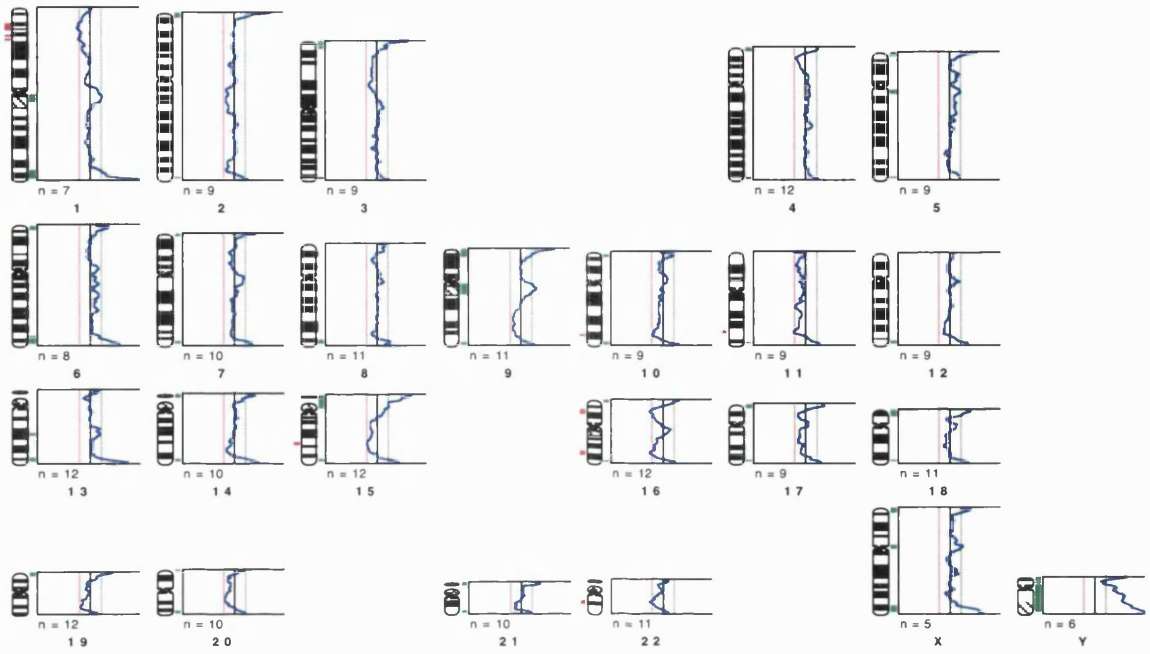


Figure 3.8: IN1265 amplified by Speicher's method and labelled by Feuerstein's method. The profiles are relatively smooth but none of the five CNAs seen in DNA labelled by nick translation (Figure 3.6a) can be seen.

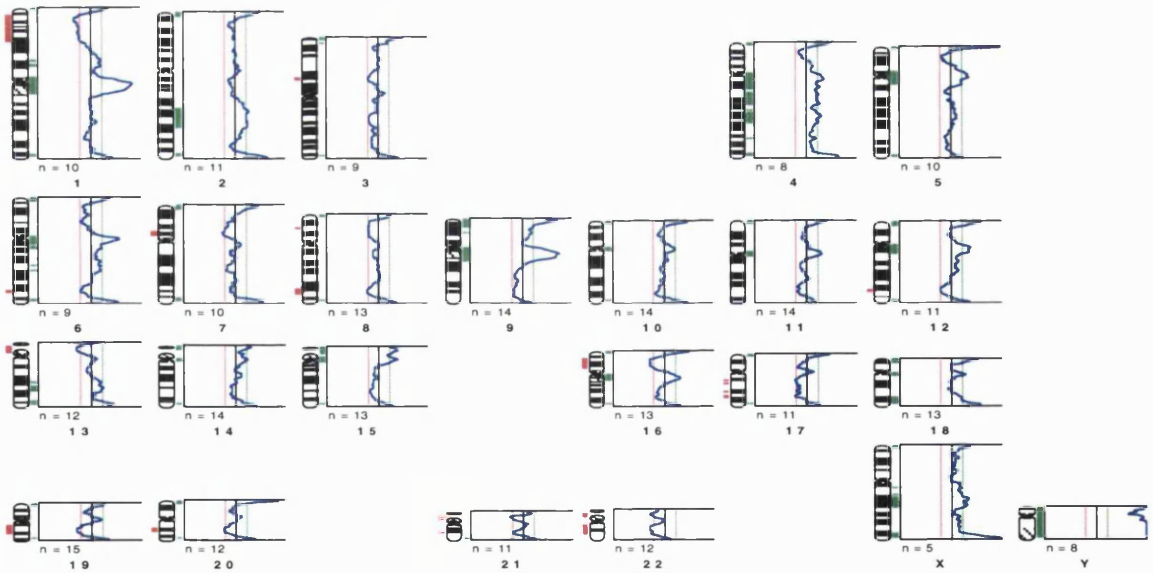


Figure 3.9: IN2376 amplified by Speicher's method and labelled by Feuerstein's method. Some of the CNAs observed in experiments with nick translated DNA (Figure 3.7a) can be seen in this composite. These include gain 2q, gain 9p, loss 16p, loss 17q and loss 22. However this method did not prove sensitive enough to correctly detect the patient's sex - as the patient is in fact male the X chromosome should show loss, i.e. only 1 copy.

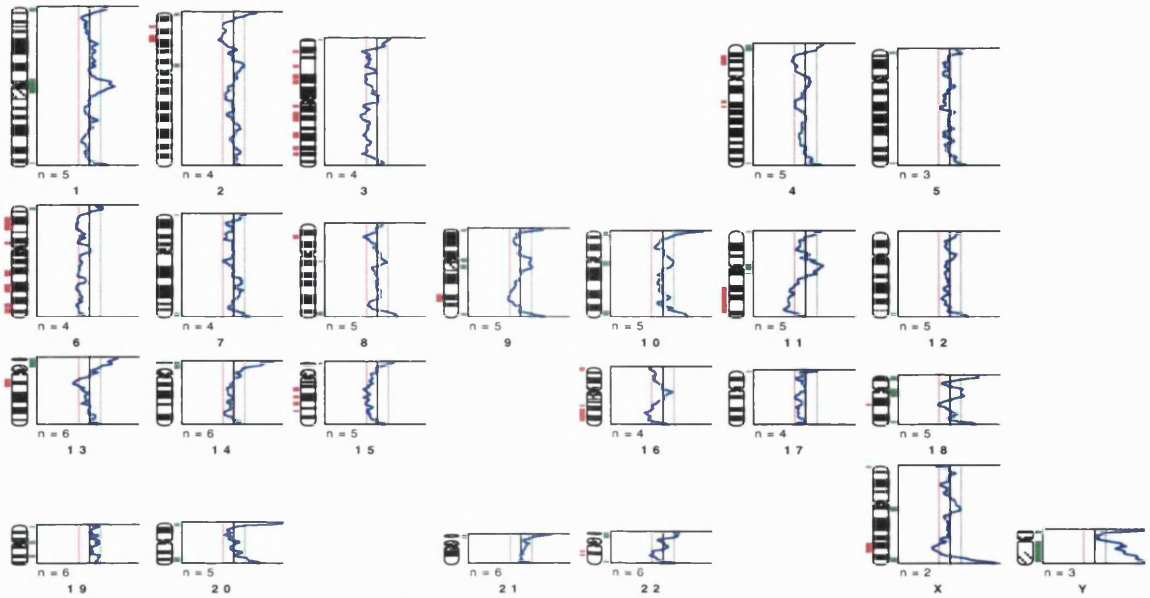


Figure 3.10: IN859 amplified by Speicher's method and labelled using Feuerstein's method. The profiles are not very smooth. None of the alterations seen in nick translated experiments (Figure 3.5a) can be seen in this composite. There appear to be regions of loss on chromosomes 3, 6 and 11 that were not seen in previous experiments, but this are probably CGH artefact.

Paraffin DNA & Kreatech Universal Linkage System (ULS)

DOP-PCR amplification of DNA extracted from paraffin embedded material often resulted in DNA that was too small to be labelled using nick translation or PCR labelling methods. The Kreatech universal linkage system offered a non-enzymatic alternative. DNA from normal pooled blood and IN2809 were used as controls to optimise the technique. The DNA samples were sonicated for 3 x 20 seconds before being labelled with d-Green. The labelled DNA was initially co-hybridised with SpectrumRed-labelled normal reference DNA to normal metaphase slides. As "homo-hybridisations" had produced better results in the DOP-PCR experiments it was decided to co-hybridise d-Green ULS-labelled tumour with rhodamine ULS-labelled normal DNA. Despite a number of attempts a successful hybridisation could not be produced. After much discussion with the technical department at Kreatech a problem with the reagents was discovered, which resulted in new packaging being produced for the ULS reagents. The problem arose because plasticisers were being leeched from the plastic used to manufacture the lids of the tubes the reagents were

packaged in, and contaminating the labelling reagents. This led to inhibition of the labelling step. Even after new reagents had been provided successful results could not be obtained. IN859 & IN1265 were also used to evaluate the method, but the hybridisation nearly always resulted in "normal" CGH profiles being obtained (see Figures 3.11 and 3.12).

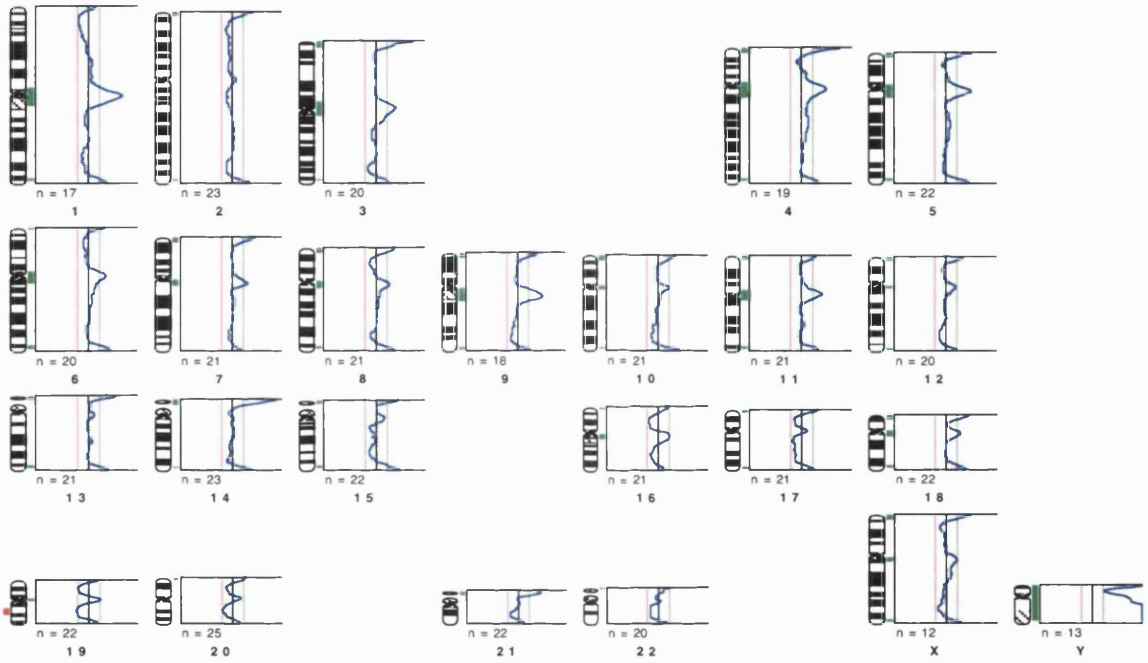


Figure 3.11: IN859 sonicated and labelled with d-Green ULS. The red reference DNA was normal pooled blood DNA sonicated and labelled with rhodamine ULS. This method did not show any of the CNAs seen with the nick translation technique.

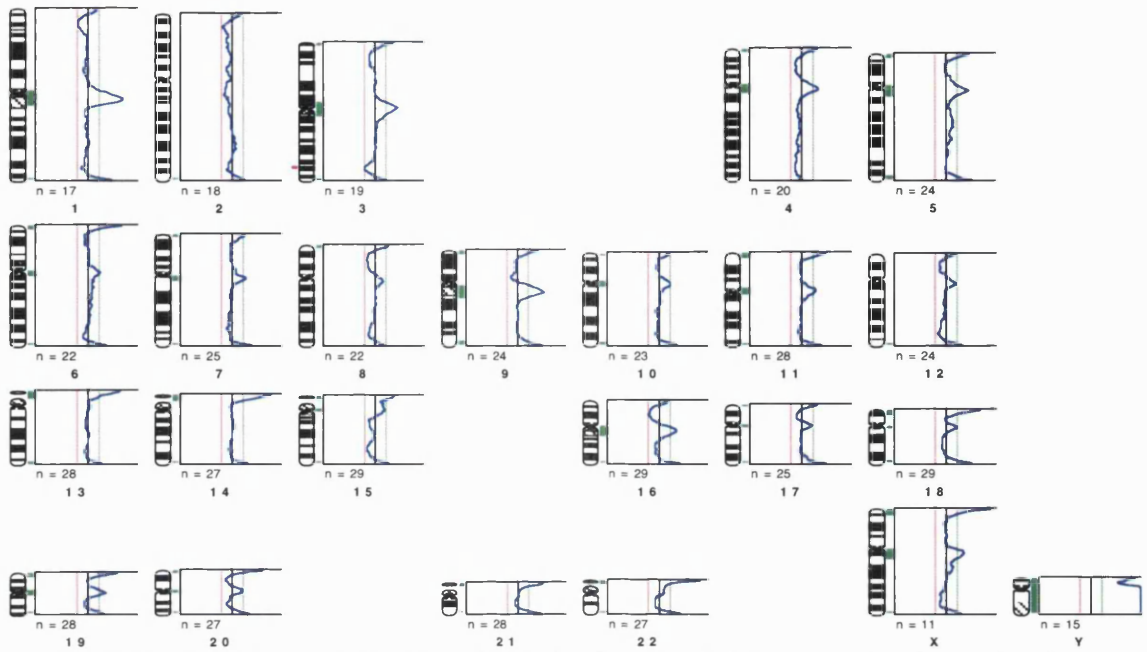


Figure 3.12: IN1265 sonicated and labelled with d-Green ULS. The red reference DNA was normal pooled blood DNA sonicated and labelled with rhodamine ULS. This method did not show any of the CNAs seen with the nick translation technique.

After personal communications with Judith Jeuken in Nijmingen, Holland it was felt the reaction could be inhibited by contaminating RNA in the sample or by DNA cross links. A new extraction method was used which is based on a salting out procedure and incorporated sodium thiocynate treatment to destroy cross links and RNase treatment to destroy residual RNA. This technique resulted in DNA that was large enough to be nick translated. DNA that had been extracted by earlier methods was treated with RNase before being labelled with ULS as before.

U38 was pre-treated with RNase before being labelled with d-Green ULS and co-hybridised with rhodamine ULS -labelled normal DNA. The resulting profile can be seen in Figure 3.13a. There appeared to be a lot of CGH artefact and no real chromosome alterations. Though the profiles appeared to be quite smooth all regions of "alteration" showed loss and no regions of gain. The regions of loss were present at chromosomal positions where previous nick translation

experiments of other ependymoma had shown gain or amplification, e.g. 1q, 4 and 7q. This patient is also female and the X chromosome is approaching the 0.8 threshold (indicating loss) rather than being on the 1.0 threshold which is what would be expected for a sample from a female patient. A CGH experiment using U38 DNA labelled by nick translation resulted in a CGH profile with no aberrations (see Figure 3.13b)

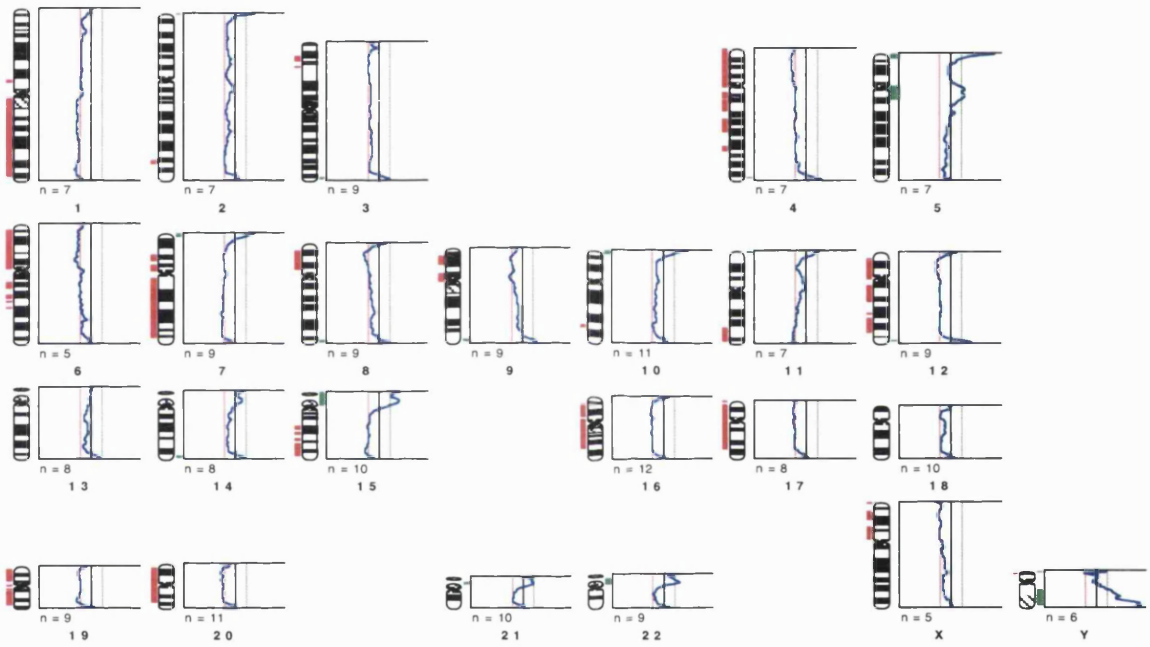


Figure 3.13a: U38 labelled with d-Green ULS and co-hybridised with rhodamine ULS labelled normal pooled blood DNA. The profiles show what appears to be a large amount of genomic loss from chromosomes 1, 4, 6, 7, 12, 15, 16, 17, 19 and 20. However these alterations were not seen in DNA that had been labelled by the nick translation technique and were taken to be artefacts of the hybridisation.

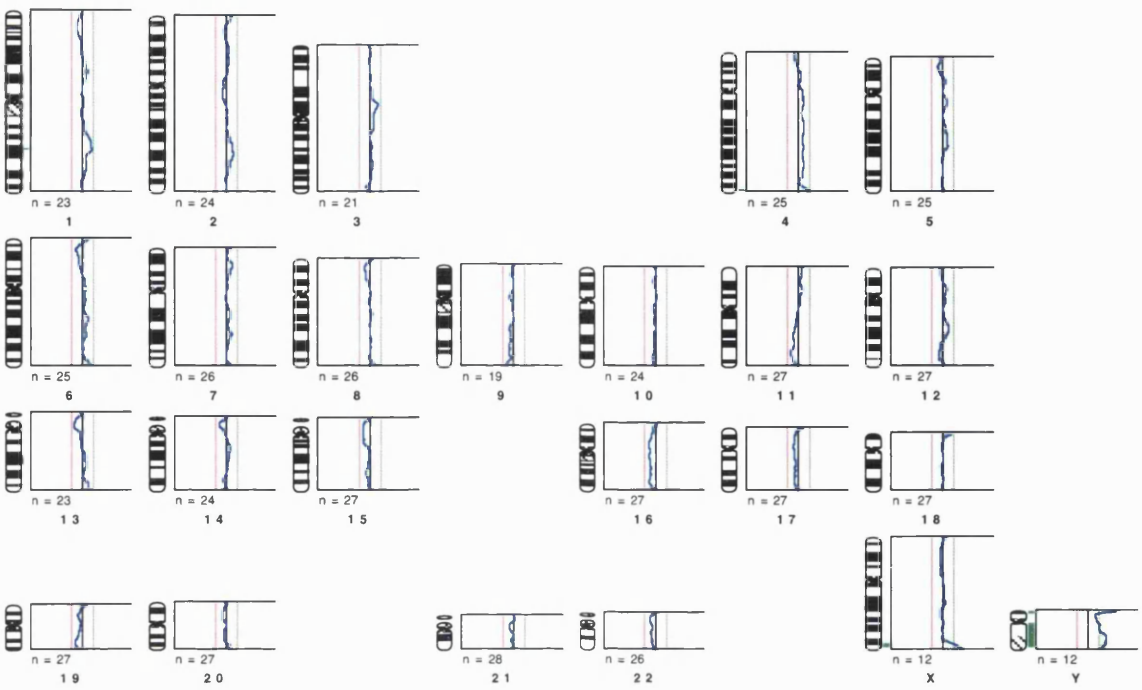


Figure 3.13b: U38 labelled by nick translation and co-hybridised with nick translated normal reference DNA. The profile shows a normal karyotype. The X chromosome profile is centred along the 1.0 threshold line indicating the patient is female.

AMH97/590 DNA was extracted from paraffin embedded sections using Jeuken's method. The DNA was considered too large to label using the ULS procedure and was labelled by nick translation. The resulting profile can be seen in Figure 3.14. No CNAs could be seen in this tumour but as the X chromosome was consistent with the patient's sex (male) this was taken to be a true representative composite for this tumour.

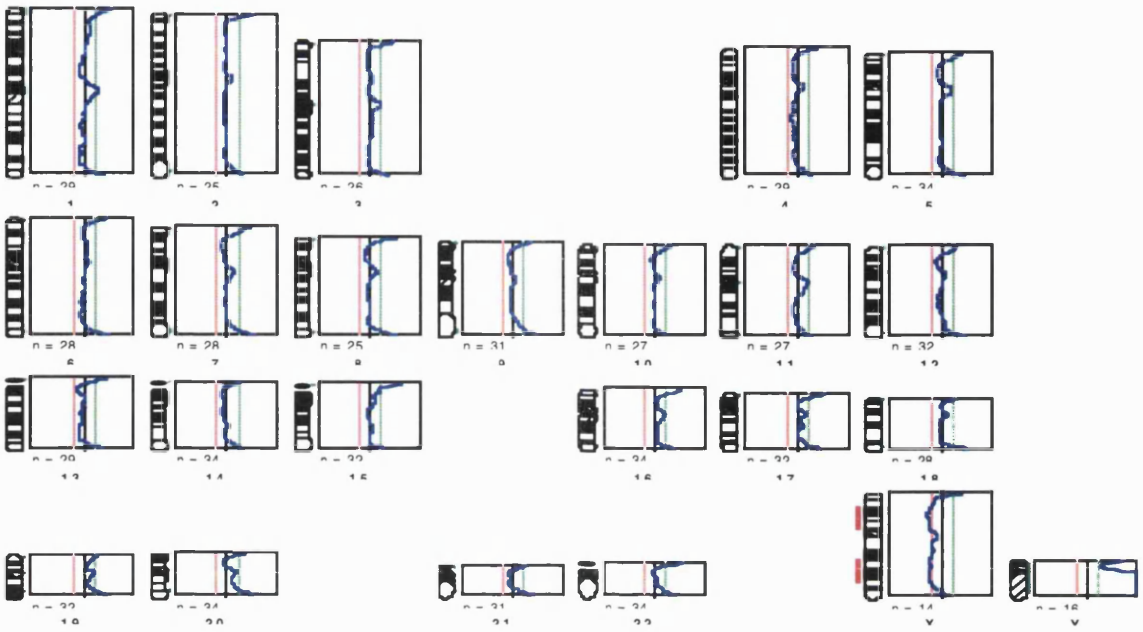


Figure 3.14: AMH97/590 labelled with SpectrumGreen dUTP by nick translation and co-hybridised with SpectrumRed labelled normal DNA. There are no CNAs but the X chromosome is true to the patient's sex so this composite was taken to be a true representation of the tumour.

DISCUSSION

The standard protocol for CGH requires 1-2μg of DNA from each tumour sample. In some cases in this study smaller amounts of DNA were available for analysis. This is a common problem in laboratories around the world and being able to produce non-specific amplification of the whole genome from a small amount of starting material is of great value. The degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) was developed by Telenius et al. in 1992, and is an efficient and reliable means of amplifying the whole genome. DOP-PCR-CGH analysis of tumour DNA has been reported by a number of investigators (Aubele, Mattis et al, 1999; Griffin, Sanoudou et al, 1998; Huang, Schantz et al, 2000; Speicher, du Manoir et al, 1993; Speicher, Howe et al, 1995; Zitzelsberger, Kulka et al, 1998).

This study evaluated the reliability of six different DOP-PCR protocols to amplify tumour DNA to use for CGH. Two of the protocols (Cheung, 1996; Kuukasjarvi, Tanner et al, 1997) did not produce any amplification products and were disregarded from further evaluation. The remaining four protocols all resulted in amplified DNA that could be used in CGH experiments. All of the protocols resulted in successful CGH hybridisations, in that a signal could be detected. In most instances, copy number aberrations could be detected but these did not always mirror the aberrations seen with DNA labelled by nick translation. For example, IN 2809 is a glioblastoma that has a number of CNAs. The protocols described by James and Feuerstein (personal communications) did not result in the detection of all of these aberrations. In fact, James' protocol did not identify any of the aberrations and Feuersteins method only identified two regions of high copy number amplification and two of the regions of low level gain, though the regions of gain on 1p and 2p could be deemed to be present but covered a smaller region than seen with nick translation. The method described by Huang et al. (2000) was a modification of the protocol developed by Kuukasjärvi et al. (1997). This protocol appeared to be more reliable at identifying chromosomal gains than losses. This protocol produced CGH profiles with results similar to those seen with nick translated DNA and for this reason this method was used to amplify and label DNA from tumours to be included in the CGH analysis. All the protocols seemed to have difficulty in correctly identifying the sex of the patients, in that quite often the X chromosome registered as normal when it should have been below the normal threshold level in male patients. This may be due to a high level of background caused by inefficient labelling of the DNA probes.

In agreement with the findings of other published data (Huang et al, 2000), homo-hybridisations, where tumour and normal DNA were both labelled under the same conditions, generated the most reproducible results with relatively good quality of hybridisation. Hetero-hybridisations, where the tumour DNA was labelled using a DOP-PCR protocol and the reference DNA was labelled using nick translation, were associated with uneven hybridisations and high levels of background.

One explanation for the poor reliability of this technique lies in the use of a partially degenerate primer, which binds during several low-temperature annealing cycles at many sites throughout the genome. After the low stringency cycles, the annealing temperature is then increased to allow amplification of the fragments that are tagged with the specific part of the primer sequence (Kittler et al, 2002). For optimal efficiency, these numerous different binding sites would in reality require equally numerous different specific PCR conditions, which would be impossible to achieve in a single reaction. The DOP-PCR procedure is very sensitive to contamination, as the degenerate primers in the reaction will amplify DNA from any source present in the tube. The low temperatures in the reaction can result in primers attaching to one another and fusing to form a double stranded product (Verhagen et al, 2000). It many instances this can also lead to the formation of short length products.

Recently a method has been described that produces long DOP-PCR products (0.5 to 7kb) allowing the amplification of long sequence targets in the subsequent PCR. This method has been further adapted to produce long amplification products from picogram quantities of genomic DNA by using a proof-reading enzyme and an increased annealing and extension time during the PCR. This protocol has been called LL-DOP-PCR (long products from low DNA quantities) and has been found to have enhanced sensitivity and coverage compared to other DOP-PCR protocols (Kittler et al, 2002). This method can also amplify degraded DNA if the DNA fragments contain two priming sites. One way of improving the coverage of DOP-PCR for degraded DNA samples might be to increase the amount of template in the reaction, thereby increasing the number of templates with two priming sites. This method might be useful for the amplification of DNA from paraffin embedded material, which is often of poor quality.

One way to overcome the multiple binding site problem in conventional DOP-PCR would be to completely digest the entire genome with a restriction enzyme such as *Mse*I. This results in the whole genome being transferred to a high complexity representation with a fragment size < 2 kilobases. The DNA is then amplified using ligation mediated PCR, where the amplification conditions are nearly optimal for all adapter-ligated sequences selected. This method has been

utilised to amplify DNA isolated from archival formalin-fixed paraffin embedded tissues. As this protocol has allowed the amplification of a single cell for use in CGH, it has been termed SCOMP (single cell comparative genomic hybridisation) (Stoecklein et al, 2002). One surprising finding of this protocol was the increased sensitivity and higher resolution of the CGH experiments with smaller cell numbers. This suggests that in some cases where CGH profiles have been normal, alterations may be masked by clonal heterogeneity or by contaminating DNA from normal cells.

Extraction of DNA from archival material

Due to the interactions of formalin between DNA and proteins, formalin fixation is a major limiting factor in the analysis of DNA extracted from archival material. Often fixation has been carried out with unbuffered formalin and exact fixation times are unknown. There have been a number of reports in which tissues fixed with unbuffered formalin have shown severe degradation. Once tissues have been embedded in paraffin, the DNA integrity of the tissues is well preserved even after long periods of storage (Speicher et al, 1993). We evaluated two different methods for the extraction of DNA from formalin fixed paraffin embedded material. One method used a salting out procedure and the second used a commercially available kit. The salting out procedure resulted in high molecular weight DNA. The Qiagen kit also resulted in high molecular weight DNA being extracted but there was a larger proportion of contaminating low molecular weight DNA. The high proportion of low weight DNA makes it difficult to determine the concentration of high molecular weight DNA that is suitable for labelling. Several attempts were made to label the DNA by nick translation but due to the difficulties in determining the exact concentration of the DNA the majority of these hybridisations failed. A number of samples that had been extracted using the Qiagen kit had DNA within a size range where it was possible to label the DNA using nick translation. In order that the DNA did not become over-digested by the nick translation enzyme, these samples were incubated with the enzyme for one hour instead of the usual two hours. A control experiment using DNA from IN2376 that had been incubated with the enzyme for one hour was included in order to determine that enough

fluorochrome had been incorporated into the DNA to allow the tumours alterations to be detected. This period of incubation gave results comparable to those achieved with longer incubation times and was therefore utilised for the labelling of the samples that had been extracted using the Qiagen kit. In order to used DNA extracted using the salting out method of Jeuken for CGH it is necessary to determine the concentration of the high molecular weight DNA alone. This could be achieved using serial dilutions of the DNA measured against a molecular weight marker where the bands correspond to known quantities of DNA. Due to the small volumes of DNA extracted using this method it was not possible to do this for this study.

It has been shown that DNA from 2000 cells, and in some cases as little as 5 to 10 cells (in the case of paraffin embedded material) are sufficient for reproducible DOP-PCR and subsequent CGH (Hirose et al, 2001; Speicher et al, 1993). These findings combined with the data from Stoecklein et al. (2002) suggest that it may in fact be of benefit to use DNA extracted from a small number of sections for DOP-PCR and subsequent CGH.

Universal Linkage System

Degradation of DNA is a major hindrance to the use of archival DNA in CGH. Quite often the bulk of the DNA is in the 400-750bp range. Enzymatic labelling of this DNA by nick translation results in further reduction of the fragment size. The size of archival DNA labelled by nick translation is often <300bp, resulting in CGH with too low a signal intensity. A commercial kit has become available (Universal Linkage System, ULS) that uses a special platinum compound that has two free binding sites, one of which is used to bind a marker group. The other site is used to link the platinum/marker complex to the DNA, where it binds preferentially to guanine groups. The resulting labelled probes can be used in a variety of techniques, including in situ hybridisation and filter hybridisation. In the literature, there have been two reports of the use of the ULS for labelling archival DNA to be used in CGH (Alers et al, 1999; van Dekken et al, 2001). This study attempted to utilise the ULS to label archival DNA for use in CGH analysis. As the system requires DNA fragments to be < 1kb in size DNA from the blood of a normal healthy donor was sonicated to produce fragments of the

required size. In contrast there was usually only a small volume of the paraffin DNA available for analysis and this was too small to sonicate. It was difficult to get successful hybridisations using archival DNA, and there was quite often a large amount of background noise and large fluorescent spots on the chromosomes. The high background interfered with the image analysis of the samples. This was also a frequent finding in the report of ULS by Alers et al. (1999). It has been suggested that this is caused by the presence of labelled DNA corresponding to the largest fragments of DNA. Nick translation would reduce these fragments to a size that would not hinder the hybridisation. It would appear that this system would be ideal for archival DNA that is in the range of 300bp to 1kb but not for samples larger than this. Treating archival DNA with RNase to remove any contaminating RNA that might have been co-extracted and with sodium thiocynate, to remove any DNA cross links may enhance the efficiency of the hybridisations using ULS labelled DNA.

CHAPTER 4
GENETIC ANALYSIS OF EPENDYMOMA

Interpretation of CGH Profiles

Each profile is composed of an average of individual ratio profiles from between eight and seventeen metaphase spreads. Each chromosome is represented in the form of a graph where the central black line represents a ratio of 1. Profiles that run along or very close to this line have an equal amount of tumour and normal DNA. A threshold value of 1±0.2 was used in order to determine loss and gain and this is indicated by the red and green lines on either side of the black line. The areas of the profiles that are outside this range represent regions of change in DNA sequence copy number. The bold green lines on the right of the chromosome ideogram show regions where the amount of tumour DNA is greater than the amount of normal DNA, and the bold red lines to the left of the ideogram show where there is less tumour DNA than normal DNA. High copy number amplification is represented by a threshold of 1 + 0.4. The Y chromosome is not included in the analysis due to it being a heterochromatic region and therefore will show up on the profile as above the 1.2 threshold level. This would make it difficult to determine if there had been gain of Y in the male patients, as even the female samples will register with gain of Y.

Control experiments

For each hybridisation experiment a control hybridisation using either normal DNA as both test and reference or MPE 600 DNA as test was conducted. The MPE 600 is a breast cancer cell line with known abnormalities. An example of a CGH profile for this cell line can be seen in Figure 4.1 and for the normal:normal control experiments in Figure 4.2. Experimental evidence has shown that in control experiments using normal DNA as both test and reference there is a high rate of apparently abnormal ratios at 1p32-pter, 16p, 19 and 22 (Kallioniemi, Kallioniemi et al, 1994). Caution should be used in interpreting ratio changes at these regions in composite profiles of tumours. Ratio changes at or near these regions have only been included as positive aberrations when the alteration is not confined to these regions. Other regions that should be interpreted with care include peri-centromeric and heterochromatic regions. DNA sequences in these regions are highly polymorphic in copy number

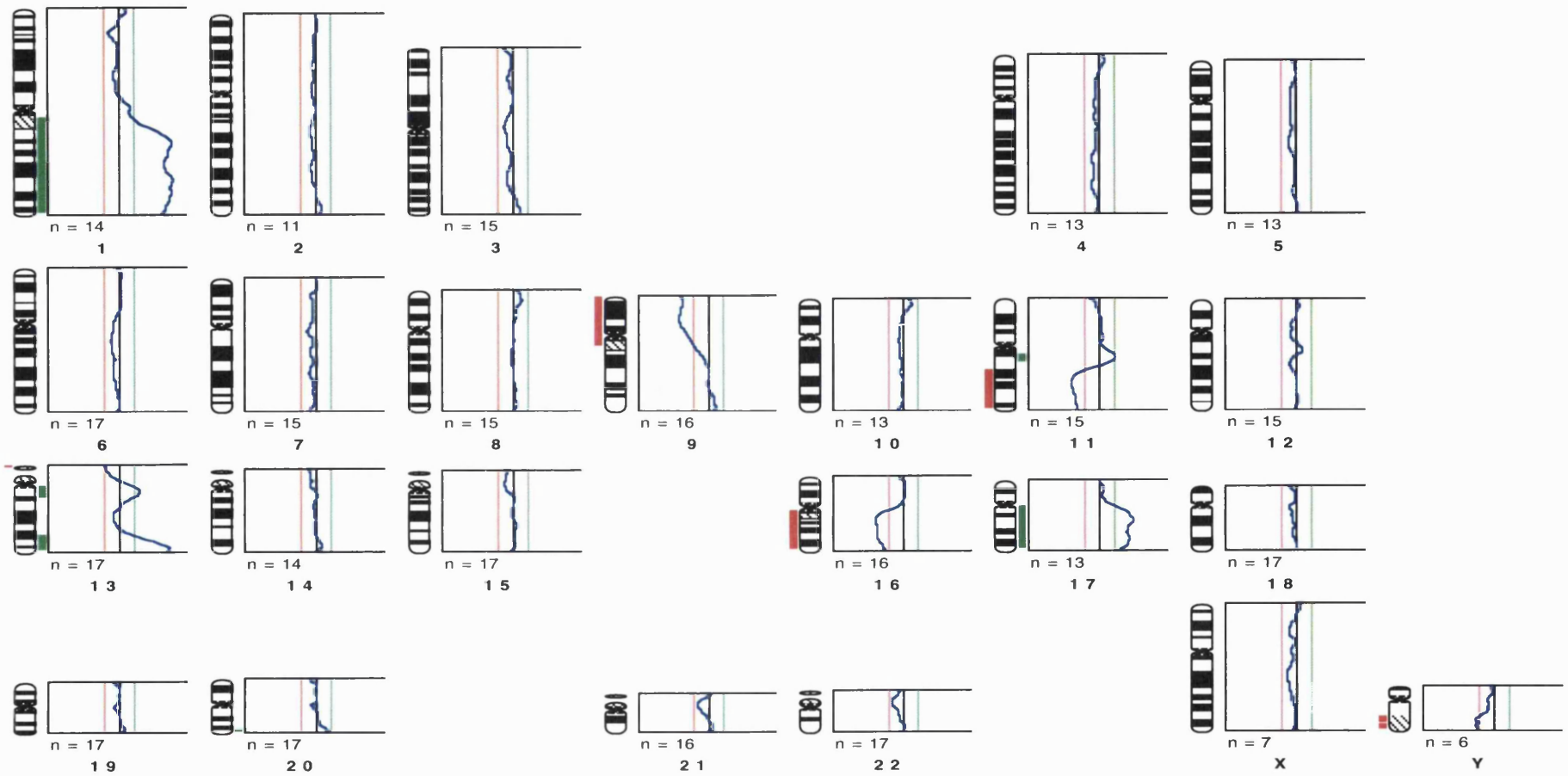


Figure 4.1: CGH composite of breast cancer cell line MPE600. The composite is compiled from the average of the individual ratios from nine metaphases. There are six regions of alteration: amplification of 1q, gain of 11q13, gain of 17q, loss of 9p and 16q, and a smaller region of deletion at 11q14-qter.

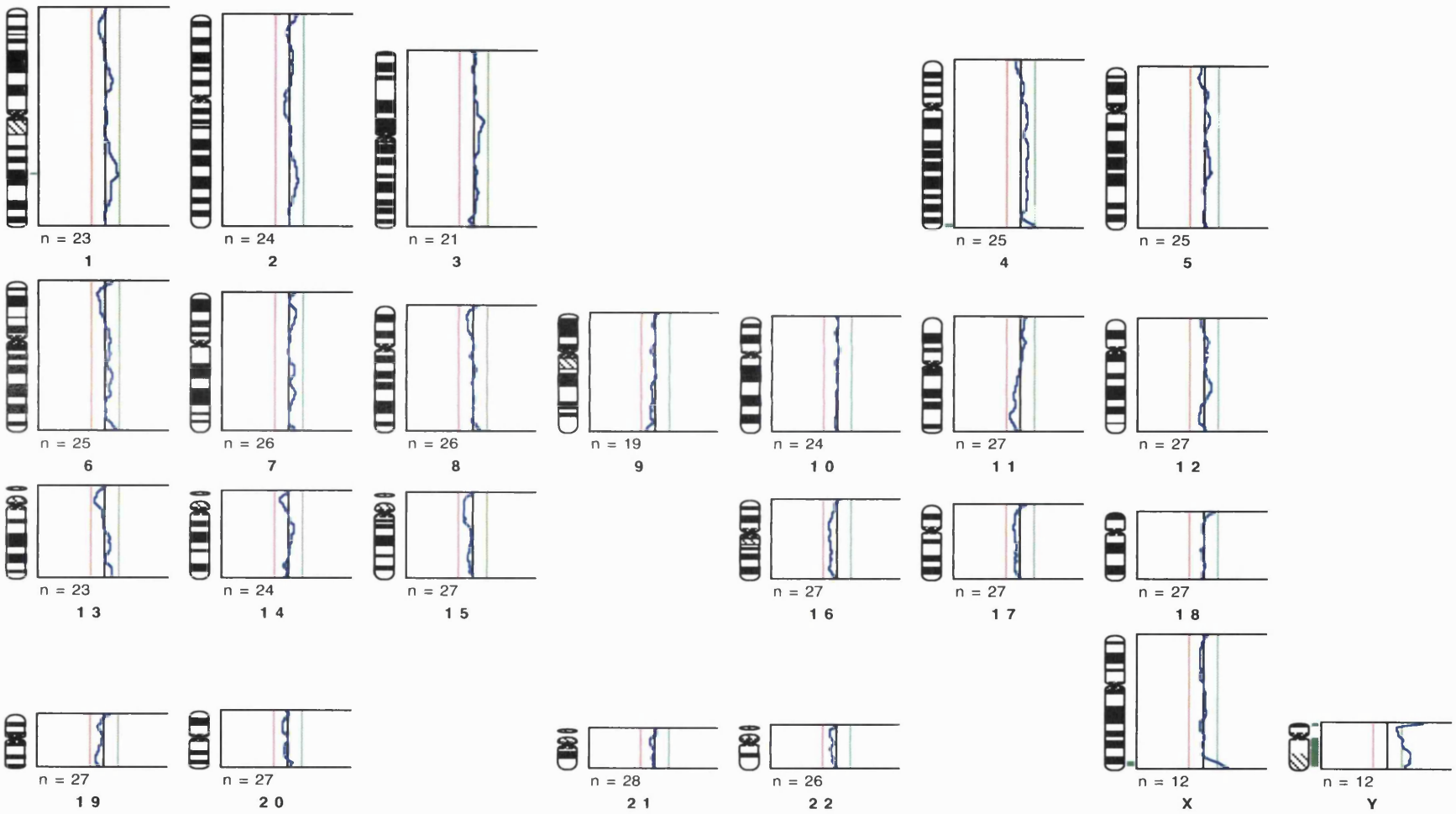


Figure 4.2: CGH composite of normal DNA labelled with SpectrumGreen co-hybridised with normal DNA labelled with SpectrumRed. Profiles are smooth and uniform with little or no deviation from the central black line, representing 1.

between individuals and are blocked to some extent in CGH experiments by the use of Cot-1 DNA. Fluorescence ratios tend to decrease at chromosome telomeres. When the absolute intensities start to approach the background fluorescence unreliable ratio changes may occur (Kallioniemi et al, 1994). Ratio changes at chromosome telomeres have only been included if the change is not confined to the telomere and is part of a larger region of alteration.

Sample details

CGH profile were obtained for fifty-one paediatric ependymoma samples. There were three subependymoma, forty ependymoma and eight anaplastic tumours. Thirty-seven of the samples were primary tumours and the remaining fourteen tumours were recurrent cases. There were six primary and recurrent paired samples and two recurrent samples from the same patient. Forty-one samples were located in the posterior fossa and ten supratentorially. The age at diagnosis ranged from 6 months to 17 years (average 5.48 years) and the male: female ratio was 26:18. There were twenty-two samples from patients aged <3 years and twenty-nine samples from patients aged >3 years. In forty cases analysis was performed using DNA extracted from biopsy samples. In seven cases the DNA was extracted from short term cell cultures at low passage (4 – 9) and the remaining four cases used DNA extracted from paraffin embedded material.

A summary of the copy number aberrations observed in these tumours can be see in Table 4.1 and Figure 4.3. CGH composite profiles for individual tumours showing copy number aberrations can be seen in Figures 4.4 – 4.28. In total 25 tumours (49%) showed detectable regions of alteration. These ranged from 1 to 20 per tumour (mean = 7.6). Four tumours had single copy number aberrations, involving whole chromosomes or individual chromosome arms. IN2944 showed monosomy 9 as a sole abnormality, U40 showed monosomy 19, IN1497 showed loss of 6q and IN2929 showed gain of 7q (see Figures 4.29 & 4.30). All chromosomes except for chromosomes 14, 15 and 21 demonstrated imbalance in at least one tumour. The most frequent aberration observed was loss of 19q, seen in 14 samples (27%). Other common abnormalities were monosomy 22, gain of 4q and 13q, each seen in twelve cases (23.5%), loss of 1p and 19p, and gain of 1q, each seen in eleven cases (21.5%), and gain of 2q and 6q, and loss 16p, each seen in ten cases (19.6%). High copy number amplification was observed at 1q21-31 (IN2904, IN2970 and IN2939), 8q21.1-23 (IN2939 and IN2970) and 9p24-q12

IN	AGE	SEX	HISTOLOGY	P/R	LOCATION	SURVIVAL	SURGERY	SOURCE	LABELLING	AMPLICONS	GAINS	LOSSES
Primary Samples												
1932	7	M	subependymoma	P	posterior fossa	4 (D)	Macroscopic removal	FF	DOP-PCR		1q X	9p
1594	1.67	M	benign	P	posterior fossa	18 (D)	Partial excision	CC	NT			
1638	1.4	M	benign	P	posterior fossa	49 (D)	Macroscopic removal	FF	NT			
2186	12.5	F	benign	P	posterior fossa	118 (A)	Macroscopic removal	CC	NT			
2242	2	F	benign	P	posterior fossa	17 (D)	Macroscopic removal	FF	NT			
2511	13	F	benign	P	posterior fossa	35 (A)	Partial excision	CC	NT			
2536	7	F	benign	P	supratentorial	51 (A)	Macroscopic removal	FF	NT		4q 6q 13q	1p 19 22
2628	17	M	benign	P	supratentorial	9 (D)	Unknown	FF	NT		1q 2 3q 7 8q 9	
2699	11.5	F	benign	P	posterior fossa	2 (A)	Macroscopic removal	FF	NT			
2752	2.25	M	benign	P	posterior fossa	62 (A)	Partial excision	FF	DOP-PCR		1q 6q	10q
2776	6	F	benign	P	posterior fossa	71 (A)	Macroscopic removal	FF	NT		1q	16q 19
2871	1.3	F	benign	P	posterior fossa	63 (A)	Macroscopic removal	FF	NT			
2891	1.9	F	benign	P	posterior fossa	20 (A)	Sub total removal	FF	NT		2q 4q 5q 6q 9p 12 13q 18q	1p 17 19
2922	5.5	M	benign	P	posterior fossa	47 (A)	Macroscopic removal	FF	NT		1p 2q 4q 5q 6q 13q 18q	1p 16p 19 20q 22
2931	1.3	F	benign	P	posterior fossa	18 (D)	Macroscopic removal	FF	NT			
2935	9.33	M	benign	P	posterior fossa	39 (A)	Macroscopic removal	FF	NT		4q 5q 6q 13q	1p 16p 19q 20 22
2939	0.58	F	benign	P	posterior fossa	38 (A)	Macroscopic removal	FF	NT	1q21-44 8q21.1-23	1q 8 9p 11p 11q	12q 19
2941	2.58	M	benign	P	posterior fossa	24 (A)	Macroscopic removal	FF	NT			
2944	11.5	M	benign	P	supratentorial	37 (A)	Partial excision	FF	NT			9
3008	10.5	M	benign	P	posterior fossa	27 (A)	Macroscopic removal	FF	NT			
3014	6.75	M	benign	P	supratentorial	23 (A)	Macroscopic removal	FF	NT		4q 5q 6q 13q	1p 9q 12q 16p 17q 19q22
3022	1.75	F	benign	P	posterior fossa	18 (A)	Macroscopic removal	FF	NT			
3029	1.75	M	benign	P	posterior fossa	23(A)	Macroscopic removal	FF	NT			
3071	4	M	benign	P	posterior fossa	4 (A)	Macroscopic removal	FF	NT			
3087	3.75	M	benign	P	posterior fossa	5 (A)	Macroscopic removal	FF	NT			
U36	3	M	benign	P	posterior fossa	Unknown	Unknown	PEM	NT		4q 5q 6q	16p 19 20q 22
U38	1.5	F	benign	P	posterior fossa	Unknown	Unknown	PEM	NT			
U40	3	M	benign	P	posterior fossa	Unknown	Unknown	PEM	NT			19
2443	4.5	M	anaplastic	P	posterior fossa	64 (D)	Partial excision	FF	NT			
2767	1.8	F	anaplastic	P	posterior fossa	72 (A)	Macroscopic removal	FF	NT			
2827	9.25	M	anaplastic	P	supratentorial	69 (A)	Macroscopic removal	FF	NT			
Recurrent Samples												
1258	2.5	F	benign	R	posterior fossa	36 (A)	Partial excision	FF	NT			1p 6q 18q 22
1497	1.25	M	benign	R	posterior fossa	45 (D)	Macroscopic removal	CC	NT			6q
2886	5.25	M	benign	R	posterior fossa	21 (D)	Partial excision	FF	NT			
2923	7	M	benign	R	posterior fossa	20 (D)	Unknown	FF	NT		2q 4 5q 6q 12q 13q	1p 9q 12q 16p 17 19 20q 22
2855	12	F	anaplastic	R	posterior fossa	26 (A)	Macroscopic removal	FF	NT		1q 2 7 18q	22
2929	2.75	F	anaplastic	R	supratentorial	14 (D)	Sub total removal	FF	NT		7q	
Paired Samples												
772	4	M	subependymoma	P	supratentorial	72 (D)	Partial excision	CC	NT		4	1p 16p
1134	5.5	M	subependymoma	R	supratentorial	72 (D)	Partial excision	CC	NT			
1231	7	F	benign	P	supratentorial	64 (D)	Macroscopic removal	CC	NT			
U37	7	F	anaplastic	R	supratentorial	64 (D)	Macroscopic removal	PEM	NT			
1759	10	M	benign	P	posterior fossa	98 (A)	Partial excision	FF	NT		1q 2 7 9p 11 13	16q
2376	15	M	benign	R	posterior fossa	98 (A)	Partial excision	FF	NT		1q 2 7 9p 11p 13	12q 16 19p
2638	1.9	M	benign	P	posterior fossa	91 (A)	Partial excision	FF	NT		1p 1q 2q 4 5q 6q 7q 8q 9p 12q 13q 18	1p 9q 12q 16 17 19 20q 22
2887	4.5	M	benign	R	posterior fossa	91 (A)	Partial excision	FF	NT		2q 4 5q 8q 9p 13q	1p 12q 19 20q 22
2682	0.66	F	benign	P	posterior fossa	11 (D)	Macroscopic removal	FF	NT			
2766	2	F	benign	R	posterior fossa	11 (D)	Macroscopic removal	FF	NT			
2904	11.5	M	benign	R	posterior fossa	97 (D)	Macroscopic removal	FF	NT	1q22-31	1q 4q 6q 8q 9p 13q	1p 9q 12q 16p 22
2970	5.5	M	benign	R	posterior fossa	97 (D)	Macroscopic removal	FF	NT	1q11-44 8q12-23 9p24-q12	2q 4q 5q 7 9q 13q	1p 8p 10 11q 12q 16 17 19 20 22
3037	2	M	anaplastic	P	posterior fossa	21 (A)	Macroscopic removal	FF	NT			
3108	4	M	anaplastic	R	posterior fossa	21 (A)	Macroscopic removal	FF	NT			

Table 4.1: Summary of chromosomal abnormalities detected in 51 Paediatric Ependymoma using CGH.

Key to table:

Age in years
M= Male; F = female
A = alive; D = deceased
P = primary sample; R = recurrent sample
FF = fresh frozen material, CC = short term cell culture; PEM = Paraffin embedded material
NT= Nick translation; DOP-PCR = degenerate oligonucleotide primed PCR

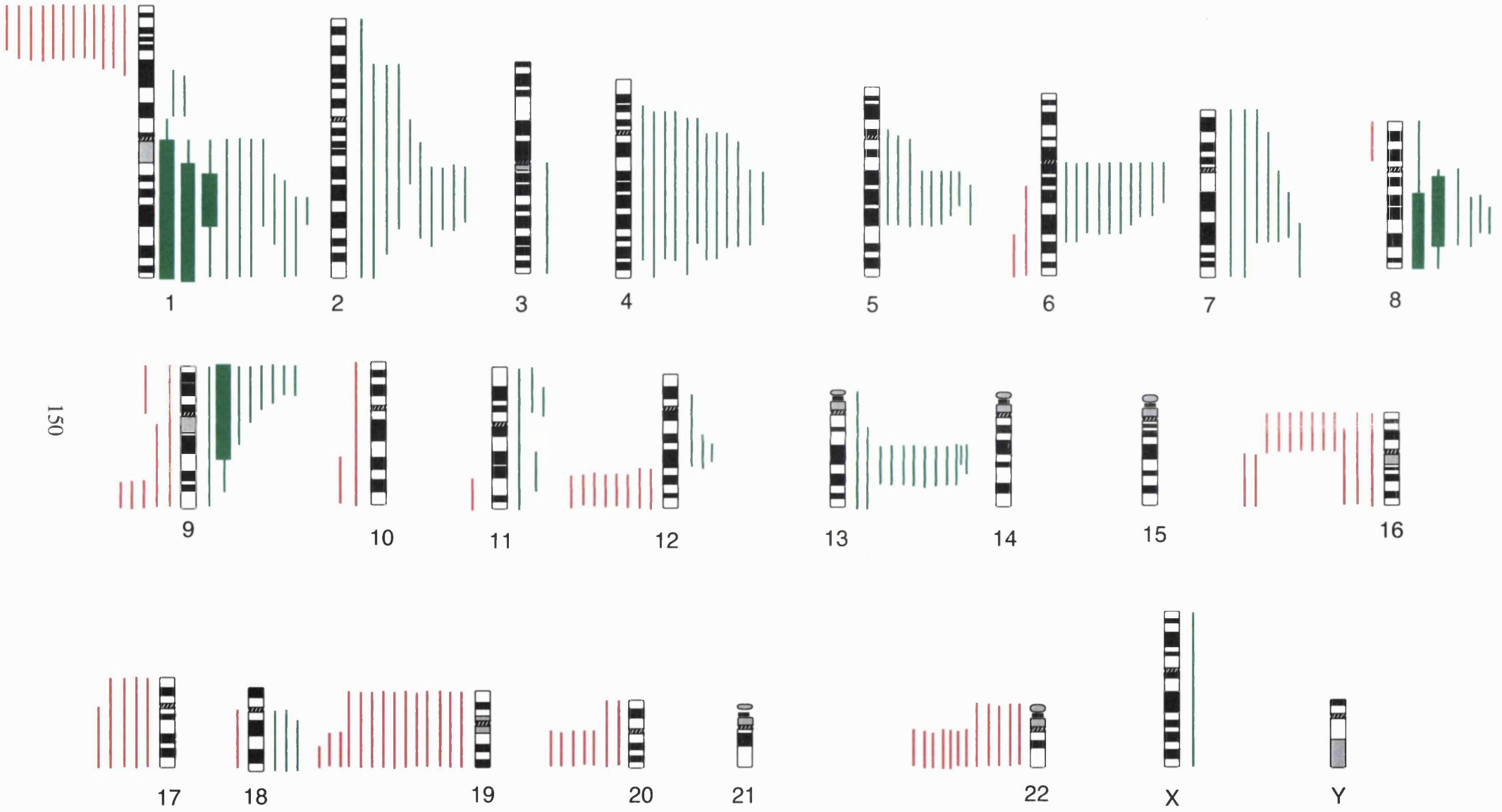


Figure 4.3: Summary of chromosomal imbalances detected in 51 paediatric ependymoma. Green lines to right of chromosomes indicate regions of gain; red lines to the left of the chromosome indicate regions of loss. Bold green blocks indicate regions of amplification.

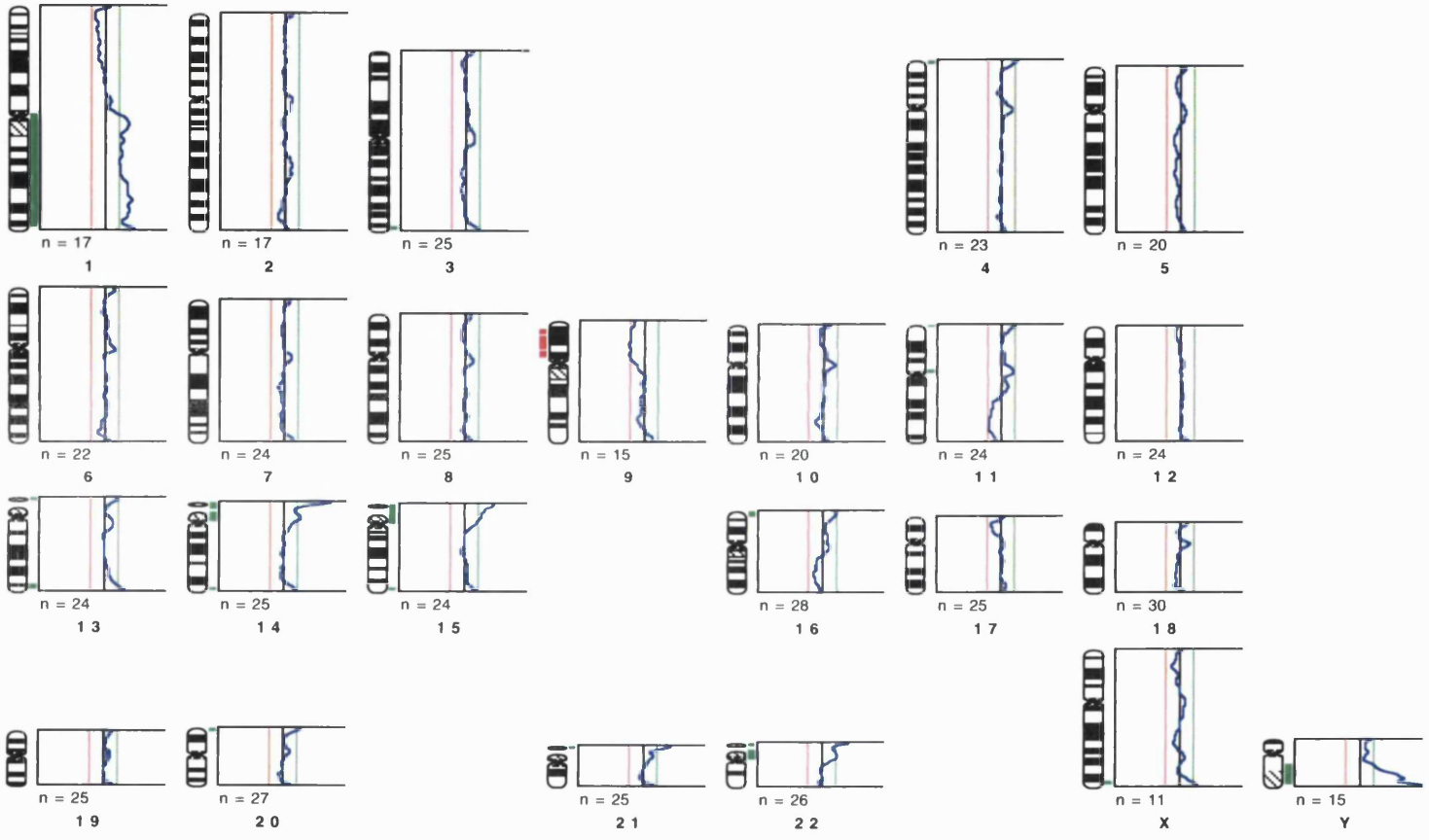


Figure 4.4: CGH composite of IN1932, a subependymoma. The composite is compiled from the average of the individual ratio profiles from fifteen metaphases. This tumour has three CNAs, gain of the entire X chromosome, gain of 1q and loss of the short arm of chromosome 9.

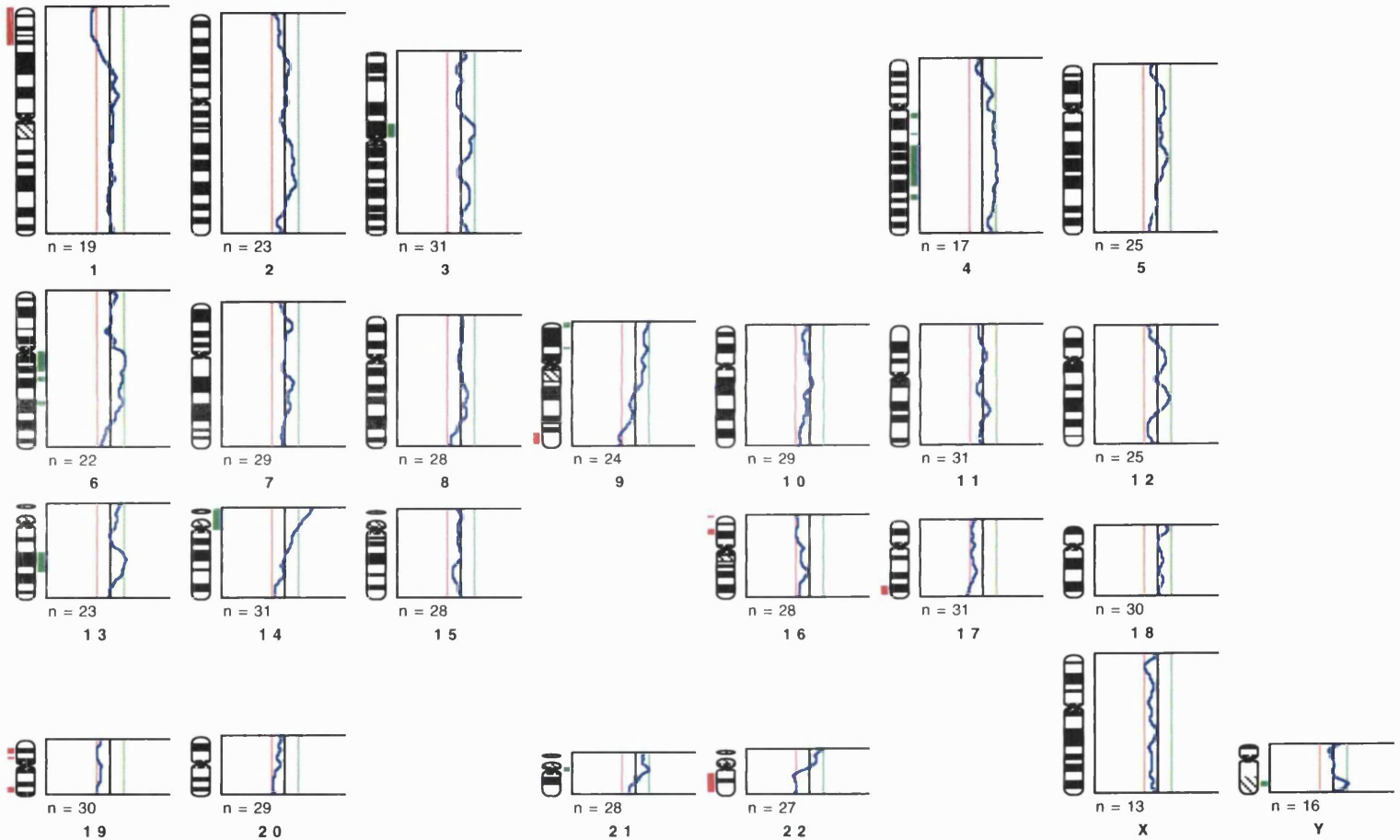


Figure 4.5: CGH composite profile of IN2536, an ependymoma. The composite is compiled from the average of the individual ratio profiles of sixteen metaphases. Thee are six CNAs. There are large regions of gain at 4q11-32, 6q11-22 and 13q21-32. There is monosomy of chromosomes 19 and 22, and a smaller region of deletion at 1p33-36.

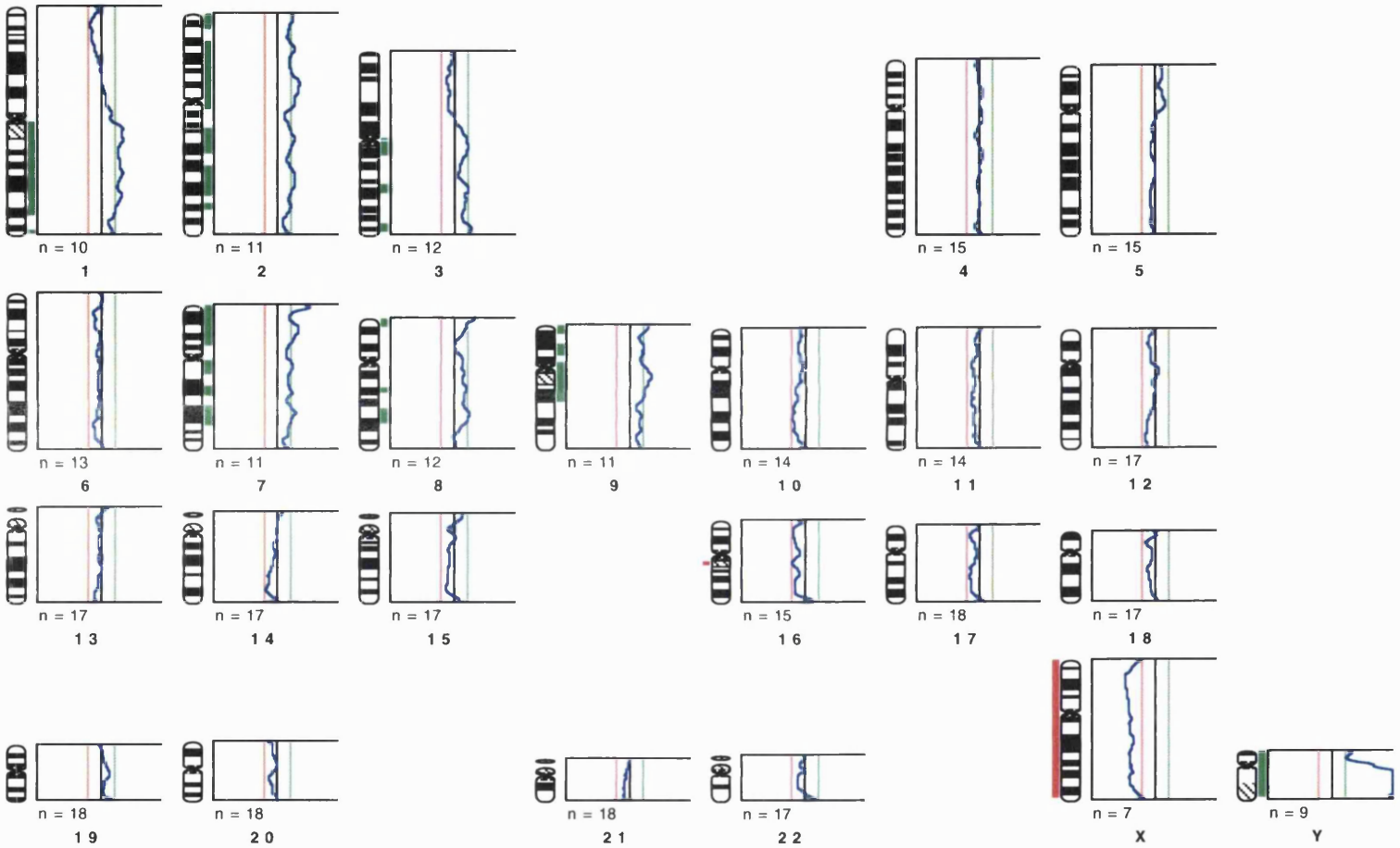


Figure 4.6: CGH composite of IN2628, an ependymoma. The composite is compiled from the average of the individual ratio profiles of nine metaphases. This tumour has six CNAs. There are gains of whole chromosomes (2, 7 and 9), as well as gains of individual chromosome arms (1q and 3q). There is also a smaller region of gain at 8q21-23. There are no genomic losses in this tumour.

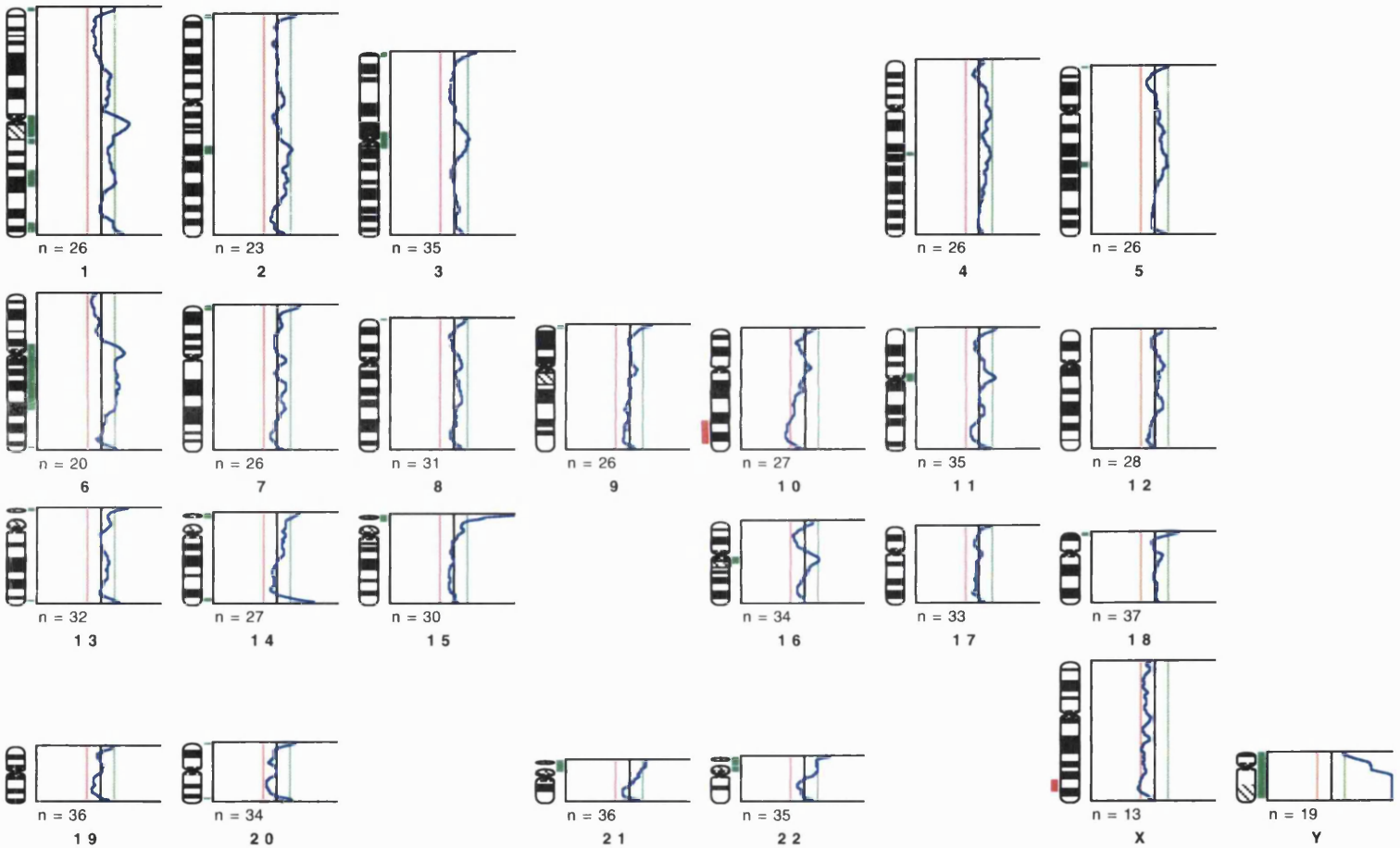


Figure 4.7: CGH composite of IN2752, an ependymoma. The composite is compiled from the average of the individual ratio profiles from twenty metaphases. This tumour has three CNAs. There is gain of 1q11-31 and 6q11-22. There is also a small region of deletion at 10q23-26.

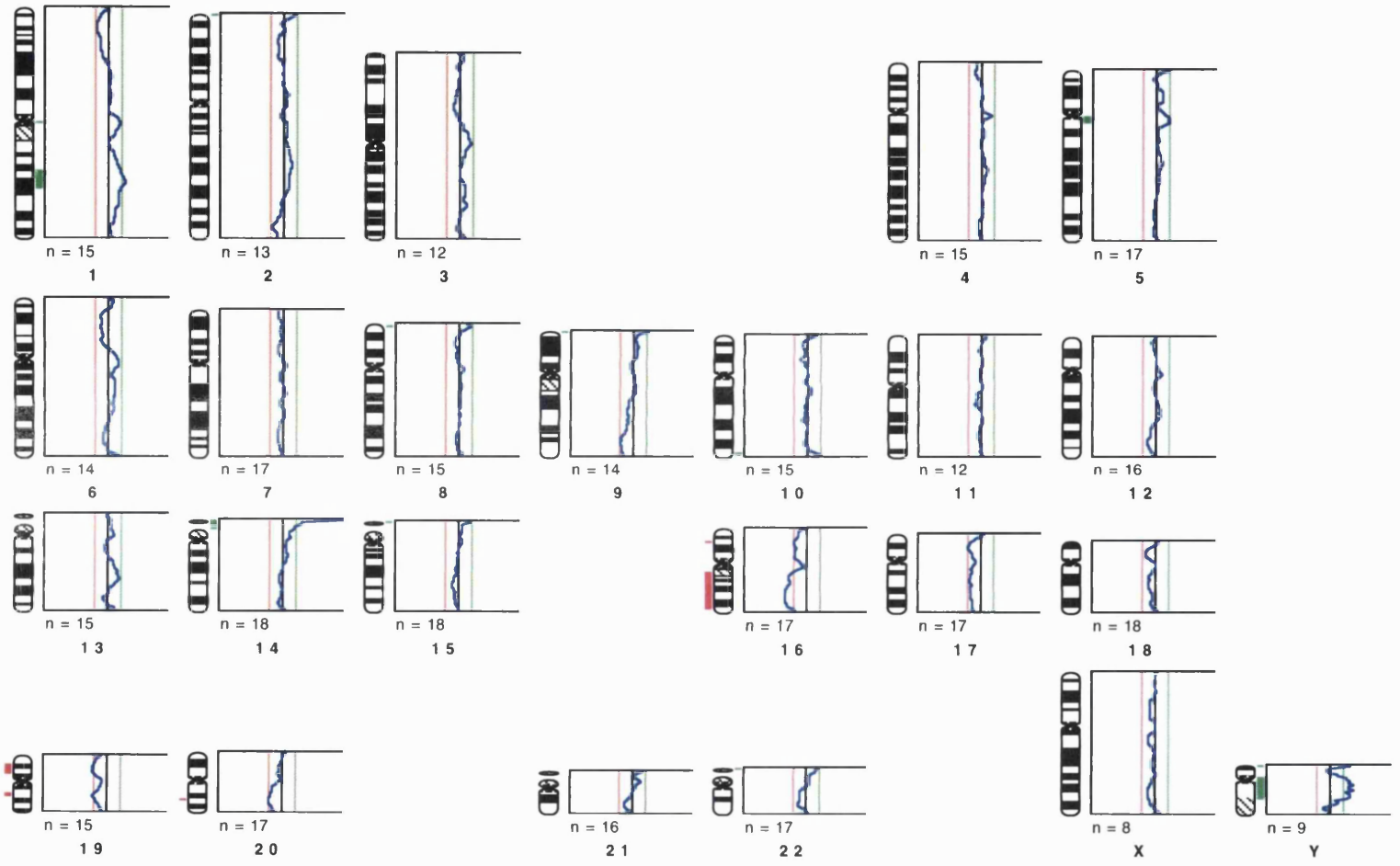


Figure 4.8: CGH composite of IN2776, an ependymoma. The composite is compiled from the average of the individual ratio profiles from nine metaphases. This tumour has three CNAs. There is gain of 1q22-32, monosomy 19 and loss of 16q.

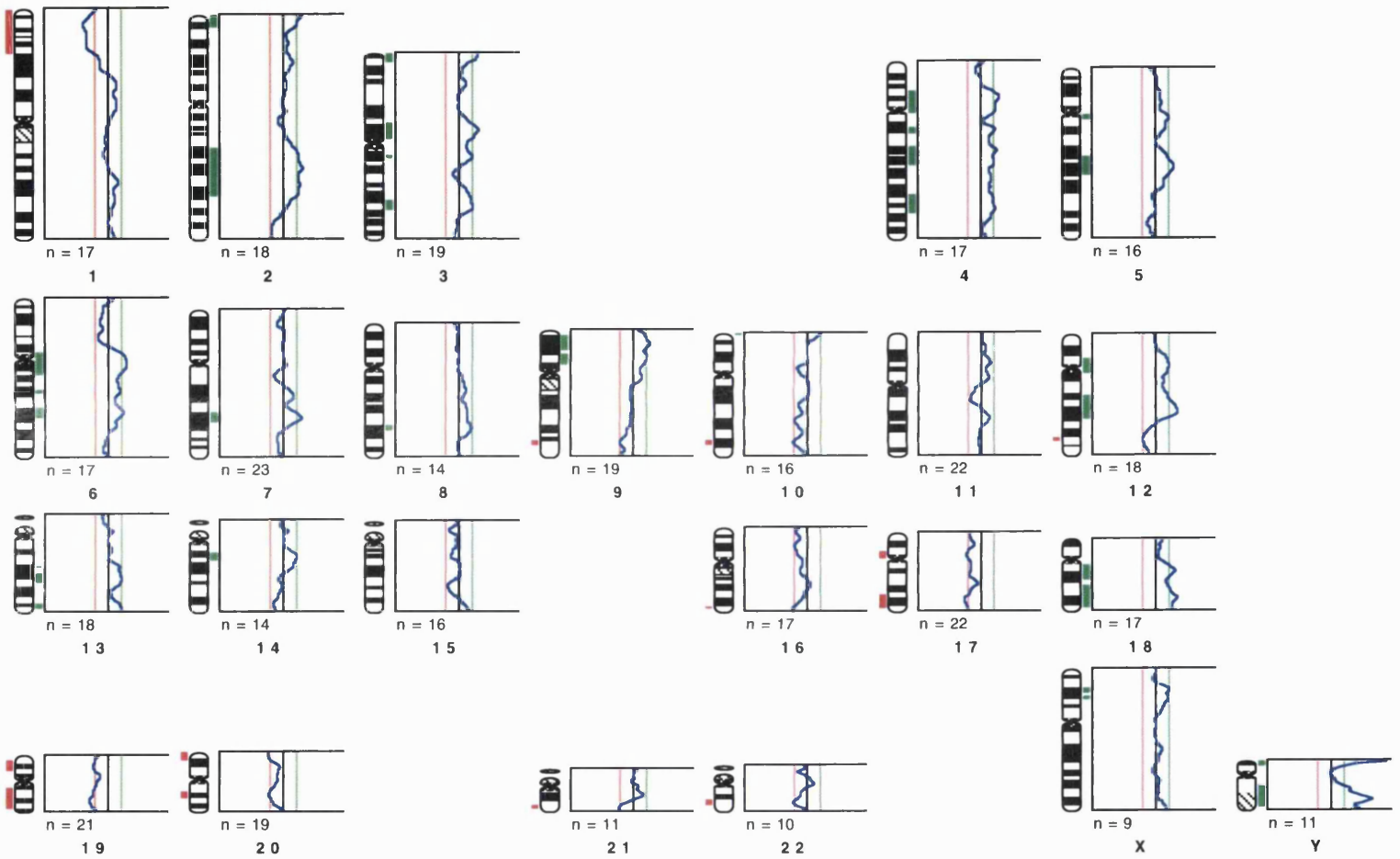


Figure 4.9: CGH composite of IN2891, an ependymoma. The composite is compiled from the average of the individual ratio profiles from eleven metaphases. This tumour has thirteen CNAs. There is gain of individual chromosome arms (4q, 9p and 18q), and smaller regions of gain at 2q22-32, 5q14-23, 6q11-23, 12p12-q21 and 13q21-31. There is monosomy of chromosomes 17 and 19 and a smaller region of loss at 1p32-36.

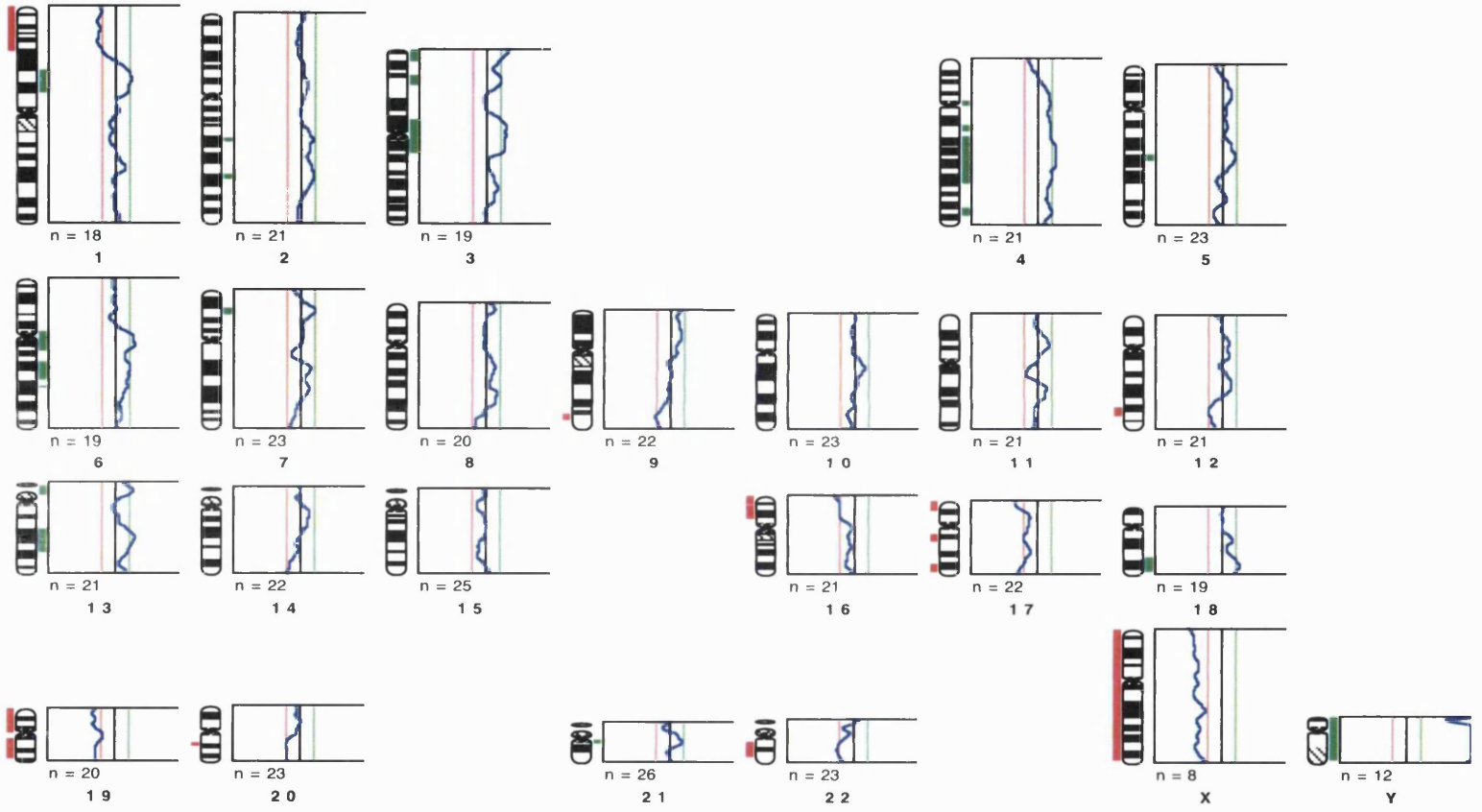


Figure 4.10: CGH composite of IN2922, an ependymoma. The composite is compiled from the average of the individual ratio profiles from twelve metaphases. This tumour has twelve CNAs. There is gain involving whole chromosome arms (4q and 18q) and smaller regions of gain at 1p13-22, 2q14-33, 5q14-21, 6q11-22 and 13q21-31. There is monosomy of chromosomes 19 and 22, loss of 16p and 20q, and a smaller region of deletion at 1p32-36.

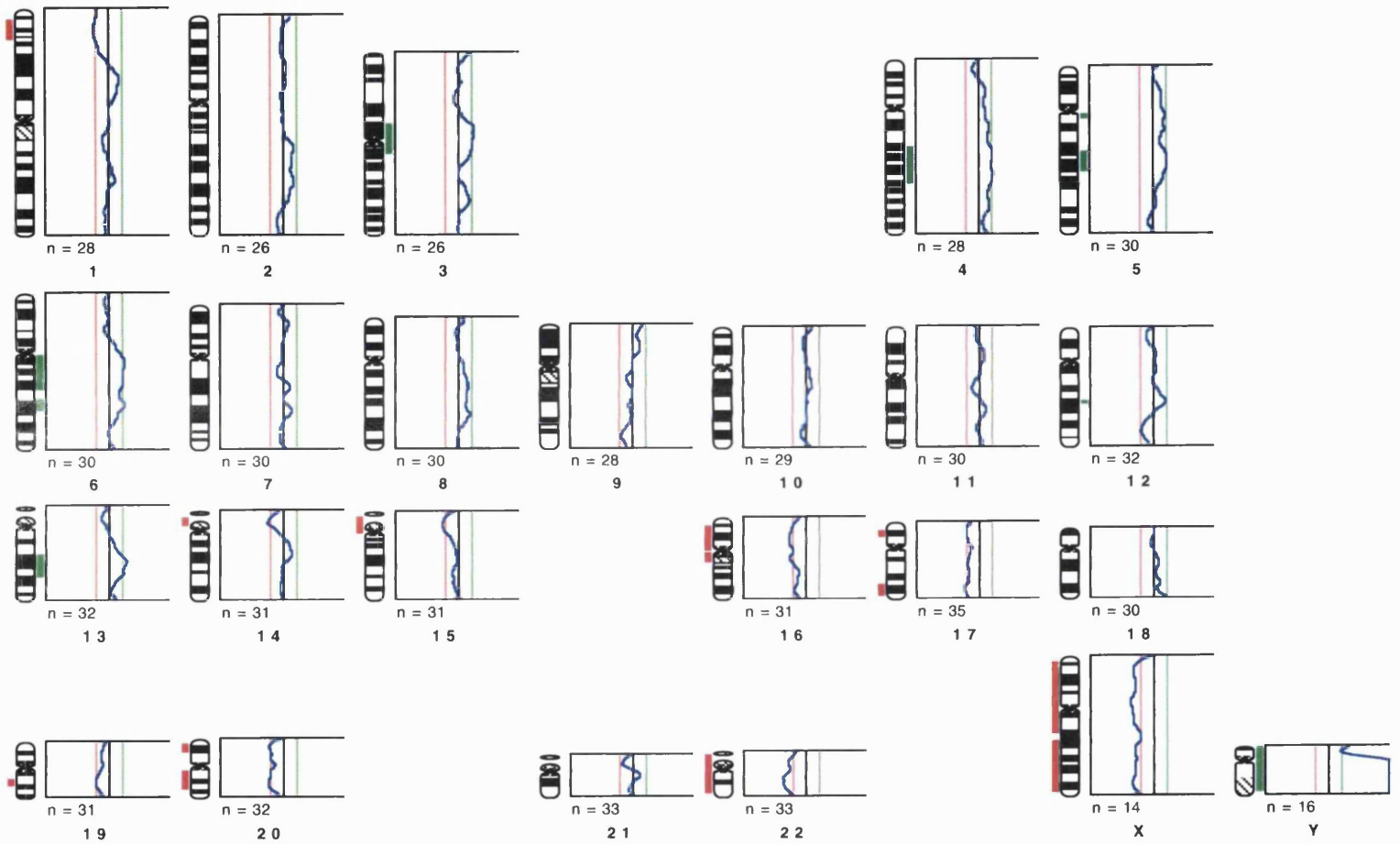


Figure 4.11: CGH composite of IN2935, an ependymoma. The composite is compiled from the average of the individual ratio profiles from sixteen metaphases. There are nine CNAs. There are regions of gain at 4q22-26, 5q14-24, 6q11-23 and 13q13-22. There is monosomy of chromosomes 20 and 22, and loss of 16p. There are also smaller regions of loss at 1p32-36 and 19q13-1-13.3.

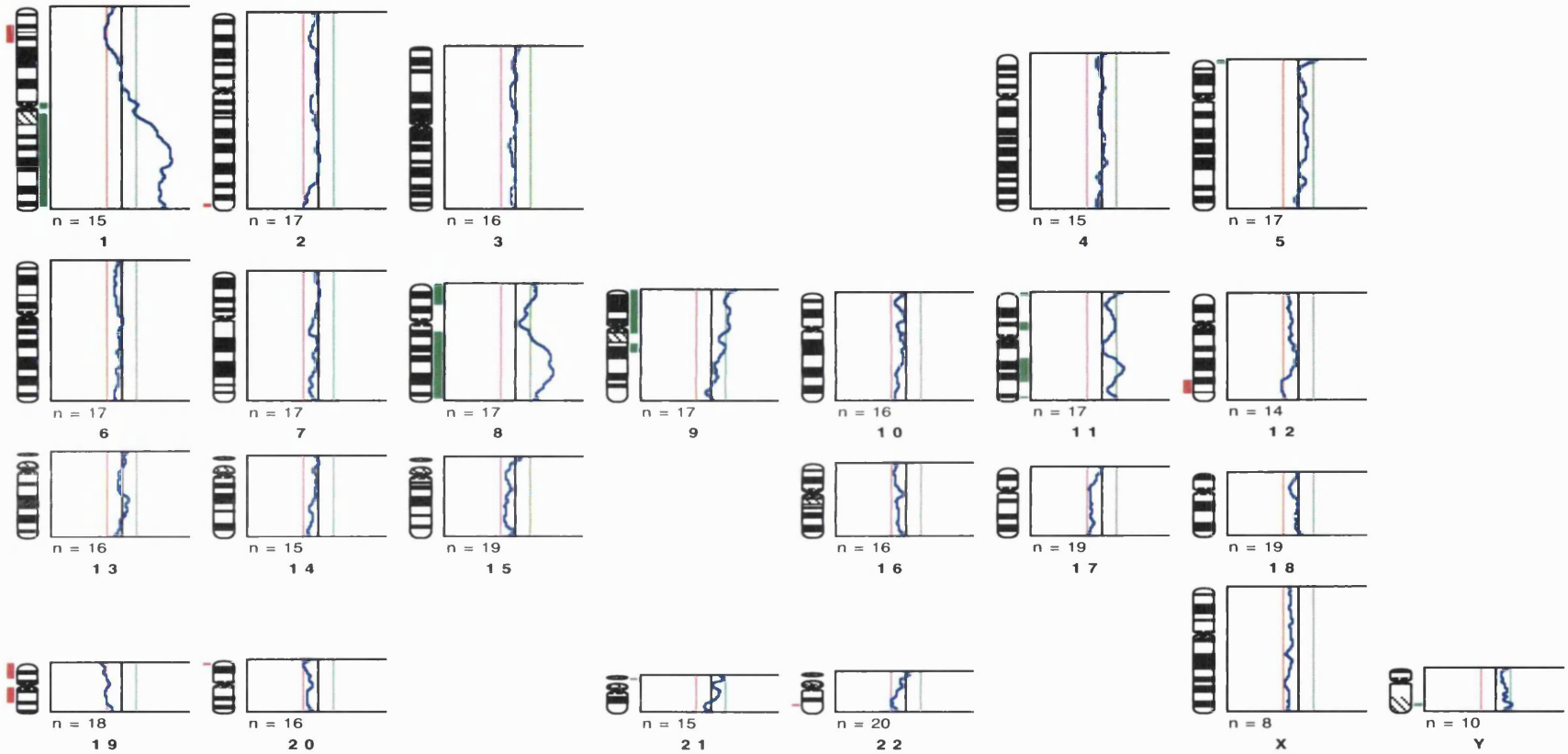


Figure 4.12: CGH composite of IN2939, an ependymoma. The composite is compiled from the average of the individual ratio profiles from ten metaphases. There are nine CNAs, including two regions of high copy number amplification at 1q21-44 and 8q21-23. There is gain of chromosome 8 as well as gain involving entire chromosome arms (1q, and 9p). There are smaller regions of gain at 11p12-14 and 11q14-24. There is monosomy 19 and a small region of deletion at 12q23-24.3.

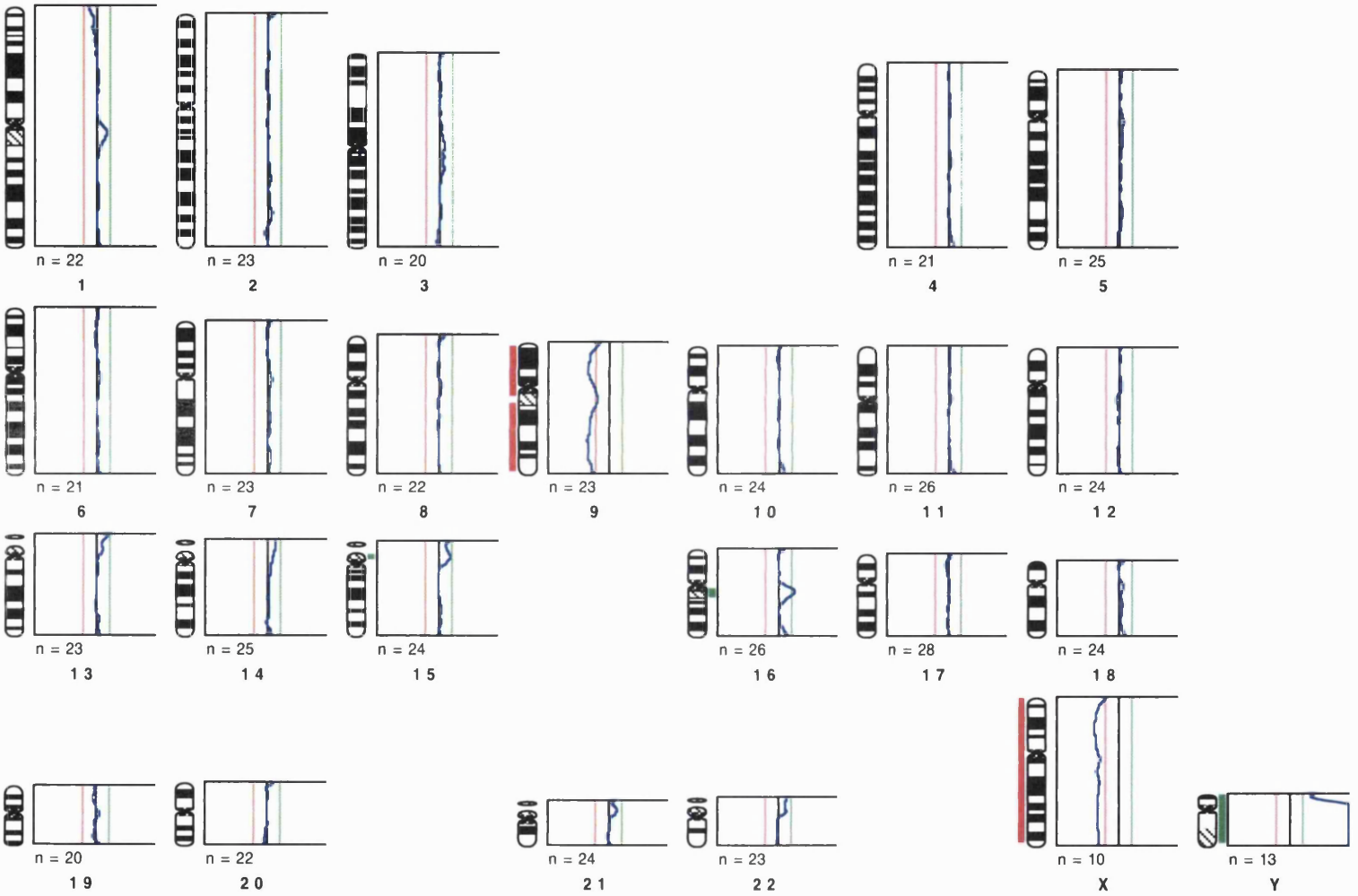


Figure 4.13: CGH composite of IN2944, an ependymoma. The composite is compiled from the average of the individual ratio profiles from fourteen metaphases. This tumour has a single aberration, which is monosomy of chromosome 9.

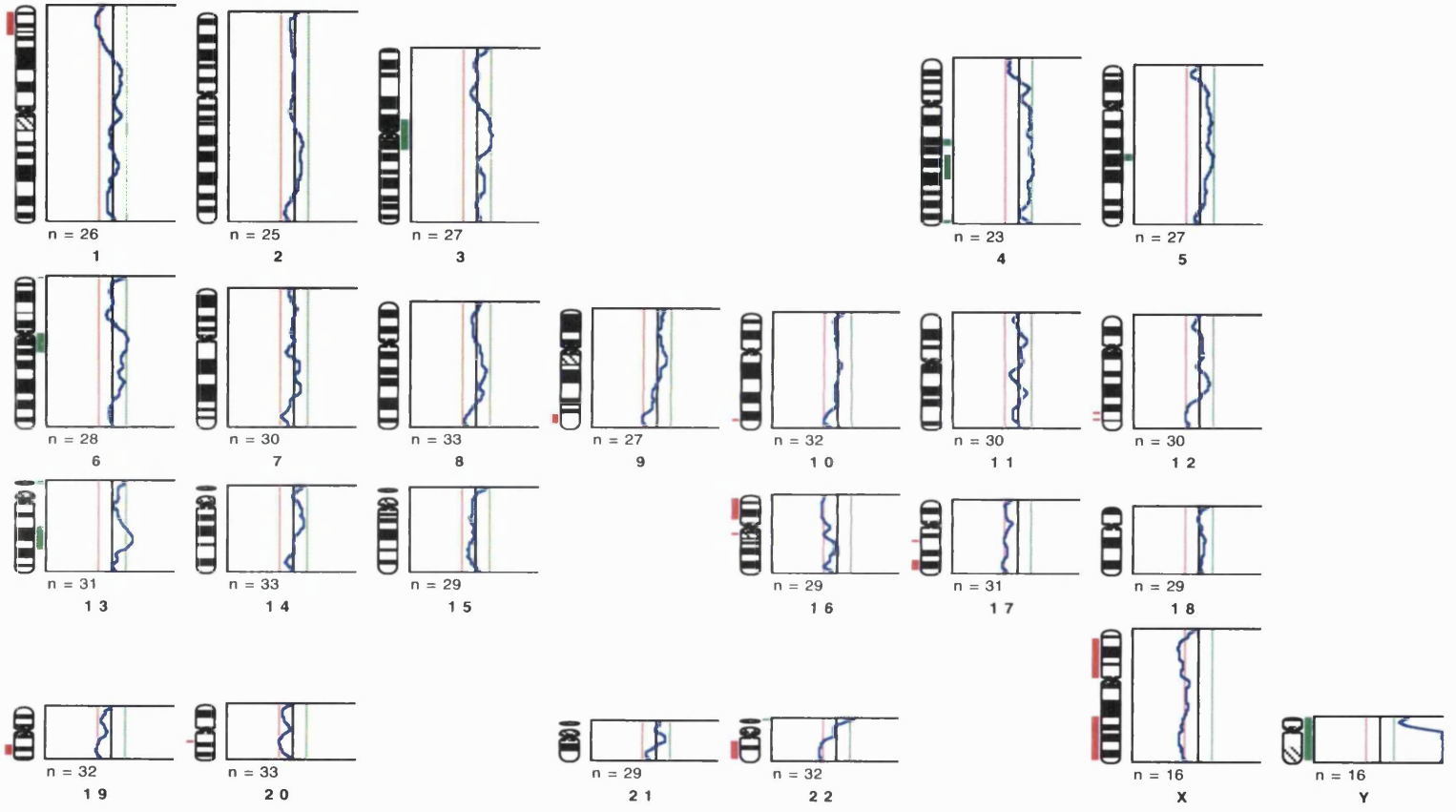


Figure 4.14: CGH composite of IN3014, an ependymoma. The composite is compiled from the average of the individual ratio profiles from sixteen metaphases. There are eleven CNAs. There are regions of gain at 4q22-32, 5q14-23, 6q11-22 and 13q21-31. There is monosomy of chromosome 22 and loss of individual chromosome arms (16p, 17q and 19q), and smaller regions of deletion at 1p32-36, 9q32-qter and 12q23-qter.

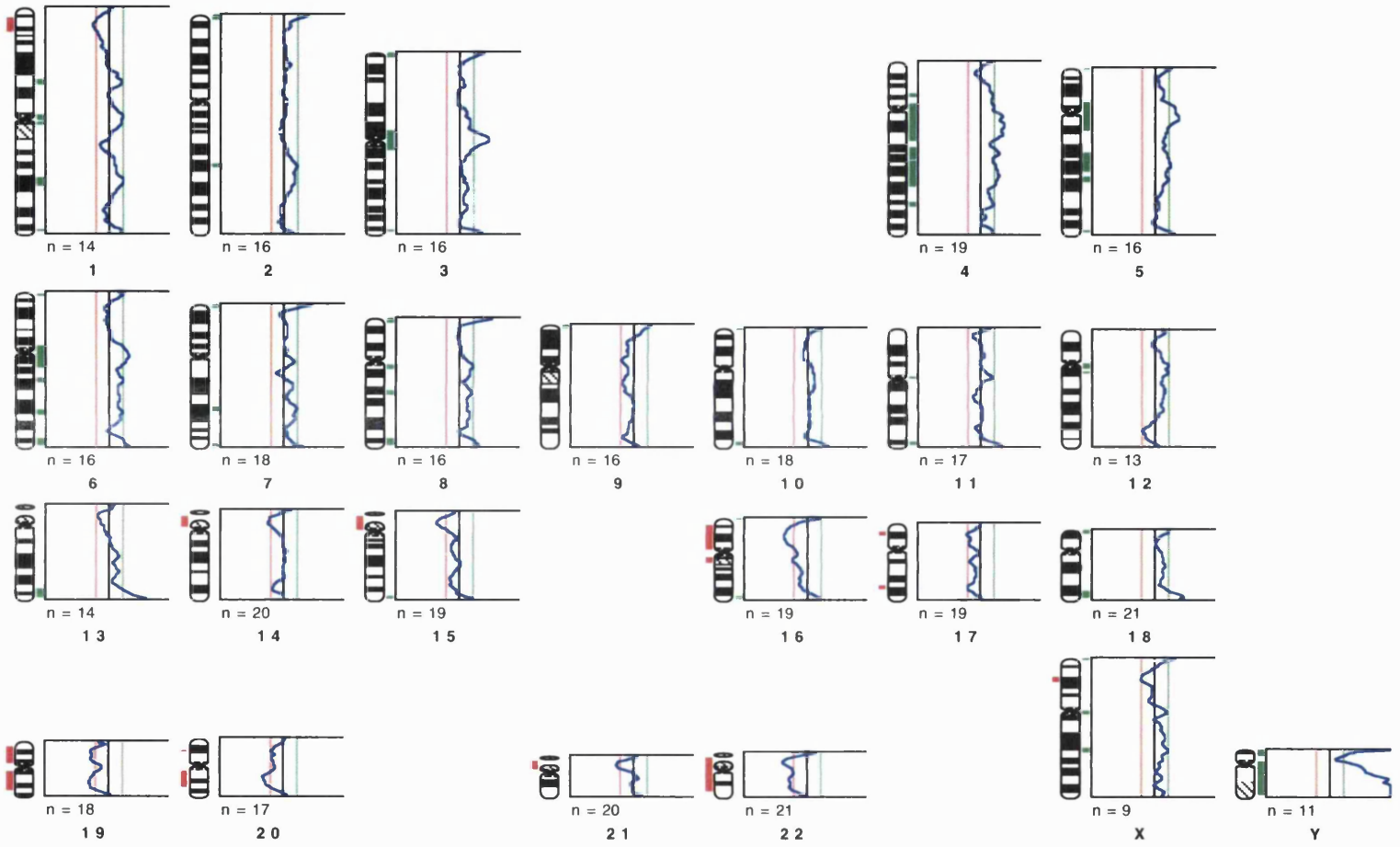


Figure 4.15: CGH composite of U36, an ependymoma. The composite is compiled from the average of the individual ratio profiles from eleven metaphases. This tumour had seven CNAs, involving gains of 4q11-32, 5q14-23 and 6q11-24. There is monosomy of chromosomes 19 and 22, as well as loss of 16p and 20q.

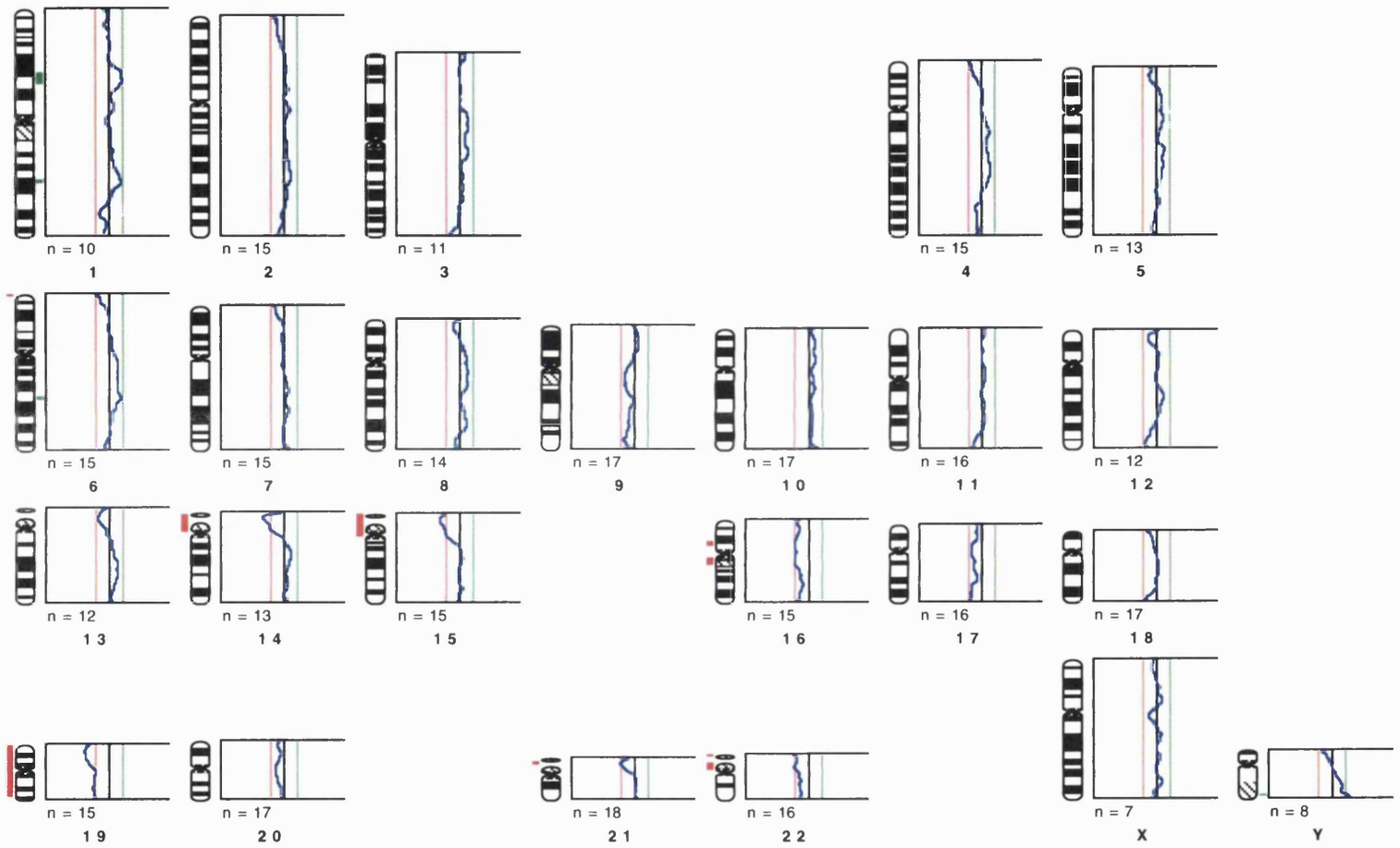


Figure 4.16: CGH composite of U40, an ependymoma. The composite is compiled from the average of the individual ratio profiles from nine metaphases. This tumour had a single aberration, which was monosomy of chromosome 19.

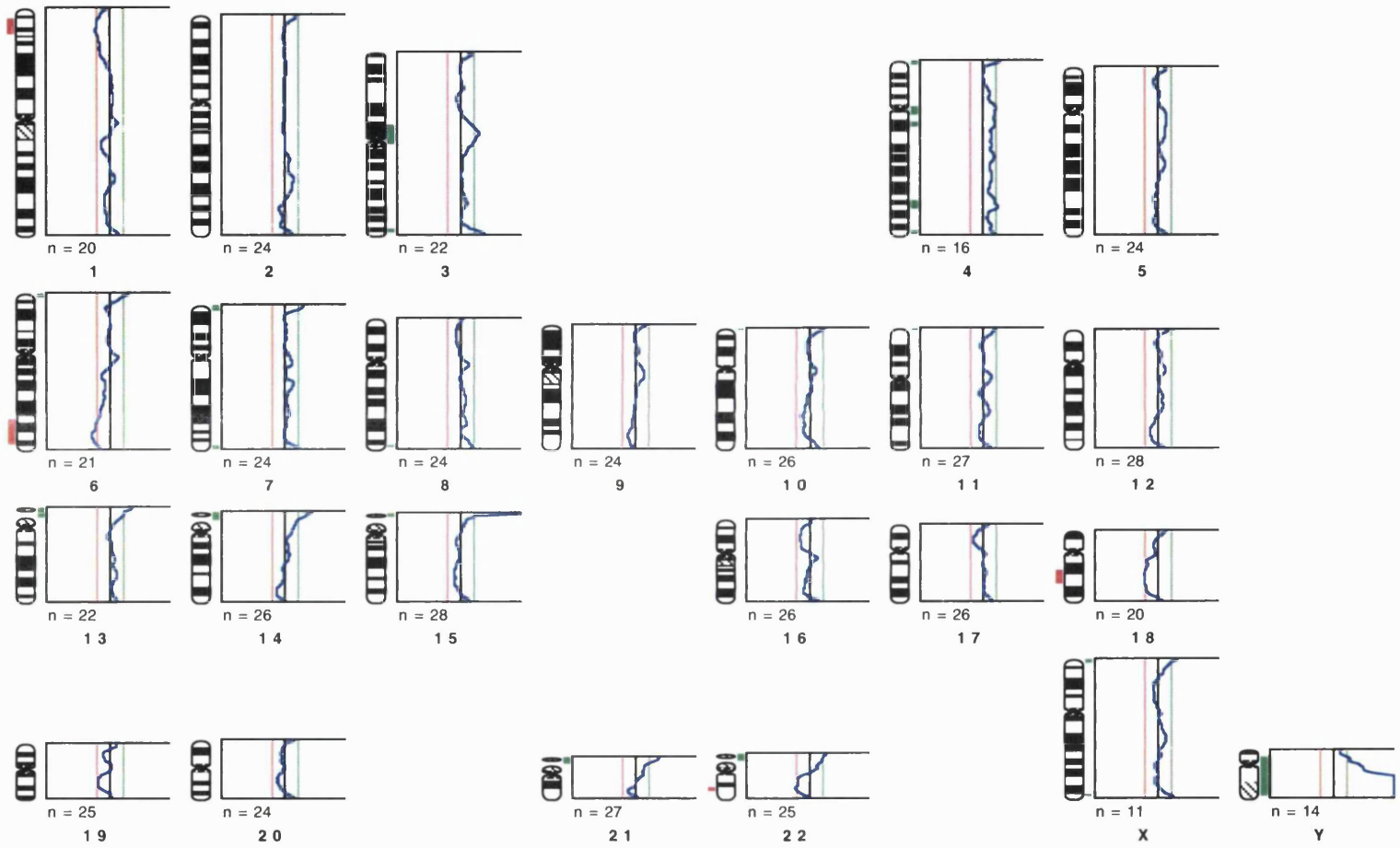


Figure 4.17: CGH composite of IN1258, a recurrent ependymoma. The composite is compiled from the average of the individual ratio profiles from fifteen metaphases. This tumour has four CNAs, all involving regions of deletion. There is loss of 1p32-36, 6q22-qter, 18q11-22 and monsomy of chromosome 22.

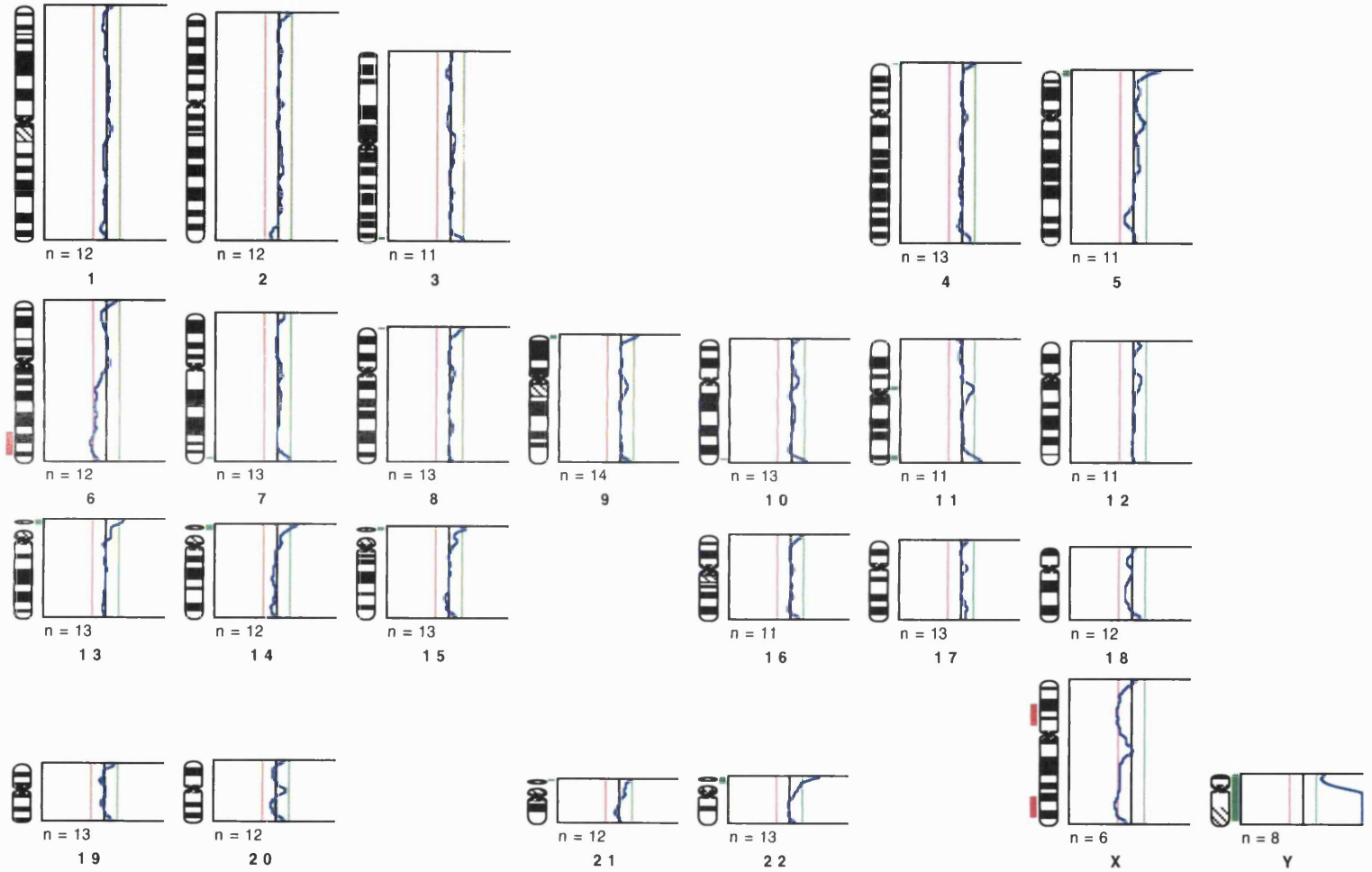


Figure 4.18: CGH composite of IN1497, a recurrent ependymoma. The profile is compiled from the average of the individual ratio profiles from eight metaphases. This tumour has a single aberration; loss of 6q16-qter.

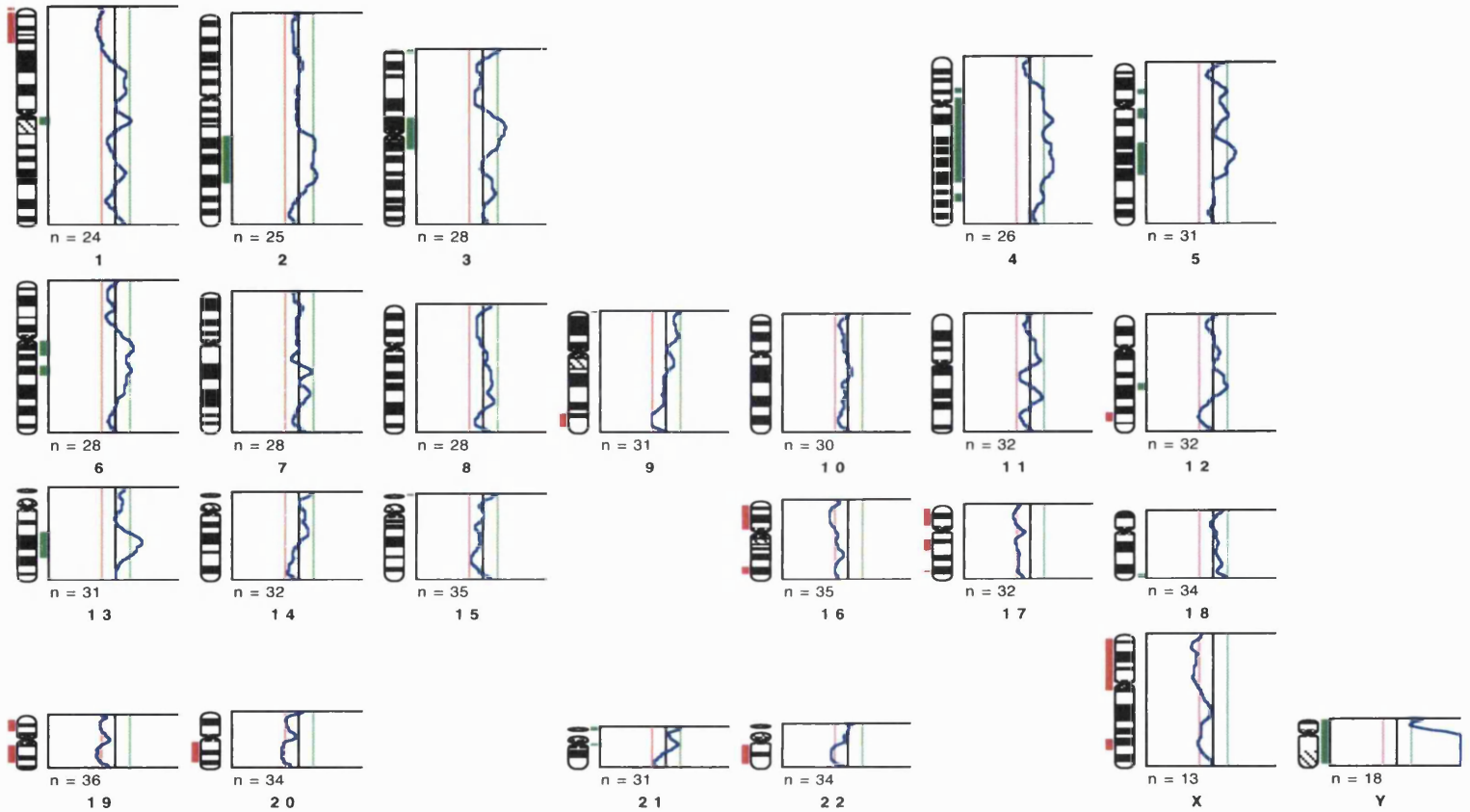


Figure 4.19: CGH composite of IN2923, a recurrent ependymoma. The composite is compiled from the average of the individual ratio profiles from eighteen metaphases. There are fourteen CNAs. There are regions of gain at 2q22-33, 4p14-q32, 5q14-23, 6q11-22, 12q14-21 and 13q14-31. There is monosomy of chromosomes 17, 19 and 22, and loss of 16p and 20q. There are also smaller regions of deletion at 1p31-pter, 9q31-qter and 12q23-qter.

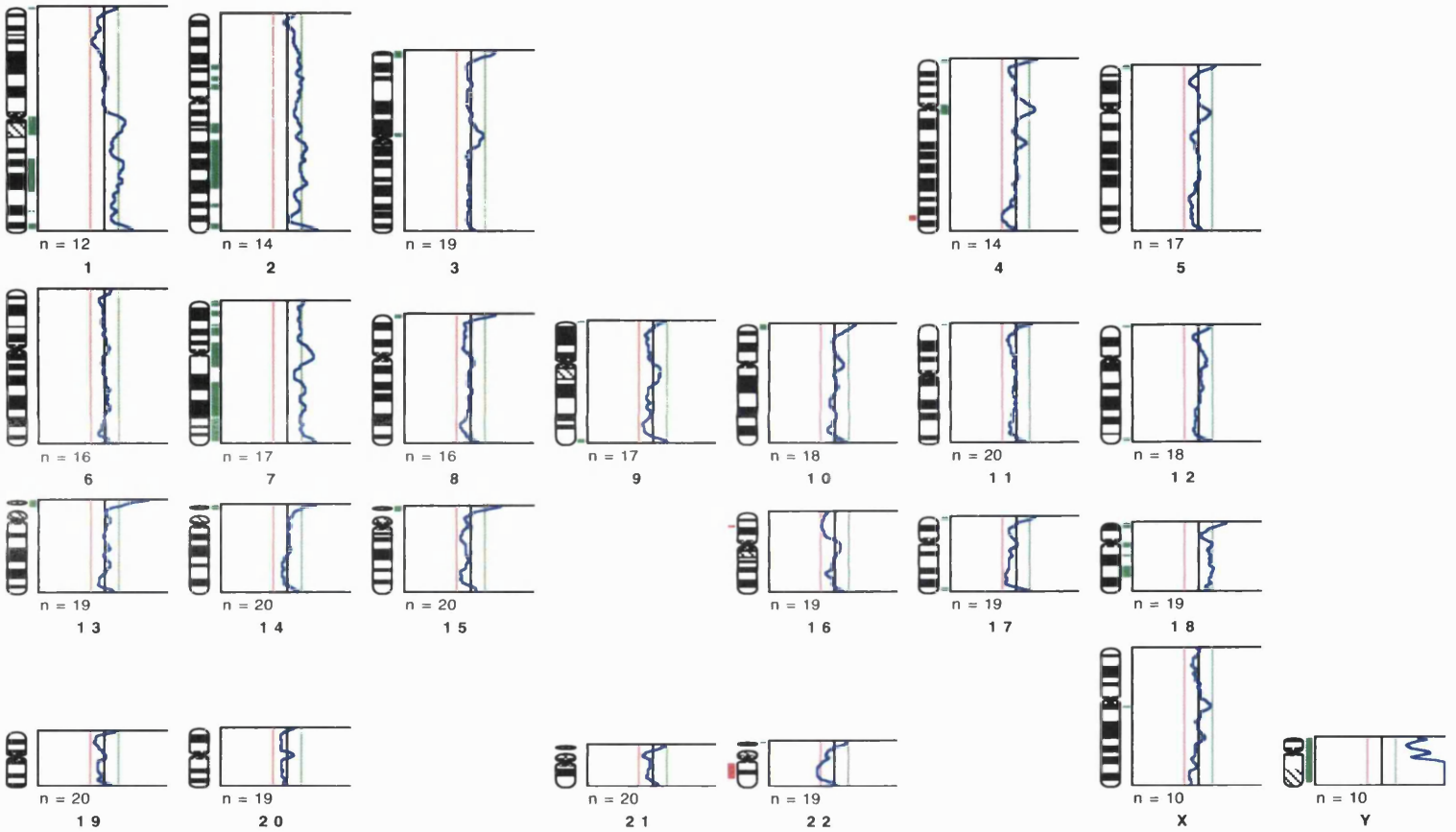


Figure 4.20: CGH composite of IN2855, a recurrent anaplastic ependymoma. The composite is compiled from the average of the individual ratio profiles from ten metaphases. This tumour has five CNAs. There are gains involving whole chromosomes (2 and 7), as well as individual chromosome arms (1q and 18q). There is also monosomy of chromosome 22.

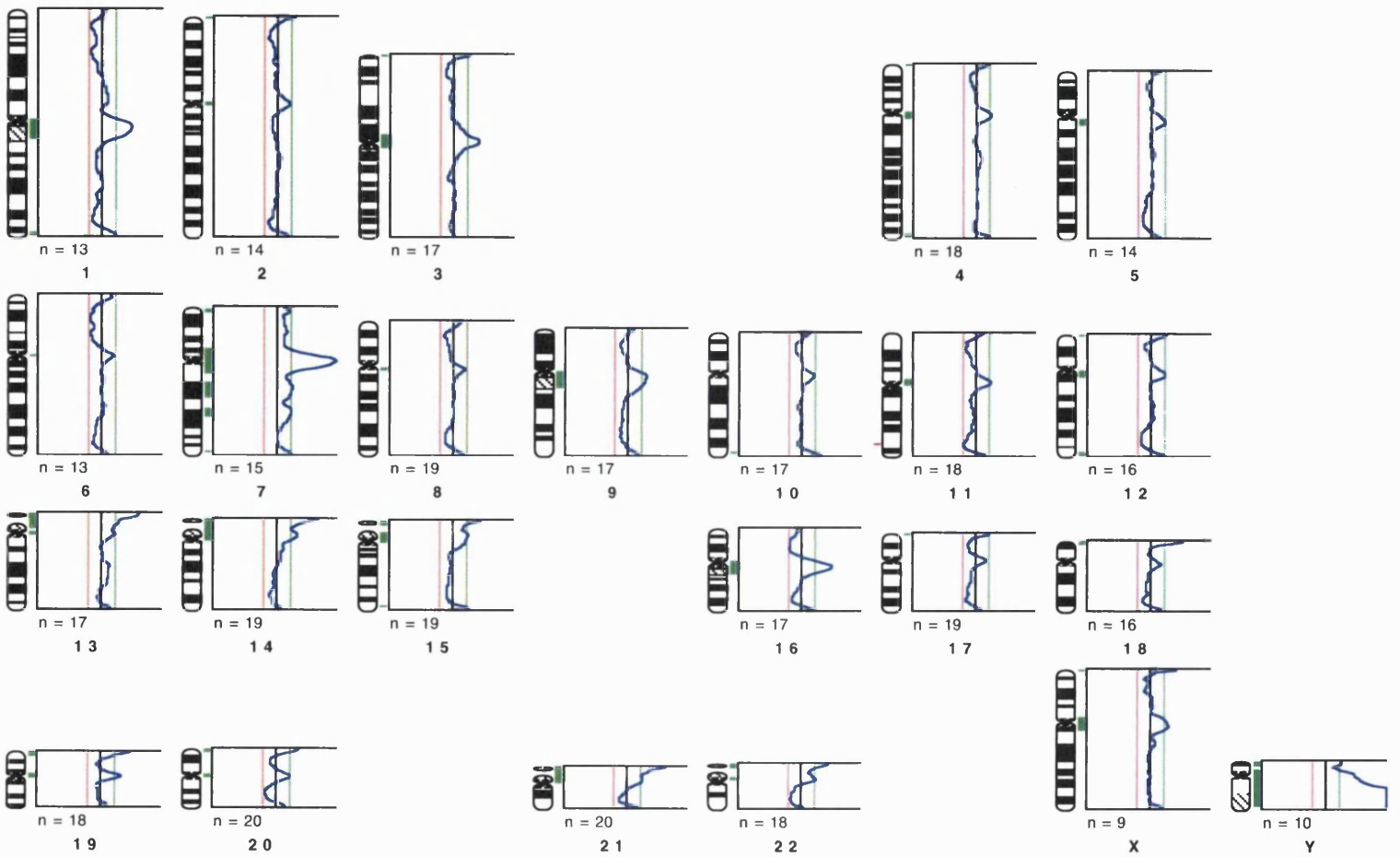


Figure 4.21: CGH composite of IN2929, a recurrent anaplastic ependymoma. The composite was compiled from the average of the individual ratio profiles from ten metaphases. This tumour had a single aberration, which was gain of 7q11-31. The apparent regions of gain at the centromeres and telomeres of most chromosomes are not counted as true aberrations, as they are a result of hybridisation artifact.

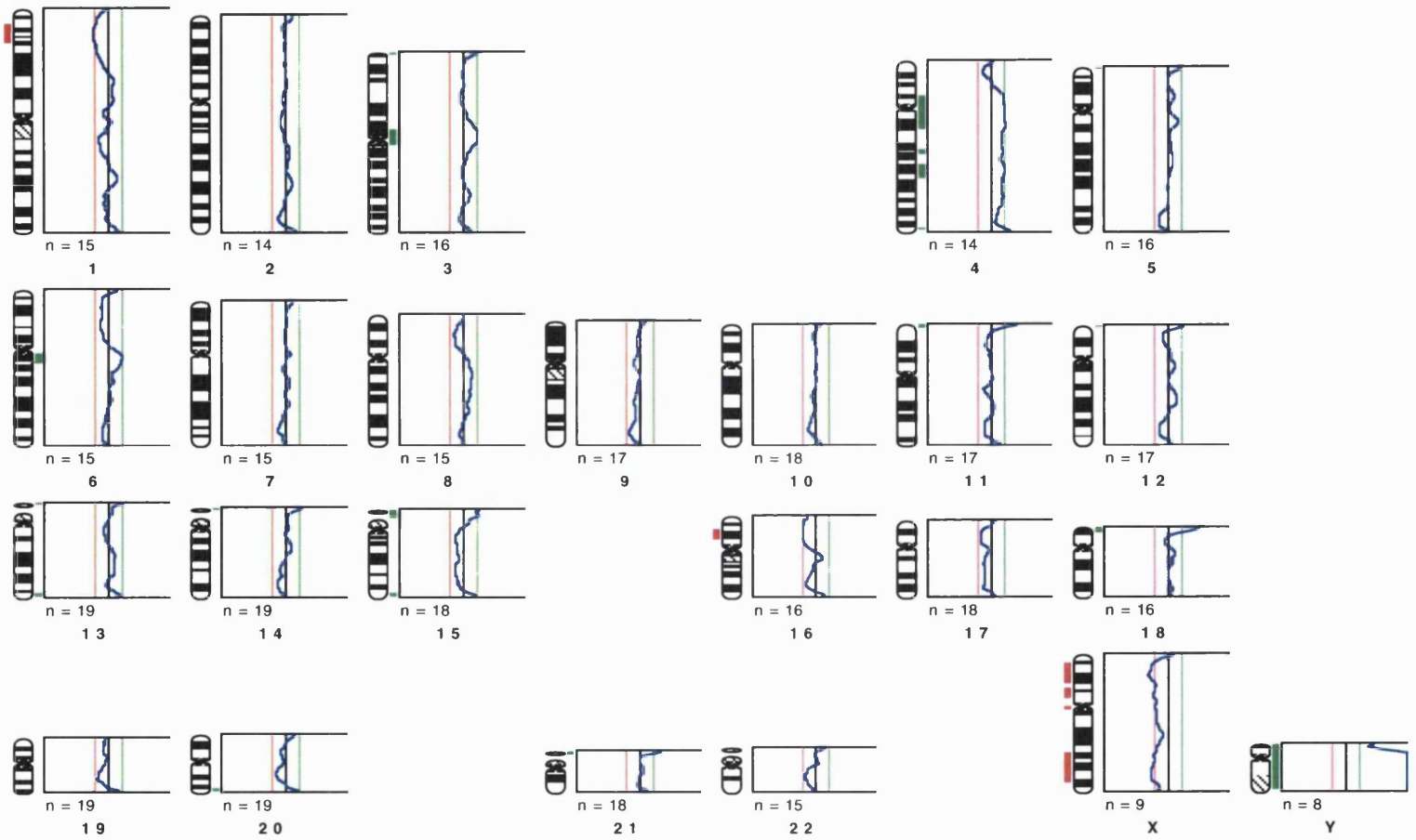


Figure 4.22: CGH composite of IN772, a subependymoma. The composite was compiled from the average of the individual ratio profiles from ten metaphases. There are three CNAs. There is gain of 4p14-q32, loss of 16p and a small region of deletion at 1p32-pter. The recurrence of this tumour, IN1134, had no alterations.

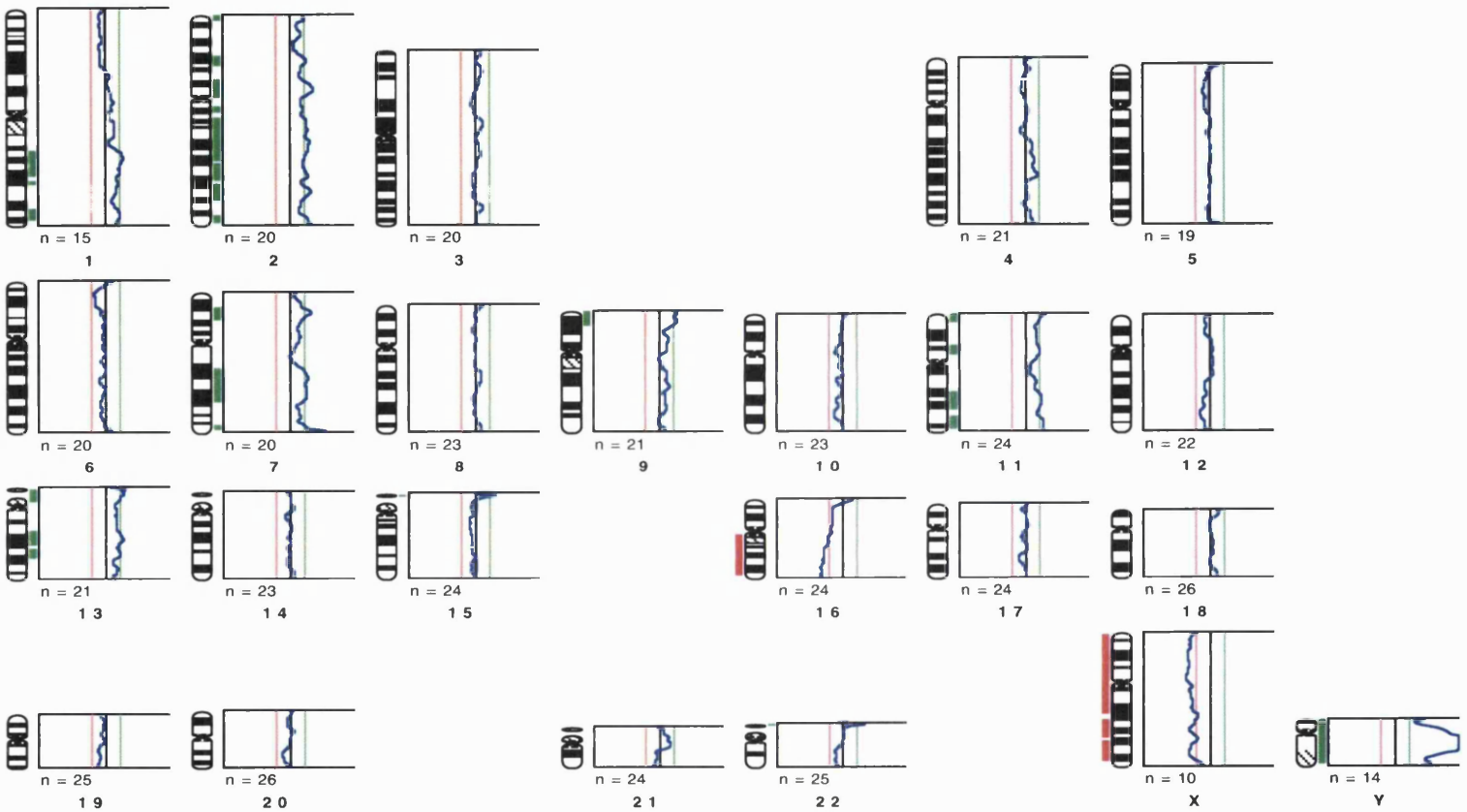


Figure 4.23: CGH composite of IN1759, an ependymoma. The composite is compiled from the average of the individual ratio profiles from fourteen metaphases. There are eight CNAs. There is gain of whole chromosomes (11 and 13), as well as smaller regions of gain at 1q22-qter, 2p16-qter, 7p13-pter, 7q21-32 and 9p13-pter. There was also loss of 16q.

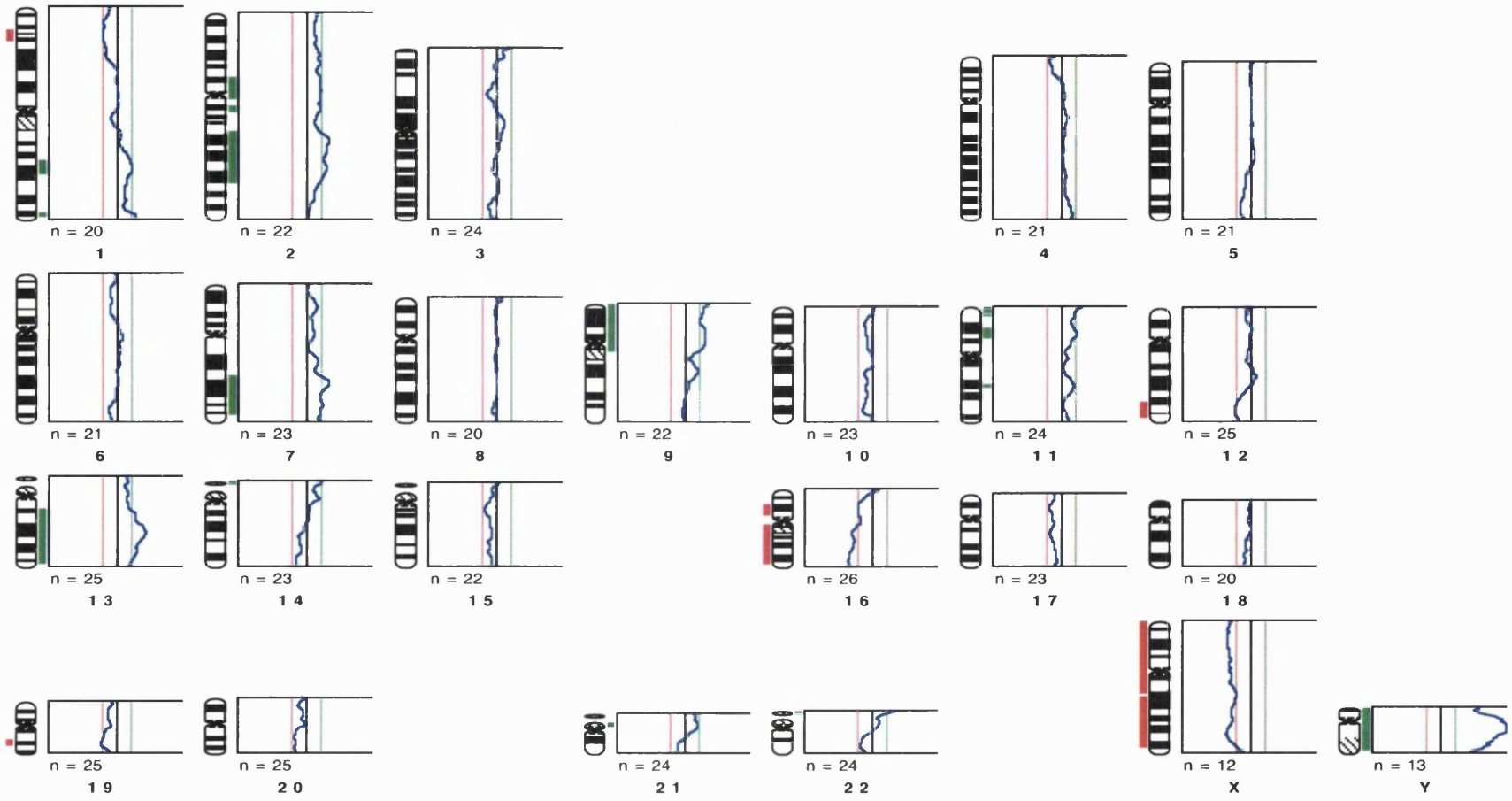


Figure 4.24: CGH composite of IN2376. This tumour is the first recurrence of IN1759. The composite is compiled from the average of the individual ratio profiles from thirteen metaphases. There are ten CNAs. There are gains of entire chromosomes (13) and of individual chromosome arms (9p and 11p). There are also smaller regions of gain at 1q24-qter, 2p14-q32 and 7q22-qter. There are also regions of deletion at 12q22-qter, 16p13.1-qter and 19p.

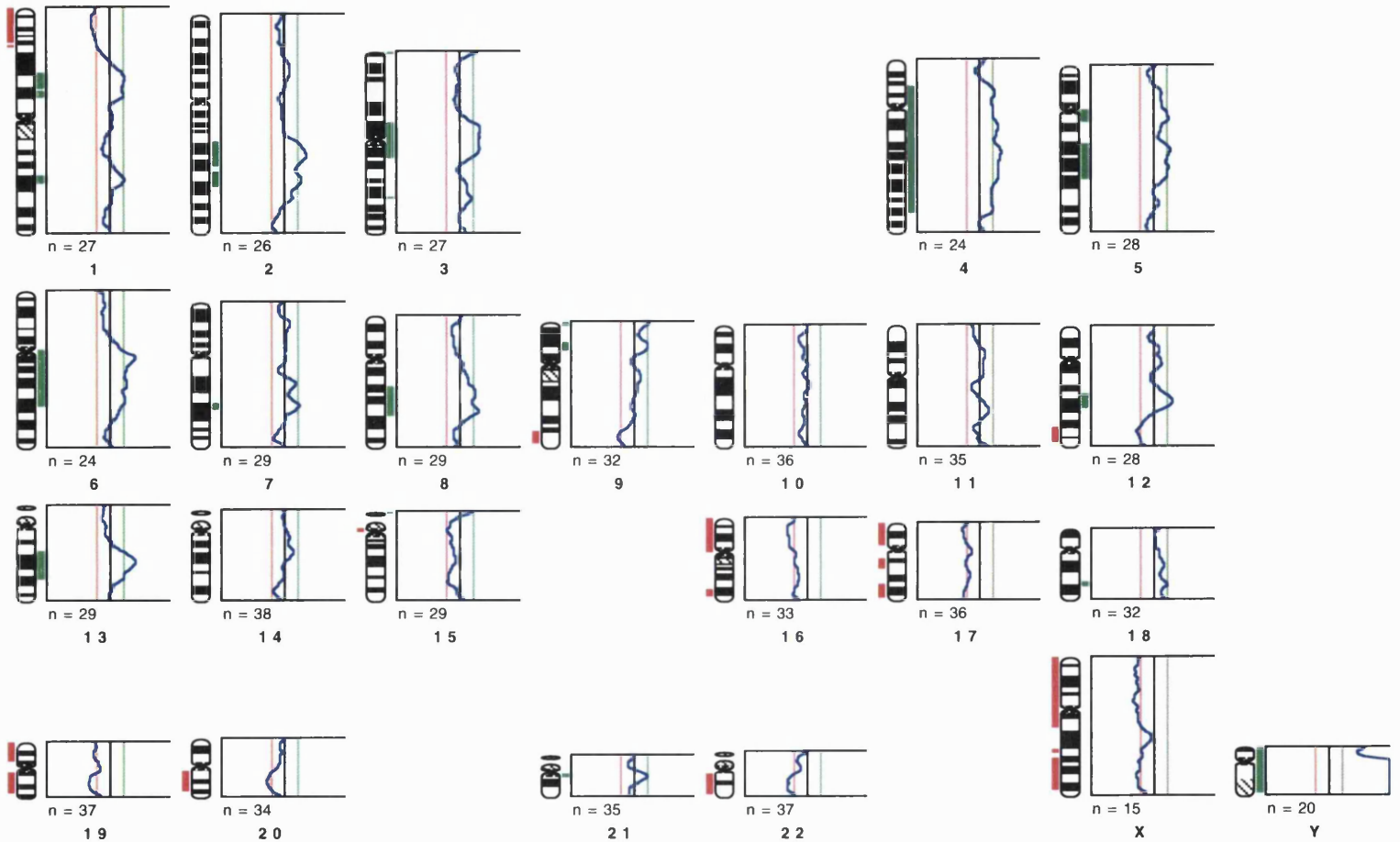


Figure 4.25: CGH composite of IN2638, an ependymoma. The composite is compiled from the average of the individual ratio profiles from twenty metaphases. There are twenty CNAs. There is gain of 1p22-31, 1q24-31, 2q22-32, 4p14-q32, 5q11-23, 6q11-22, 7q21-31, 8q21-23, 9p, 12q14-21, 13q21-31 and 18q12-qter. There is monosomy of chromosomes 16, 17, 19 and 22. There is also loss of 20q and small regions of deletion at 1p32-pter, 9q32-qter and 12q23-qter.

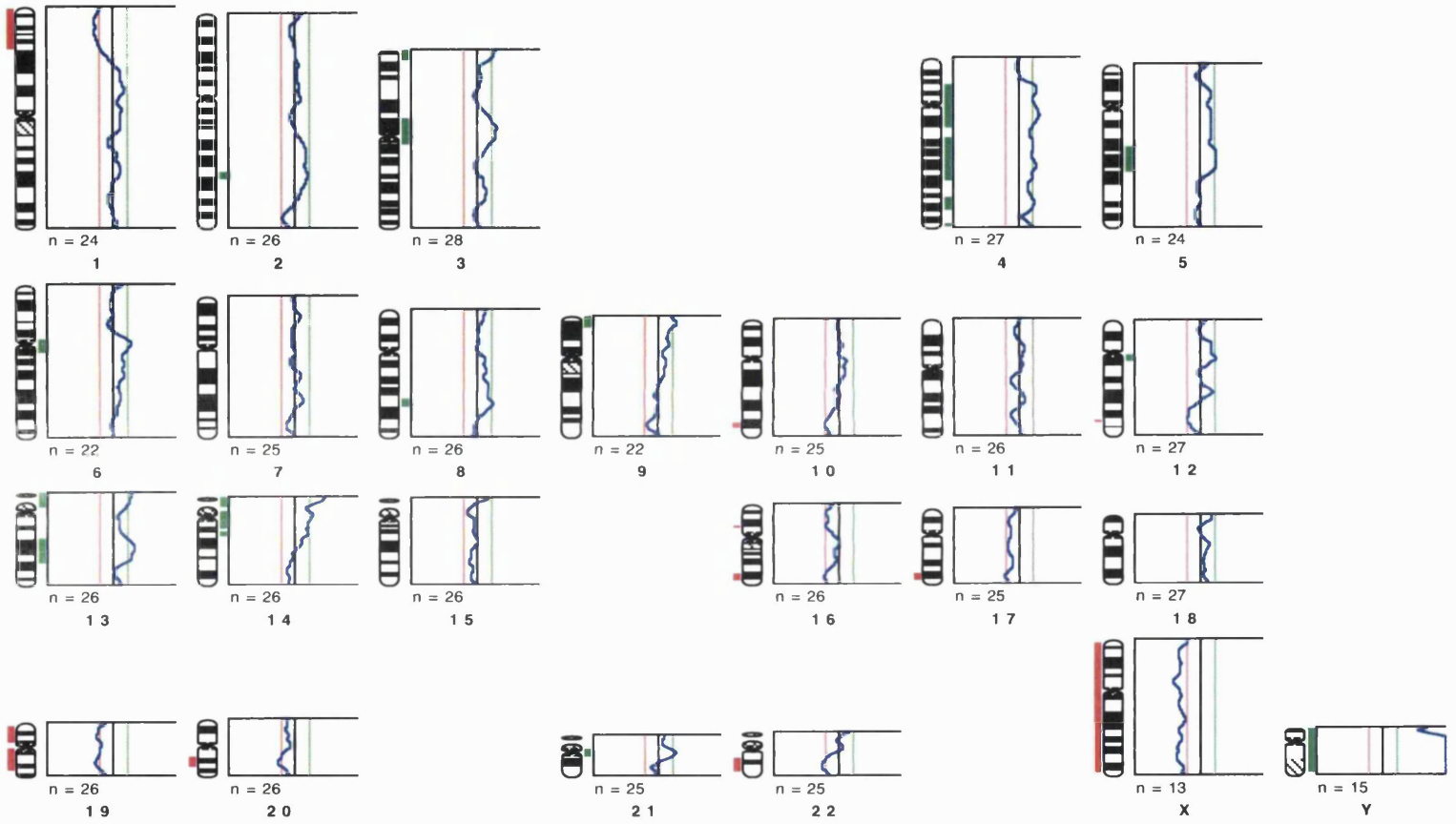


Figure 4.26: CGH composite of IN2887, the first recurrence of IN2638. The composite is compiled from the average of the individual ratio profiles from fifteen metaphases. There are eleven CNAs. There are regions of gain at 2q22-32, 4p14-q33, 5q14-23, 8q21-23, 9p and 13q21-31. There is monosomy of chromosomes 19 and 22, and smaller regions of deletion at 1p32-pter, 12q23-24 and 20q.

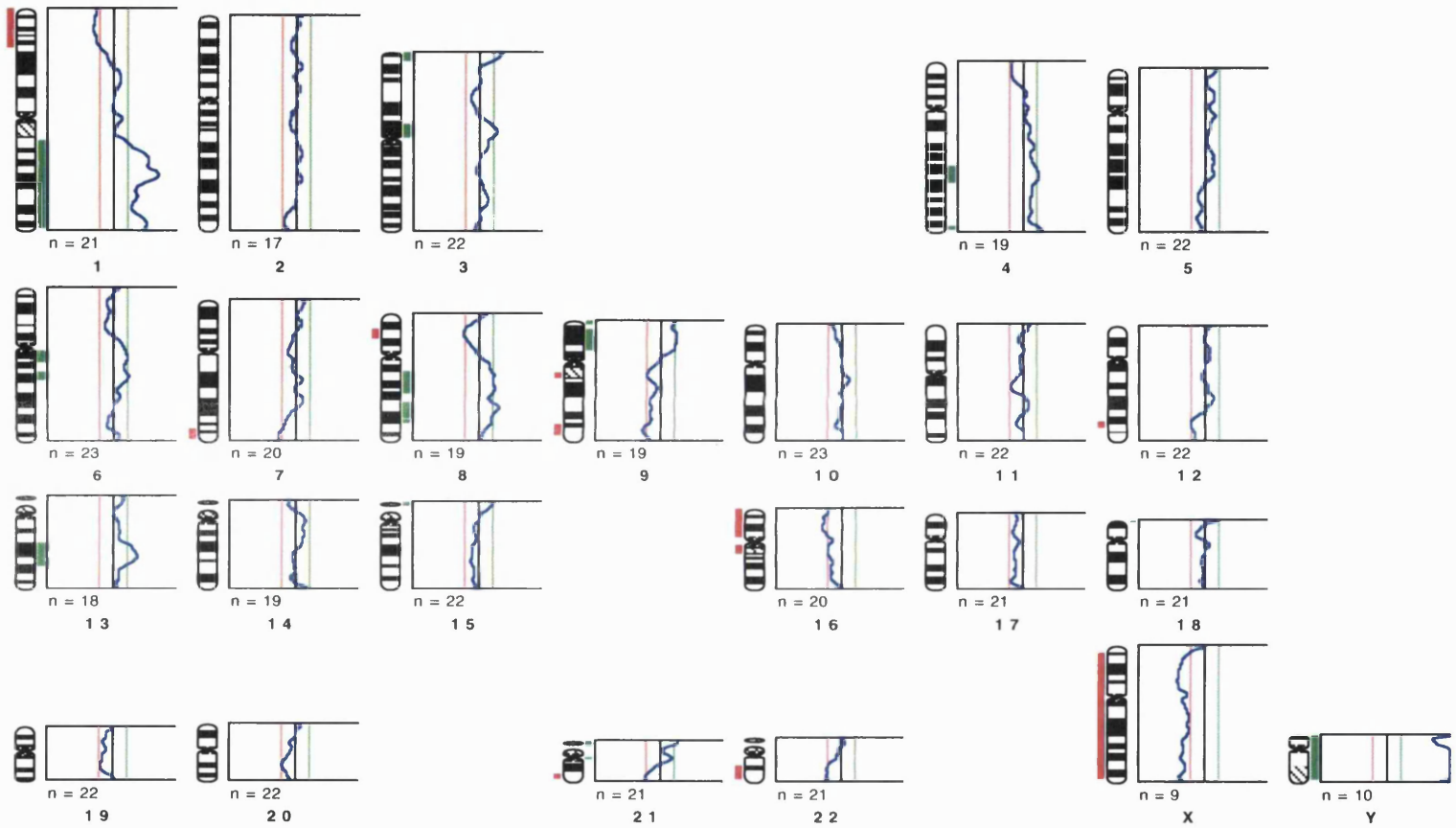


Figure 4.27: CGH composite of IN2904, a recurrent ependymoma. The composite is compiled from the average of the individual ratio profiles from eleven metaphases. There is one region of high copy number amplification at 1q22-31 and eleven other CNAs. There are gains of individual arms (1q and 9p) and smaller regions of gain at 4q23-27, 6q11-21, 8q12-23 and 13q21-31. There is monosomy of chromosome 22, loss of 9q and 16p and a smaller region of loss at 1p32-pter.

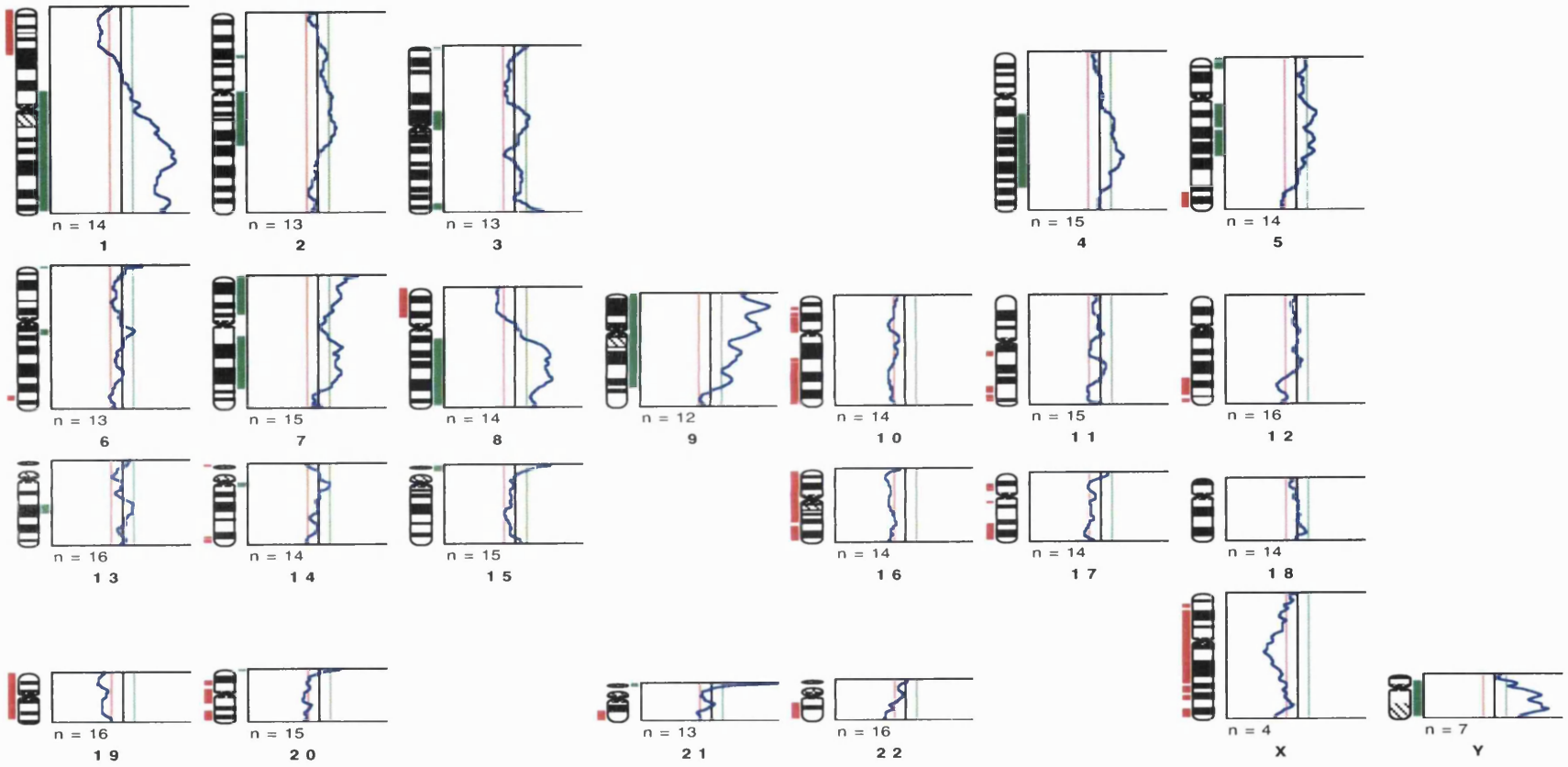


Figure 4.28: CGH composite of IN2970, the second recurrence of IN2904. The composite is compiled from the average of the individual ratio profiles from eight metaphases. There are three regions of high copy number amplification at 1q11-44, 8q12-23 and 9p24-q12, as well as sixteen other CNAs. There are regions of gain at 2q11-23, 4q13-31.3, 5q11-23, 7pter-q32, 9q22-33 and 13q14-22. There is monosomy of chromosomes 10, 16, 17, 19, 20 and 22, and smaller regions of loss at 1p31-pter, 8q, 11q22-qter and 12q21-qter.

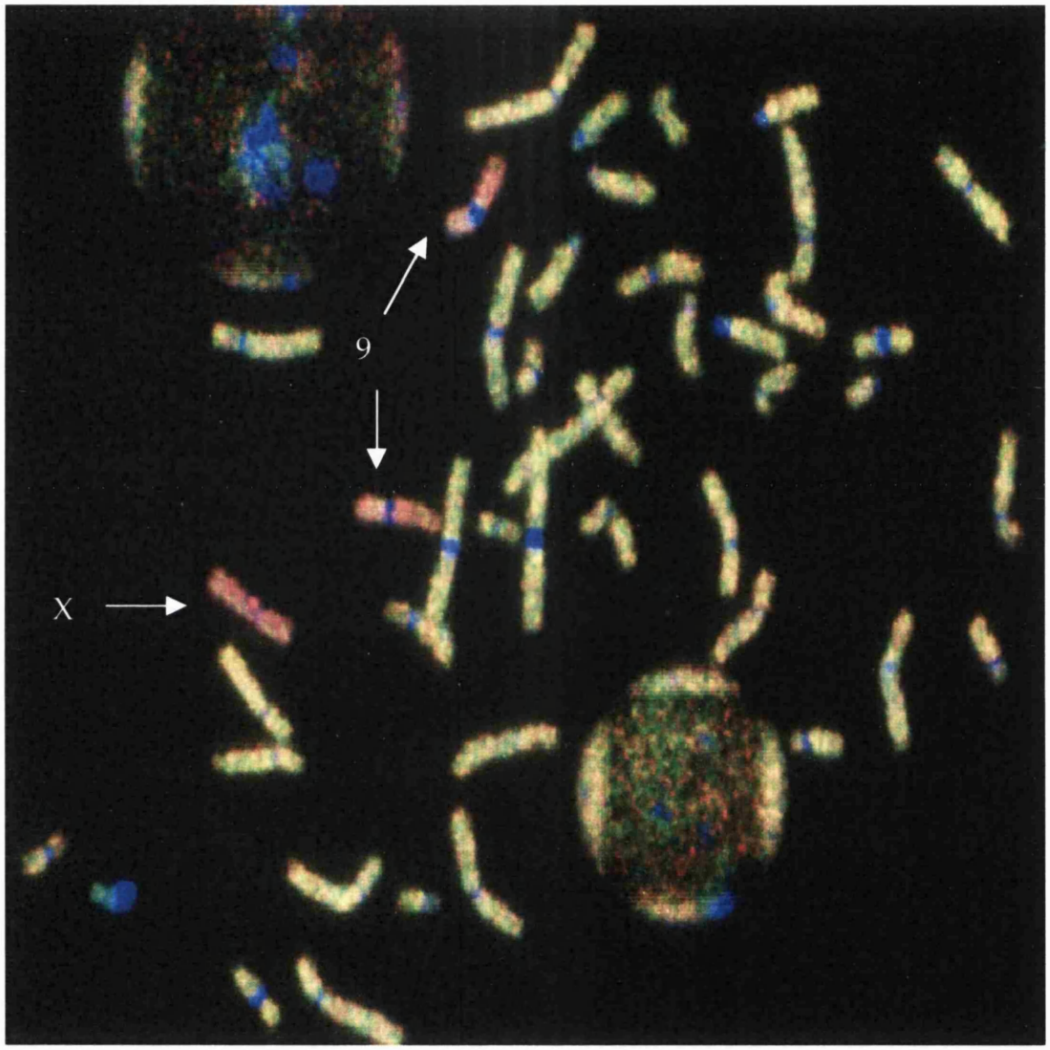


Figure 4.29: IN2944, an ependymoma showing the sole abnormality of loss of chromosome 9, indicated by the red chromosomes in the picture. The X chromosome also shows up red as the tumour is from a male patient who only has one copy of the X chromosome compared to the two copies present in the female reference DNA.

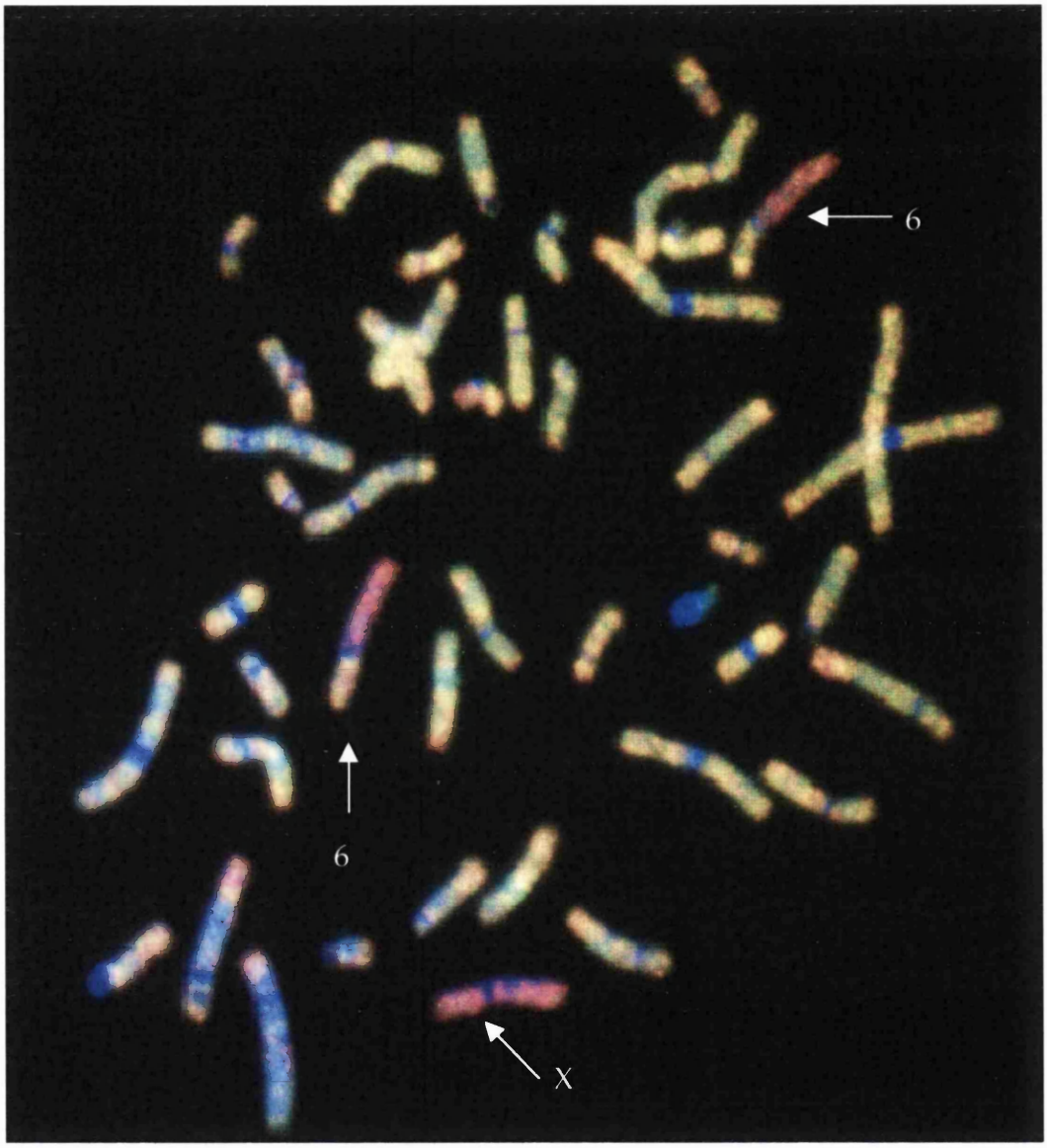


Figure 4.30: IN1497, an ependymoma with the sole abnormality of loss of 6q, indicated by the red portions of chromosome 6. The X chromosome is also red due to the tumour coming from a male patient with only one X chromosome compared to the two copies present in the female reference DNA.

(IN2970). Unlike astrocytoma, gains and losses were equally common in ependymoma.

Estimation of minimum overlapping regions

The minimum overlapping regions of alteration in ependymoma can be summarised as follows:

Chromosome 1: There was a region of loss confined to 1p32-36.6. There were two regions of gain that could be minimised to 1p21-31 and 1q25-31. The region of gain on 1q also includes the area of high copy number amplification seen in IN2904, IN2970 and IN2939.

Chromosome 2: There were two distinct regions of gain on chromosome 2, minimised to 2p11.2-16 and 2q22-32.

Chromosome 4: The minimum region of overlap on chromosome 4 could be localised to 4q22-28. Six cases also showed gain extending into the p arm of chromosome 4, which could be minimised to 4p11-14.

Chromosome 5: The minimum region of gain on chromosome 5 could be localised to 5q15-21.

Chromosome 6: There were two distinct regions of alteration on chromosome 6. There was a region of gain that could be minimised to 6q11-16 and a region of loss at 6q23-27.

Chromosome 7: There were two regions of gain on chromosome 7 that could be localised to 7p11-15 and 7q21-31.

Chromosome 8: There was a region of gain that could be minimised to 8q21.2-22. This region of gain incorporated the region of high copy number amplification seen in tumours IN2939 and IN2970.

Chromosome 9: There were two distinct regions of alteration on chromosome 9. These were a region of gain at 9p21-24, that included the region of high copy number amplification seen in tumour IN2970, and a region of loss at 9q32-qter.

Chromosome 10: There was a region of loss on chromosome 10 that could be localised to 10q23-qter.

Chromosome 12: There were two distinct regions of alteration on chromosome 12, a region of gain at 12q15-21 and a region of loss at 12q23-qter.

Chromosome 13: The minimum region of gain on chromosome 13 could be localised to 13q21.

Chromosome 16: There are two region of loss on chromosome 16 that can be localised to 16p11-12 and 16q11-qter.

Chromosome 18: There was a region of gain on chromosome 18 that could be minimised to 18q12-qter.
Chromosomes 17, 19, 20 and 22: The regions of deletion on these chromosomes were quite large and spanned the majority of the chromosome, making it difficult to estimated a minimum region of overlap.

Association between CNAs and clinicopathological criteria

All statistical analysis was performed using GraphPad Prism® software.
Two-way contingency tables were assessed by Fishers exact test (Rees, 1994) in order to determine whether any CNA could be associated with clinicopathological features such as tumour grade, location, recurrence, patient age and sex and survival. Tables of all p values can be seen in Appendix III. There were only two CNAs that could be associated with any of the features examined and these were gain of 7q ($p = 0.041$) and loss 12q ($p = 0.027$), which were seen at a higher frequency in recurrent tumours compared to primary tumours. Individual CNAs could not be associated with tumour location or patient age and sex. Loss 19q in benign tumours was approaching statistical significance ($p = 0.088$). However, the fact that no individual abnormality could be associated with tumour grade may be due to the relatively small number of anaplastic tumours in this study group.

Survival data was available for forty-one of the forty-four patients in this study and was analysed using the Log-rank test to determine whether any individual CNA or clinicopathological feature influenced survival in these patients. The five year overall survival for these patients was 62% and the ten year survival was 29% (see Figure 4.31).

Clinicopathological criteria including patient age, sex, tumour location, tumour grade or recurrence had no effect on patient survival. The extent of tumour resection (macroscopic v partial removal) also had no effect on survival. The only criteria that influenced survival for the patients in this study was the treatment regimen. Patients who underwent surgery alone had the worst survival (all patients dead 18 months post-operatively). Patients who were treated with surgery and radiotherapy had the best outcome, with the 5 year survival for this group of patients being 90%. Those patients who had been treated with surgery and chemotherapy and a combination of surgery, radiotherapy and chemotherapy had a 5 year survival of 60%. Ten year survival data was only

available for those patients who had received surgery and radiotherapy (75%). All patients in the group who had received surgery radiotherapy and chemotherapy had died by 96 months post-diagnosis (see Figure 4.32).

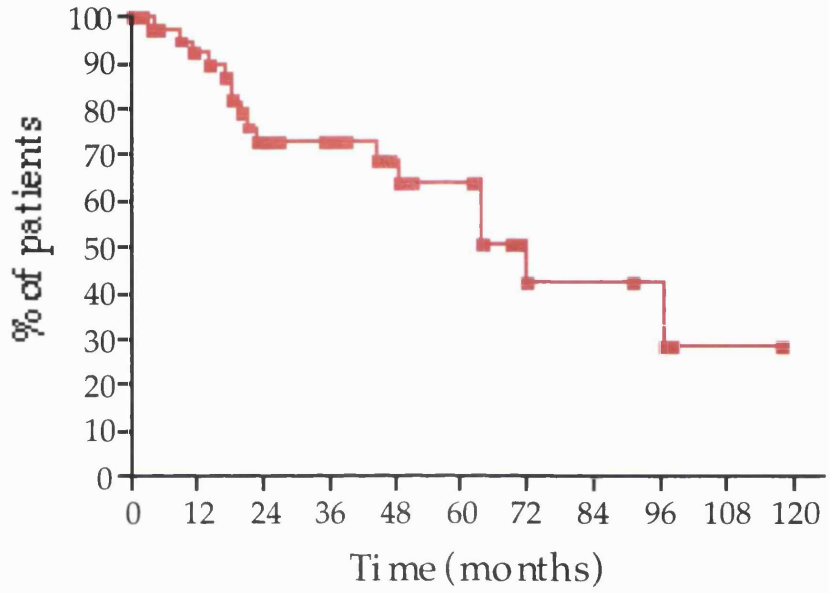


Figure 4.31: Overall survival in 41 paediatric ependymoma patients. Survival is measured in months from the time of initial diagnosis.

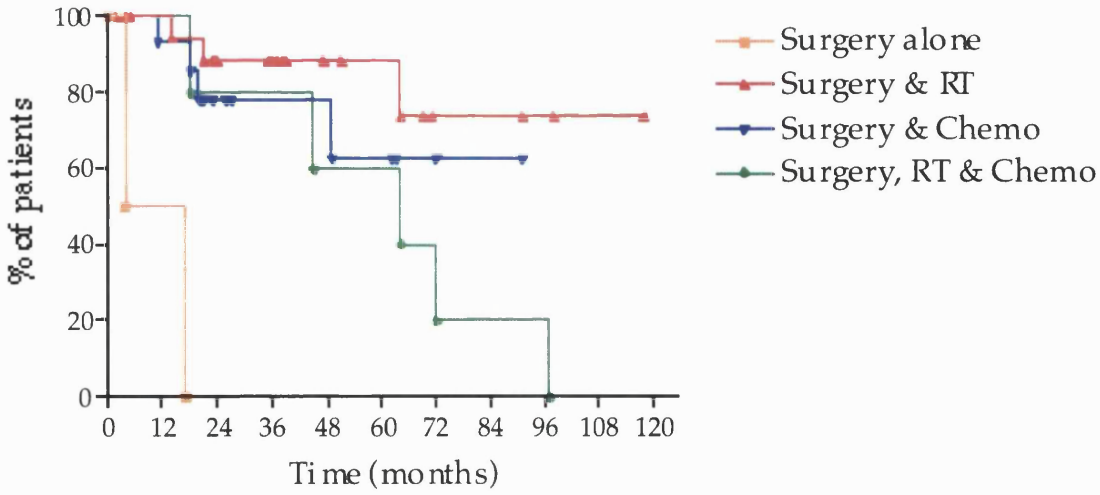


Figure 4.32: The effects of treatment regimens on the survival of patients with ependymoma. Survival was significantly associated with treatment regimens involving surgery in combination with either chemotherapy or radiotherapy (log-rank test, $p = <0.0001$).

Only one aberration appeared to influence survival and this was loss of chromosome 19 (p = 0.037). Patients who had this aberration had a better outcome than those with no alteration of chromosome 19. The five-year survival for patients with this abnormality was 90% compared to 50% in those patients with no loss of chromosome 19 (see Figure 4.33).

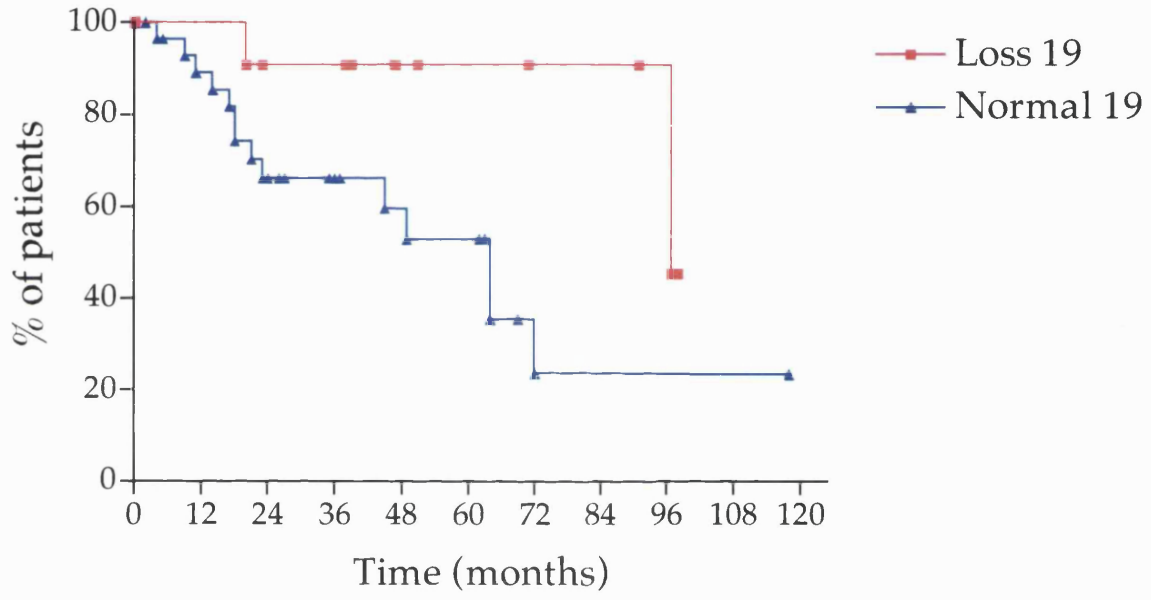


Figure 4.33: Survival of ependymoma patients with loss of chromosome 19 compared to those patients with no loss of chromosome 19. Survival is measured in months from the time of initial diagnosis (Log-rank test, p = 0.0370).

Association between copy number aberrations

Data analysis demonstrated that a large number of aberrations appeared to occur in conjunction with one another (see Table 4.2). Fishers exact test was used to identify pairs of aberrations and the Mantel-Haenszel test (Kirkwood, 1998) identified larger groups of aberrations that occurred together. From the analysis it was clear that these ependymoma could be split into three groups depending on their CNAs. Three tumours had alterations that appeared in both the group 1 and group 2 tumours and have therefore been included in the analysis of both groups.

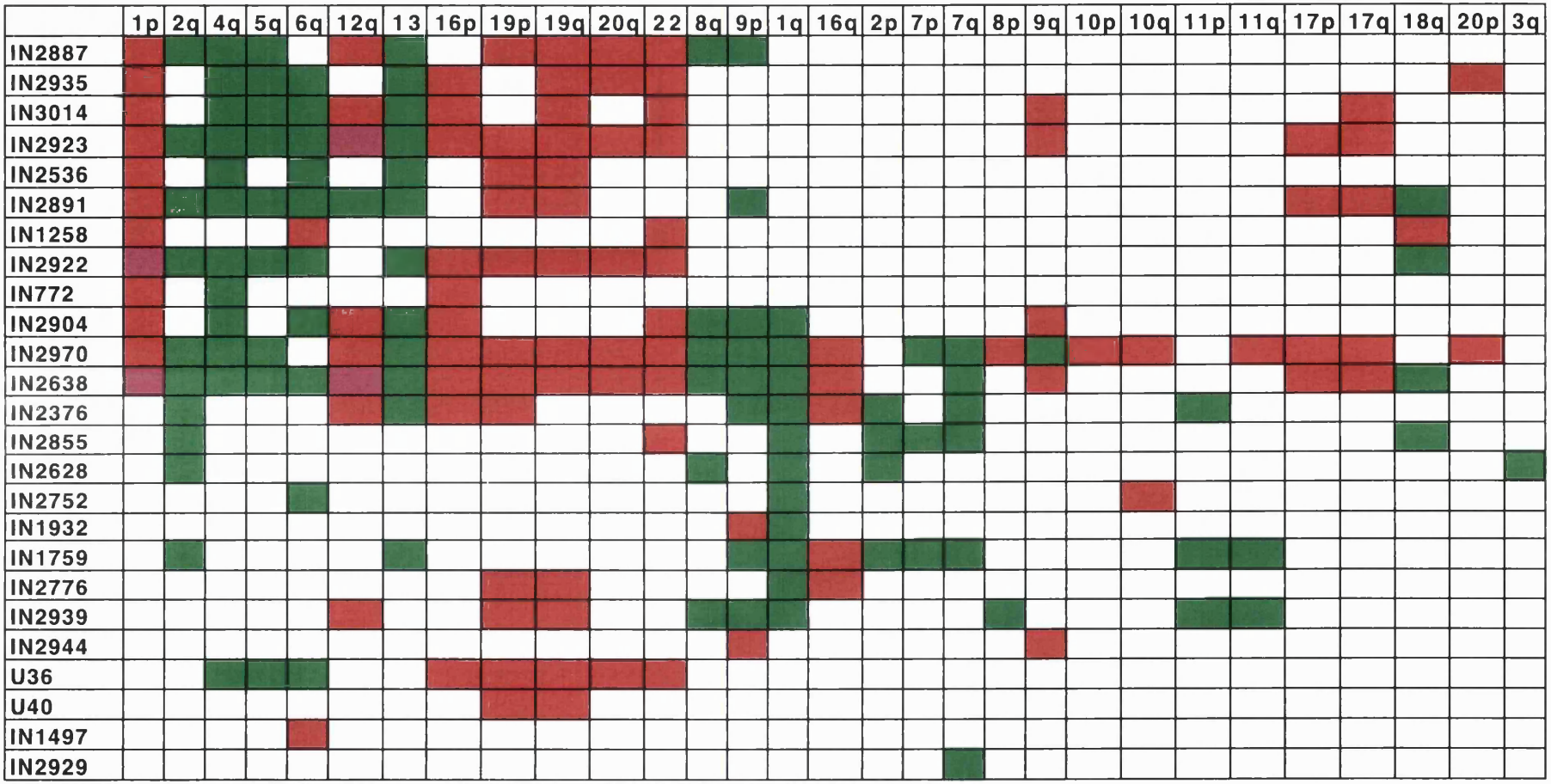


Table 4.2: Association between CNAs in ependymoma samples. Samples had either loss of 1p and associated aberrations or gain of 1q and associated aberrations.

The first group of tumours (n = 12) had seventeen CNAs that appeared to occur together. The primary alteration was loss of 1p and this was seen together with gain 4q (11 cases), gain 13 (10 cases), loss 19q (9 cases), loss 22 (9 cases), gain 5q (8 cases), gain 6q (8 cases), loss 16p (8 cases), loss 19p (7 cases), gain 2q (6 cases), loss 20q (6 cases), loss 12q (6 cases), loss 17q (5 cases), gain 9p (5 cases), loss 17p (4 cases), gain 8q (4 cases), gain 18q (3 cases), and loss 20p (2 cases). This association was statistically significant at the 95% confidence level (p = 0.008). Within this group of associated CNAs there were pairs of alterations occurring together that were also statistically significant. Details of these can be seen in Table 4.3.

The second group of tumours (n = 11) had eleven CNAs that occurred together. The predominate alteration was gain of 1q (11 cases), seen in conjunction with gain 2q (6 cases), gain 9p (6 cases), gain 7q (5 cases), gain 8q (5 cases), loss 12q (5 cases), gain 13 (5 cases), loss 16q (5 cases), loss 19p (5 cases), loss 19q (4 cases), gain 6q (3 cases) and gain 11p (3 cases). The association of all of these alterations was approaching statistical significance (p = 0.0832). An association between gain of 1q occurring in conjunction with gain 2q, 6q, 7q, 8q, 9p, 11p, 13 and loss of 12q and 16q was statistically significant at the 95% confidence level (p = 0.0462). Within this second group of alterations there were a number of pairs of alterations that were seen together that were statistically significant (see Table 4.4).

The third group of tumours (n = 26) was complied of the tumours that had no detectable CNAs (n = 21) and five tumours (IN2944, IN1497, IN2929, U36 & U40) that had sole abnormalities or did not fit into either of the two previous groups.

Analysis of survival data did not show a significant difference in patient survival for these three groups of tumours (see Figure 4.34). The association of each group with clinicopathological features was also analysed . There was no association with tumour grade (p = 0.2683) or tumour location (p = 0.4884). There was a significant association between the group 3 tumours and younger patient age (p = 0.0543).

Alteration	No. tumours with alteration	*p* - value
Loss 1p & gain 4q	11	<0.0001
Loss 1p & gain 5q	8	0.0036
Loss 1p & gain 6q	8	0.0154
Loss 1p & gain 13	10	0.0012
Loss 1p & loss 16p	8	0.0154
Loss 1p & loss 17p	4	0.0391
Loss 1p & loss 17q	5	0.0149
Loss 1p & loss 19q	9	0.0472
Loss 1p & loss 22	9	0.0048
Gain 4q & loss 19q	10	0.0048
Loss 19q & loss 20q	7	0.0052
Gain 5q & gain 13	8	0.0036
Gain 13 & gain 9p	7	0.0112
Gain 4q & loss 22	10	0.0002
Gain 4q & loss 16p	9	0.0010
Gain 4q & loss 17p	4	0.0391
Gain 4q & loss 17q	5	0.0149
Gain 4q & gain 5q	8	0.0036
Gain 5q & gain 6q	6	0.0036
Gain 4q & gain 13	10	0.0012
Loss 16p & gain 13	8	0.0051
Gain 5q & loss 22	8	0.0021
Loss 16p & loss 22	7	0.0486

Table 4.3: Pairs of CNAs occurring in the group 1 tumours that are statistically significant at the 95% confidence level, as determined by Fishers exact test.

Alteration	No. tumours with alteration	*p*- value
Gain 1q & gain 7q	5	0.0561
Gain 1q & gain 8q	5	0.0561
Gain 1q & loss 16q	5	0.0087
Gain 2p & gain 2q	4	0.0101
Gain 2q & gain 7q	4	0.0230
Gain 7q & gain 9p	4	0.0593
Gain 7q & loss 16q	4	0.0055

Table 4.4: Pairs of CNAs occurring together in group 2 tumours that are statistically significant at the 95% confidence level, as determined by Fishers exact test.

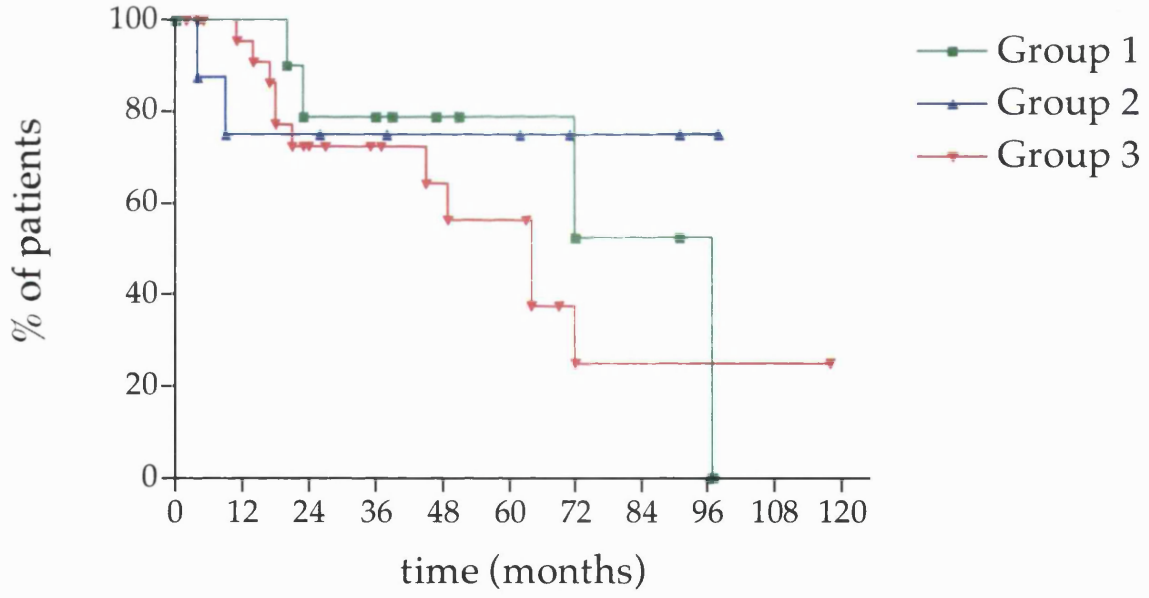


Figure 4.34: Survival in ependymoma patients grouped according to their CNAs (Log-rank test, p = 0.4551). Patients in group 1 had loss of 1p and associated alterations, patients in group 2 had gain of 1q and associated alterations and patients in group 3 had either sole abnormalities or no detectable abnormalities. Survival is measured in months from the date of initial diagnosis.

Tumour recurrence

There were six primary and recurrent paired samples in this study and two recurrent samples from the same patient. No primary tumour was available for analysis in this case. Three of the paired samples (IN1231 & U37, IN2682 & IN2766, and IN3037 & IN3108) had no detectable CNAs in either the primary or the recurrent tumour. IN772 had three CNAs but the recurrent sample, IN1134, had no regions of alteration. IN1759 had seven CNAs and the recurrent sample, IN2376, had nine CNAs. The major difference was the recurrent sample, IN2376, had two regions of loss (12q and 19q) that were not present in the primary sample. IN2638 had twenty CNAs compared to eleven in the recurrent sample, IN2887. The primary sample had extra regions of alteration involving gain of 1, 6q, 7q 12q and 18q and regions of loss at 9q 16 and 17. IN2904 was a first recurrence of a benign ependymoma and had twelve CNAs (see Figure 4.35). The second recurrence, IN2970, had nineteen CNAs. IN2970 had three regions of high copy number amplification (1q11-44, 8q12-23 and 9p24-q12) compared to only one (1q22-31) in IN2904. The extra regions of alteration in IN2970 were gain of 2q, 7, and 9q, as well as loss of 9p, 10, 11q, 16q, 17, 19 and 20. IN2904 also had some alterations that were not seen in IN2970, including gain of 6q and loss of 12q. An example of some of these differences can be seen pictorially in Figure 4.36.

Tumour recurrence was analysed in two ways. The first analysis compared the aberrations seen in primary tumour samples with those seen in recurrent samples in order to determine whether any one particular CNA could be associated with recurrent samples. A summary of the CNAs in these two groups of tumours can be seen in Figure 4.37. Two CNAs were seen at significantly higher frequencies in recurrent tumours than in primary tumours. These were gain of 7q ($p = 0.044$) and loss of 12q ($p = 0.0278$). Two further abnormalities that were observed at a higher frequency in recurrent tumours were approaching statistical significance: loss of 6q ($p = 0.074$) and loss of 8p ($p = 0.074$). High copy number amplification was also seen more frequently in recurrent tumours than in primary tumours. ($p = 0.018$). There was no significant difference in survival between these two groups (see Figure 4.38).

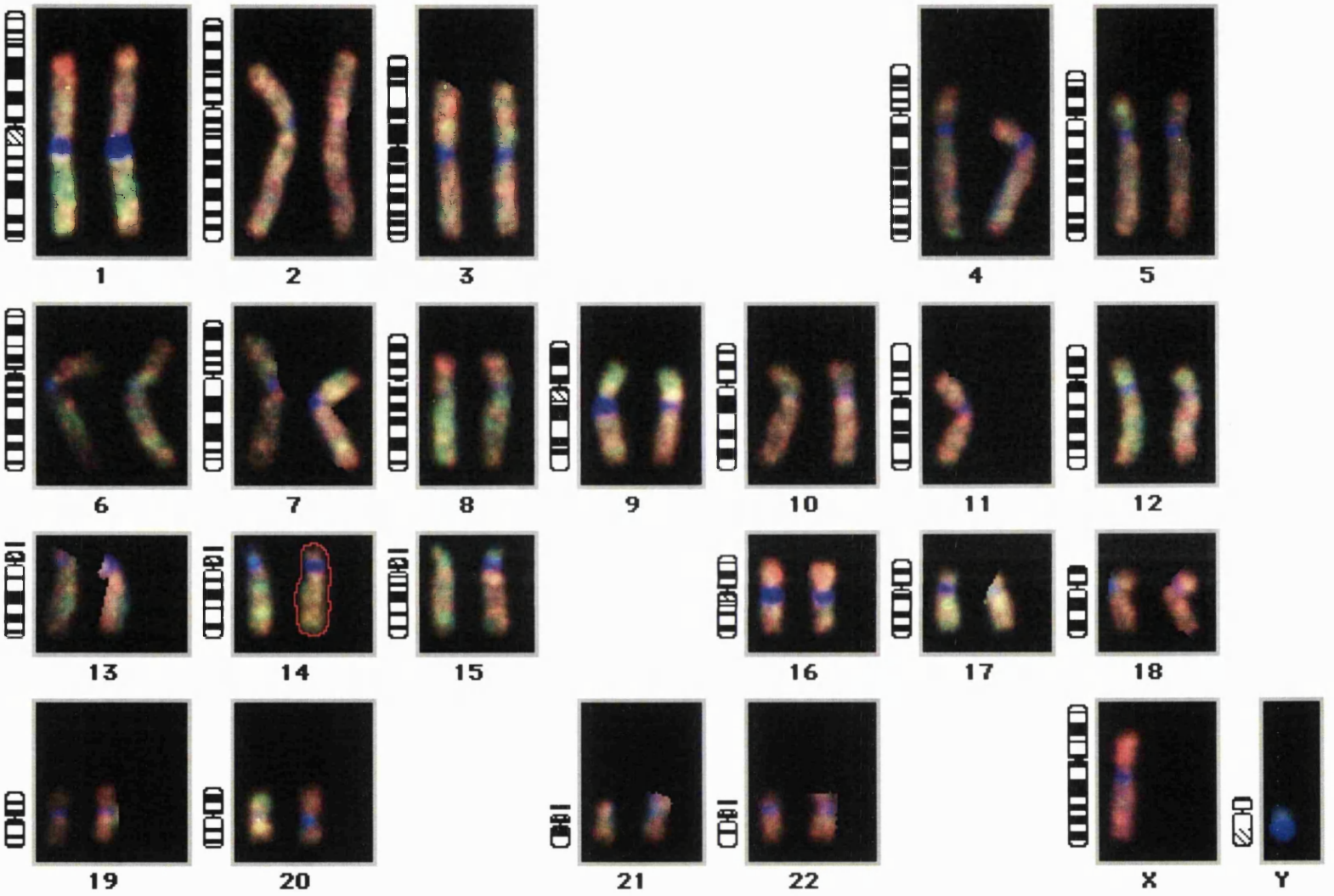


Figure 4.35: IN2904, a recurrent ependymoma, showing multiple CNAs including, amplification of 1q22-31, loss of 8p and gain of 8q, gain of 9p and loss of 9q, monosomy of chromosomes 16 and 22.

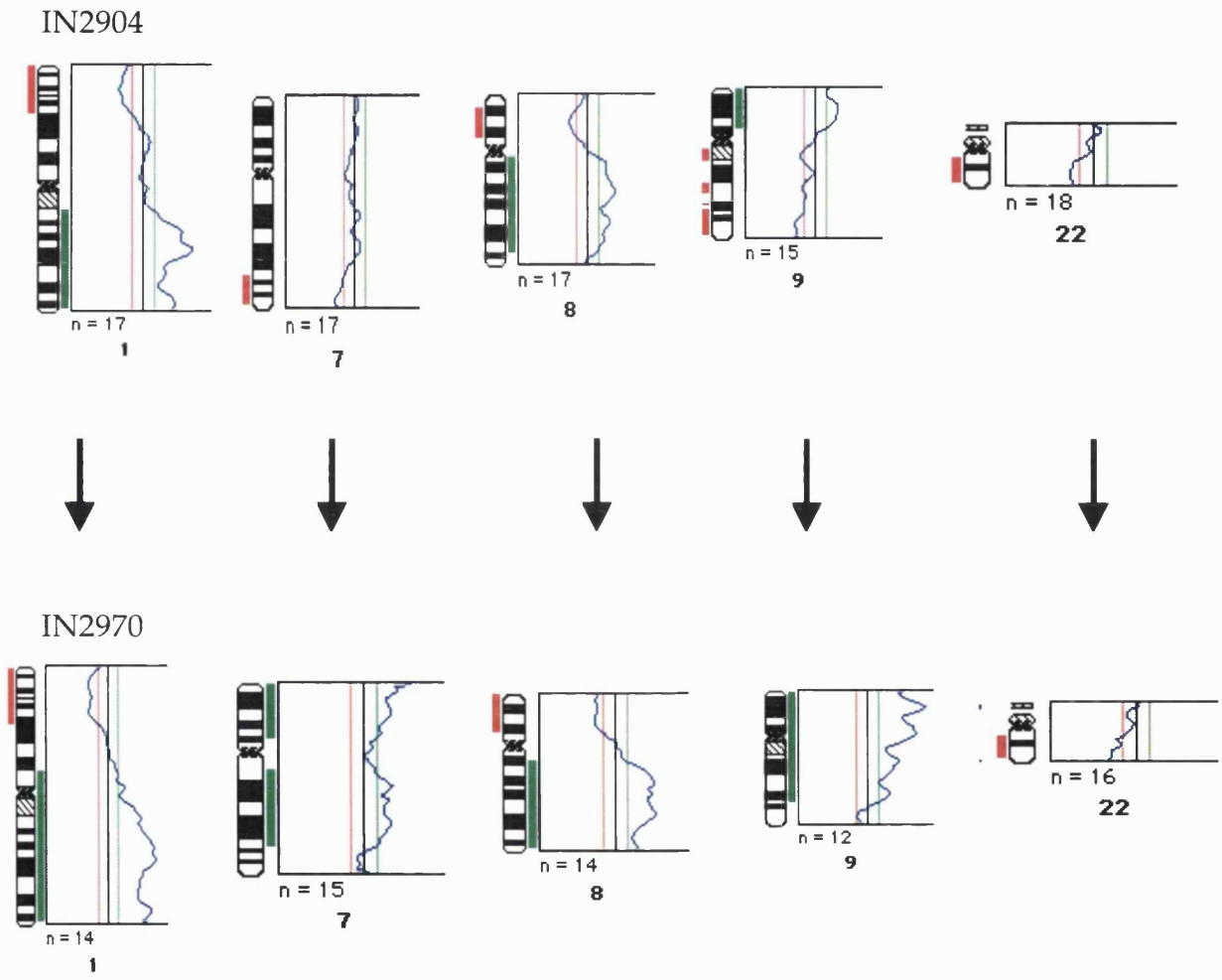


Figure 4.36: Accumulation of CNAs with tumour recurrence. The top row shows alterations in the 1st recurrence IN2904, the bottom row shows alterations in the 2nd recurrence, IN2970. There is no change in chromosomes 1 and 22. IN2904 shows no alteration of chromosome 7, whereas IN2970 has gain of chromosome 7. IN2904 has gain of 8q and IN2970 shows high copy number amplification of 8q. IN2904 has gain of 9p and loss of 9q, and IN2970 shows high copy number amplification of 9p and gain of 9q.

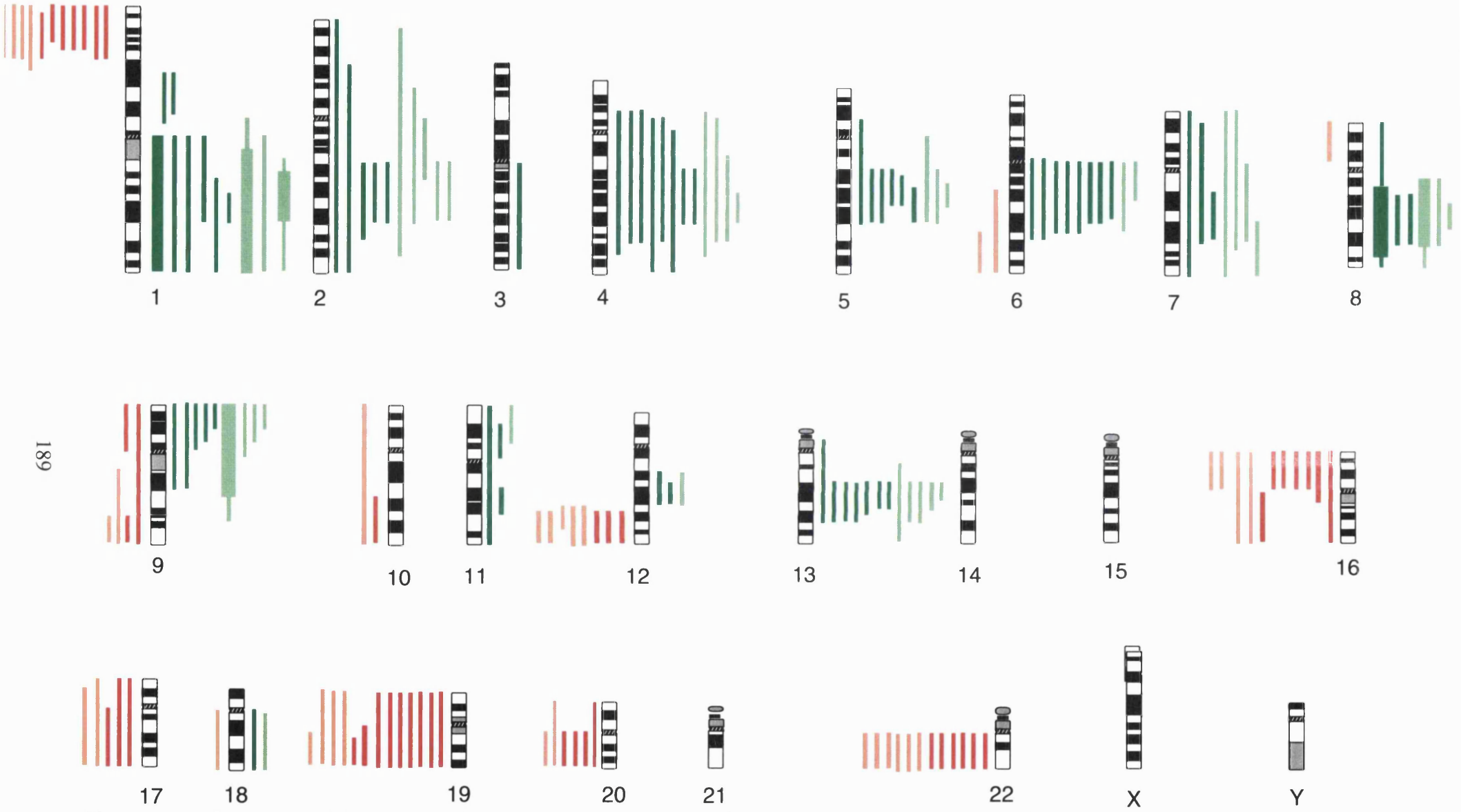


Figure 4.37: Summary of CNAs in primary and recurrent ependymoma samples. Dark green lines indicate regions of gain in primary tumours; light green lines indiacte regions of gain in recurrent samples. Bold lines indicate regions of high copy number amplification. Red lines indicate regions of loss in primary tumours, pale orange lines indicate regions of loss in recurrent tumours.

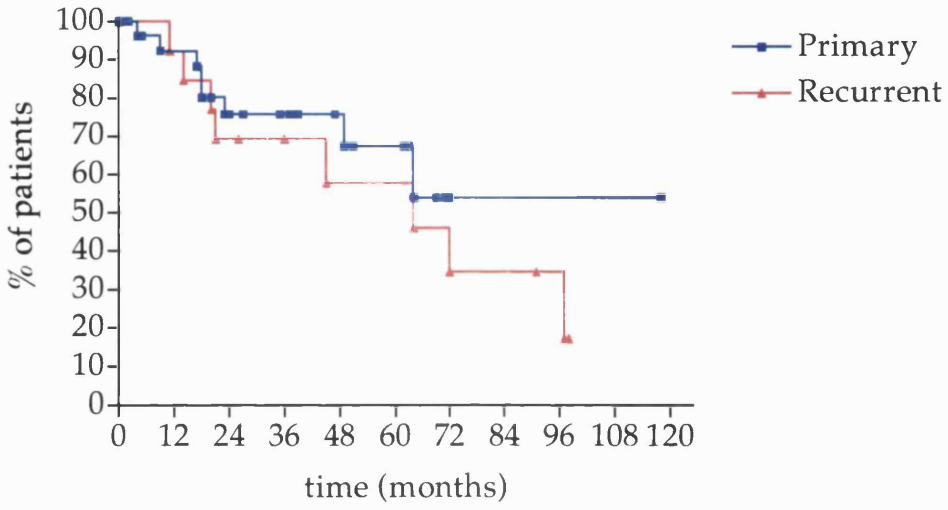


Figure 4.38: Comparison of survival times in samples where the primary sample was analysed compared to samples where the recurrent sample was analysed. Survival is measured in months from the date of initial diagnosis. (Log-rank test, $p = 0.418$).

The second analysis compared the aberrations seen in patients that had, at the time of this study, no reported clinical recurrence with those whose tumour had recurred. The CNAs seen in the primary samples were compared in order to establish whether any one aberration was associated with clinical recurrence (see Table 4.5 and Figure 4.39). There were twenty patients who had no reported tumour recurrence and thirteen whose tumour had recurred. No follow-up data was available for three of the primary samples, and there were six samples where the recurrent tumour was analysed and no primary tumour was available for analysis. The mean time to recurrence was 26.8 months. Of the patients who had no clinical recurrence twelve (60%) had no detectable CNAs. The remaining eight patients had between one and twelve aberrations (mean 6.6). The most common alterations in these patients were gain of 6q and loss of 19q, seen in five cases (41.6%) each, and gain of 4q and 13q, and loss of 1p and 22, seen in four cases (20%) each. Eight (61%) of the patients whose tumour had recurred had no alterations. The remaining five patients had between three and nineteen aberrations (mean 8.6). The most common alterations in this group were gain of 1q, 2q, 4q, 9p and 13q, and loss of 1p, 16q and 19, each seen in three cases (23%). Analysis using Fishers exact test revealed that the presence of CNAs was not associated with one patient group ($p = 1.00$). However, one CNA that occurred

	Survival	Time to recurrence	Amplicons	Gains	Losses
No recurrence					
IN 2699	2 (A)	n/a			
IN 3071	4 (A)	n/a			
IN 3087	5 (A)	n/a			
IN 3022	18 (A)	n/a			
IN 3029	23 (A)	n/a			
IN 2941	24 (A)	n/a			
IN 3008	27 (A)	n/a			
IN 2511	35 (A)	n/a			
IN 2871	63 (A)	n/a			
IN 2827	69 (A)	n/a			
IN 2767	72 (A)	n/a			
IN 2186	118 (A)	n/a			
IN 1932	4 (D)	n/a		1q	9p
IN 3014	23 (A)	n/a		4q 5q 6q 13q	1p 9q 12q 16p 17q 19q 22
IN 2944	37 (A)	n/a			9
IN 2939	38 (A)	n/a	1q21-44 8q21-23	1q 8 9p 11	12q 19
IN 2935	39 (A)	n/a		4q 5q 6q 13q	1p 16p 19 20 22
IN 2922	47 (A)	n/a		1p 2q 4q 5q 6q 13q 18q	1p 16p 19 20q 22
IN 2536	51 (A)	n/a		4q 6q 13q	1p 19 22
IN 2752	62 (A)	n/a		1q 6q	10q
Recurrence					
IN 2682	11 (D)	8			
IN 2242	17 (D)	16			
IN 2931	18 (D)	15			
IN 1594	18 (D)	16			
IN 3037	21 (A)	19			
IN 1638	49 (D)	27			
IN 2443	64 (D)	60			
IN 1231	64 (D)	26			
IN 2891	20 (A)	19		2q 4 5q 6q 9p 12q 13q 18q	1p 17 19
IN 2638	30 (A)	30		1 2q 4 5q 6q 7q 8q 9p 12q 13q 18q	1p 9q 12q 16 17 19 20q 22
IN 2776	71 (A)	60		1q	16q 19
IN 772	72 (D)	17		4	1p 16p
IN 1759	98 (A)	36		1q 2 7 9p 11 13	16q

Table 4.5: Copy number aberrations detected by CGH in primary ependymomas that have and have not shown a clinical recurrence of tumour.
Survival and time to recurrence are measured in months. A = alive; D = deceased

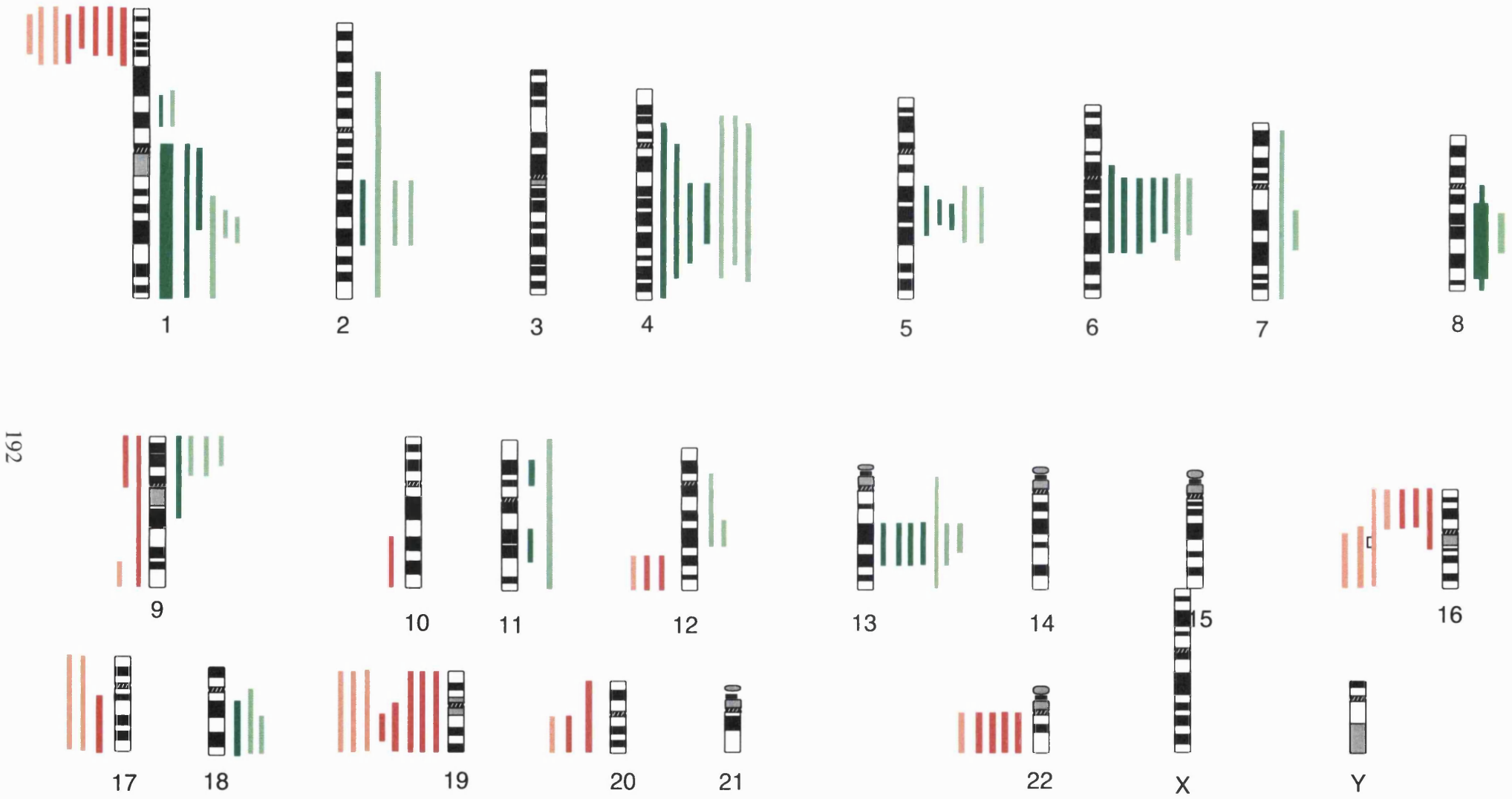


Figure 4.39: Summary of chromosomal imbalances in primary ependymoma with clinical recurrence and those without recurrence. Dark green lines indicate regions of gain in tumours with no recurrence, light green lines represent regions of gain in tumours with recurrence. Bold lines indicate regions of high copy number amplification. Red lines indicate regions of loss in tumours with no recurrence and plae orange lines indicate regions of loss in tumours with recurrence.

more frequently in the patients with tumour recurrence than in those with no recurrence was loss of 16q ($p = 0.0524$). Full details of the statistical analysis can be seen in Appendix III.

There was a significant difference in overall survival between the recurrent and non-recurrent patients ($p = 0.0117$). At five years and ten years the survival for the patients with no recurrence was 95%, compared to 55% in those patients whose tumour had recurred. Eight (61.5%) of the patients with tumour recurrence had died seventy-two months after diagnosis (see Figure 4.40). Log-rank tests did not identify any one aberration that could be associated with survival in these patients. For full statistical details see Appendix III.

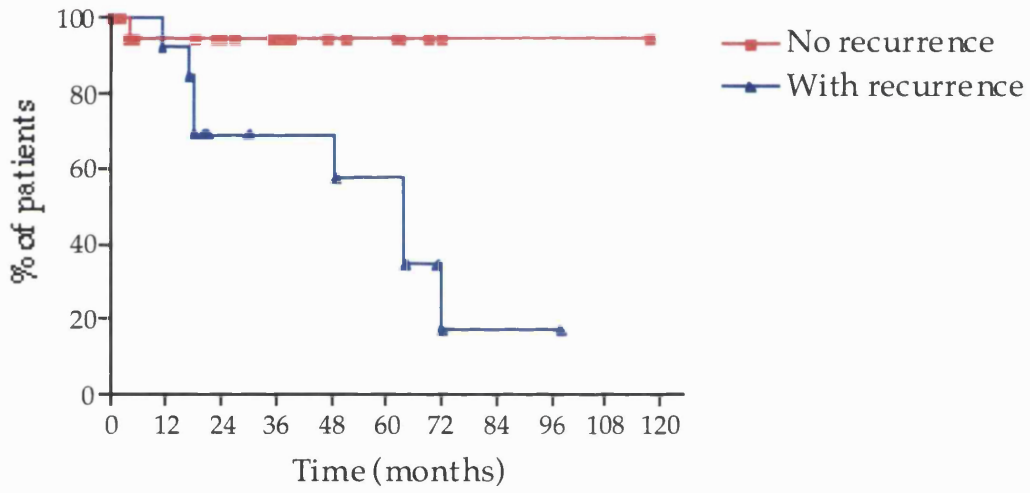


Figure 4.40: The effect of tumour recurrence on survival in 33 paediatric ependymoma. Survival is significantly associated with the absence of tumour recurrence (Log-rank test, $p = 0.0117$).

Y-PCR analysis of ependymoma samples

Y-PCR analysis of the ependymoma samples in this study was performed in order to verify the patients sex and determine whether there was any genomic loss from the Y chromosome. Samples with abnormal PCR results can be seen in Figure 4.41.

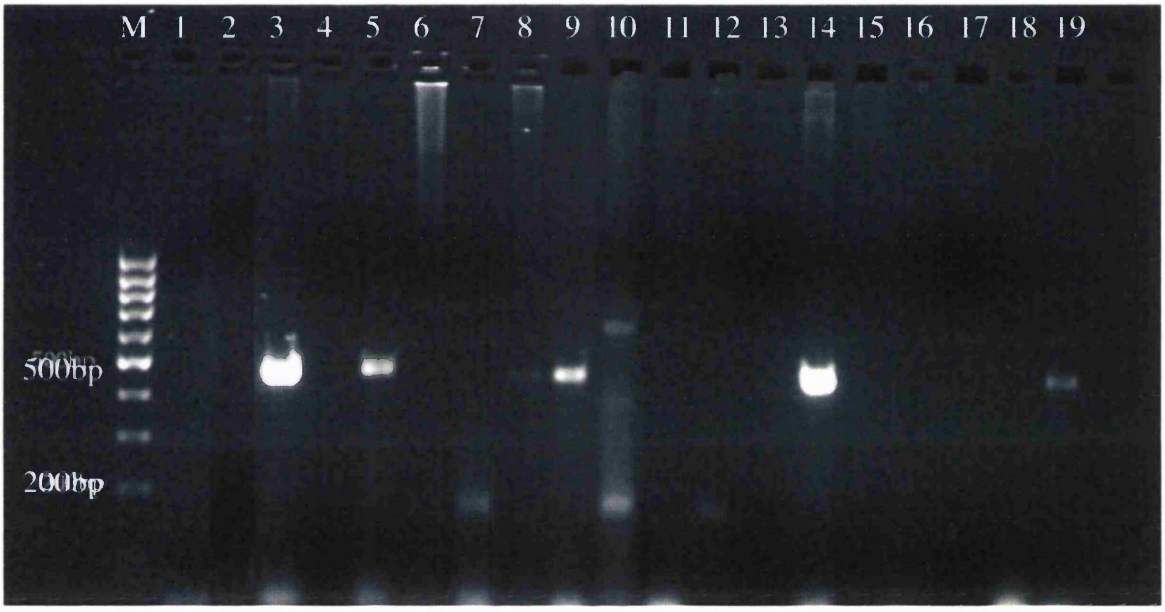


Figure 4.41: Results of Y-PCR analysis of ependymoma samples.
The marker (M) is a 100bp ladder. Lanes 1 & 2 show IN1638 blood and culture with Y1.7/Y1.8 primers; Lanes 3, 4 & 5 show IN1638 blood, biopsy and culture with SRY primers; Lanes 6 & 7 show IN2931 blood and biopsy with Y1.7/Y1.8 primers; Lanes 8 & 9 show IN 2931 blood and biopsy with SRY primers; Lane 10 shows IN2939 biopsy with Y1.7/Y1.8 primers; Lane 11 shows IN2939 biopsy with SRY primers; Lanes 12 & 13 show IN1932 blood and culture with Y1.7/Y1.8 primers; Lanes 14 & 15 show IN1932 blood and culture with SRY primers; Lanes 16 & 17 show IN1759 blood and culture with Y1.7/Y1.8 primers and Lanes 18 & 19 show In1759 blood and culture with SRY primers.

IN1638

IN1638 was a male patient who had no detectable products using the Y1.7 and Y1.8 primers. When the SRY primers were used, a product was detected in the matched normal blood sample but not the tumour sample. This infers that this patient has loss of the Y chromosome.

IN2931

IN2931 is a female patient who has a faint product using the Y1.7 and Y1.8 primers. A product was also produced in both the matched normal blood sample and the tumour sample using the SRY primers. This suggests that either the patient information is incorrect or there is a genetic abnormality in this patient that results in Y chromosome specific sequences being present in this patient.

IN2939

IN2939 is a female patient who had a detectable product when the Y1.7 and Y1.8 primers were used. When the PCR was repeated using the SRY primers no product was detected, indicating this patient did not have the sex determining region of the Y chromosome present and was indeed female.

IN1932

IN1932 was a male patient who had a detectable product for the matched normal blood sample using both sets of primers but no detectable product for the tumour sample with either set of primers. This is indicative of Y chromosome loss.

IN1759

IN1759 was a male patient who had a faint product for the tumour sample using the Y1.7 and Y1.8 primers. When the PCR was repeated using the SRY primers, a more obvious product was visible, confirming this patient is male.

MDR1 EXPRESSION IN PAEDIATRIC EPENDYMOMA

Ependymoma are often resistant to treatment with conventional chemotherapeutic agents. Such resistance to a spectrum of agents may be associated with the expression of the multidrug resistance gene, *MDR1*. In order to determine whether this gene is involved in drug resistance in paediatric ependymoma, twenty-five ependymoma were analysed for the expression of *MDR1* using two PCR primer sets specific for this gene. Primers for the β2-microglobulin (*β2-MG*) gene were used as a control for these experiments. This is as a "house keeping" gene and is present in all tissues at a constant level. The patient details for the samples in this study can be seen in Table 4.6.

IN[a]	Sex[b]	Age[c]	Histology[d]	Source[e]	Treatment[f]	Survival[g]
772	M	4	SE	CC	PE, RT, chemo	72 (D)
959	M	4	E	CC	Unknown	Unknown
1231	F	7	E	CC	MR, RT	64 (D)
1258	F	2.5	RE	FF	PE, RT	36 (A)
1497	M	1.25	RE	CC	MR, RT, chemo	45 (D)
2186	F	12.5	E	CC	MR, RT	118 (A)
2376	M	15	RE	FF	PE, chemo	98 (A)
2511	F	13	E	CC	PE, RT	35 (A)
2536	F	7	E	FF	MR, RT	51 (A)
2628	M	17	E	FF	Unknown	9 (D)
2638	M	1.9	E	FF	PE, chemo	91 (A)
2682	F	0.66	E	FF	MR, chemo	11 (D)
2767	F	1.8	AE	FF	MR, chemo	52 (A)
2776	F	6	E	CC	MR, RT	62 (A)
2798	F	15	RSGCA	FF	Unknown	Unknown
2923	M	7	RE	FF	Surgery	14 (D)
2931	F	1.3	E	FF	MR, chemo	18 (D)
2935	M	9.33	E	FF	MR, RT	16 (A)
2939	F	0.58	E	FF	MR, RT	19 (A)
2941	M	2.58	E	FF	MR, RT	24 (A)
2944	M	11.5	E	FF	PE, RT	19 (A)
3008	M	10.5	E	FF	MR, chemo	3 (A)
3014	M	6.75	E	FF	MR, RT	5 (A)
3029	M	1.75	E	FF	MR, chemo	9 (A)
3037	M	2	AE	FF	MR, chemo	6 (A)

Table 4.6: Patient details for samples in *MDR1* expression analysis.
IN[a]: Institute of Neurology assigned number; Sex[b]: M = male, F = female; Age[d]: in years; Source[e]: FF = fresh frozen material, CC = short term cell culture; Histology[f]: SE = subependymoma, E = ependymoma, RE = recurrent ependymoma, AE = anaplastic ependymoma, RSGCA = recurrent subependymal giant cell astrocytoma; Treatment[f]: PE = partial excision, MR = macroscopic removal, RT = radiotherapy, Chemo = chemotherapy; Survival[g]: measured in months from date of initial diagnosis, A = alive; D = deceased.

MDR1 expression was observed in 17 (65%) cases using both the MDR1E and MDR1F primers. All samples showed equal intensity for the control gene. Results were recorded as 0 when no MDR1 expression could be detected, 1 when a weak band could be observed, 2 when a strong band could be observed and 3 when a very strong band could be observed. Results can be seen in Table 4.7, and Figures 4.42 and 4.43. All negative controls in these experiments did not produce an amplification product proving that all products seen with tumour samples indicates true *MDR1* expression and not amplification of a contaminant in the reaction components. Negative controls are not shown in Figures 4.42 and 4.43.

There was good correlation between the amount of expression seen with the two primer sets. In the majority of cases strong expression was seen with both sets of primers if it was present, and in samples were there was little or no expression this was seen using both sets of primers. As the *MDR1* gene maps to 7q, the CGH profiles of the ependymoma were checked for gain of 7q. Three tumours (IN2376, IN2628 & IN2638) had detectable regions of gain on 7q. Only one of these samples, IN2376, showed expression with both sets of primers. IN2638 showed no expression of *MDR1* with either set of primers, and IN2628 showed only faint expression with the MDR1F primer set.

IN1497 is a recurrent sample that had been previously treated with vincristine and CCNU. This sample proved to be resistant to vincristine *in vitro* (personal communication, Tracey Collins, Department of Molecular Pathogenesis, Institute of Neurology), yet there was very low expression of MDR1 mRNA. IN2376 had also been treated with chemotherapy (agents unknown) and this sample showed moderate expression of MDR1 mRNA.

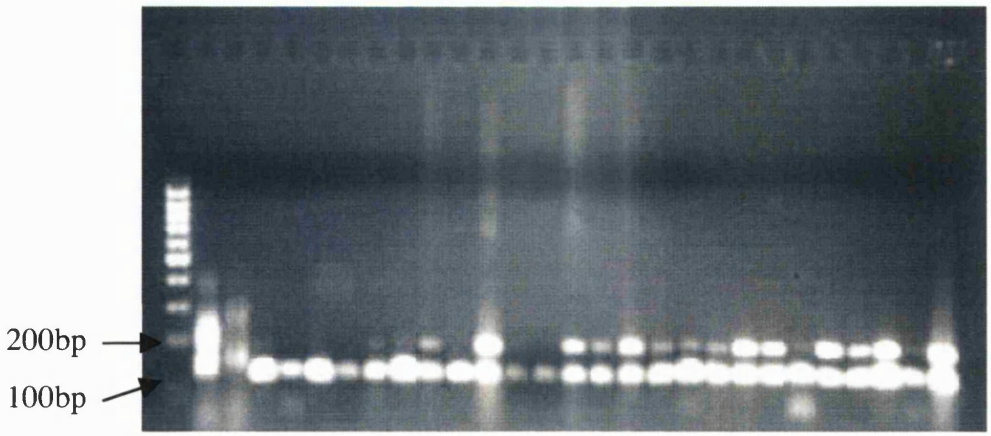


Figure 4.42: MDR1 expression in ependymoma using MDR1E primers
Lane 1 shows 100bp ladder, lane 2: normal brain, lane 3: HCT116 adenocarcinoma, Lane 3: IN772, Lane 4: IN959, Lane 5: IN1231, Lane 6: IN1258, Lane 7: IN1497, Lane 8: IN2186, Lane 9: IN2376, Lane 10: IN2511, Lane 11: IN2536, Lane 12: IN2628, Lane 13: IN2638, Lane 14: IN2682, Lane 15: IN2767, Lane 16: IN2776, Lane 17: IN2798, Lane 18: IN2923, Lane 19: IN2931, Lane 20: IN2935, Lane 21: IN2939, Lane 22: IN2941, Lane 23: IN2944, Lane 24: IN3008, Lane 25: IN3014, Lane 26: IN3029, Lane 27: IN3037. MDR1E PCR products can be seen at 179bp and the β2-MG gene product at 120bp.

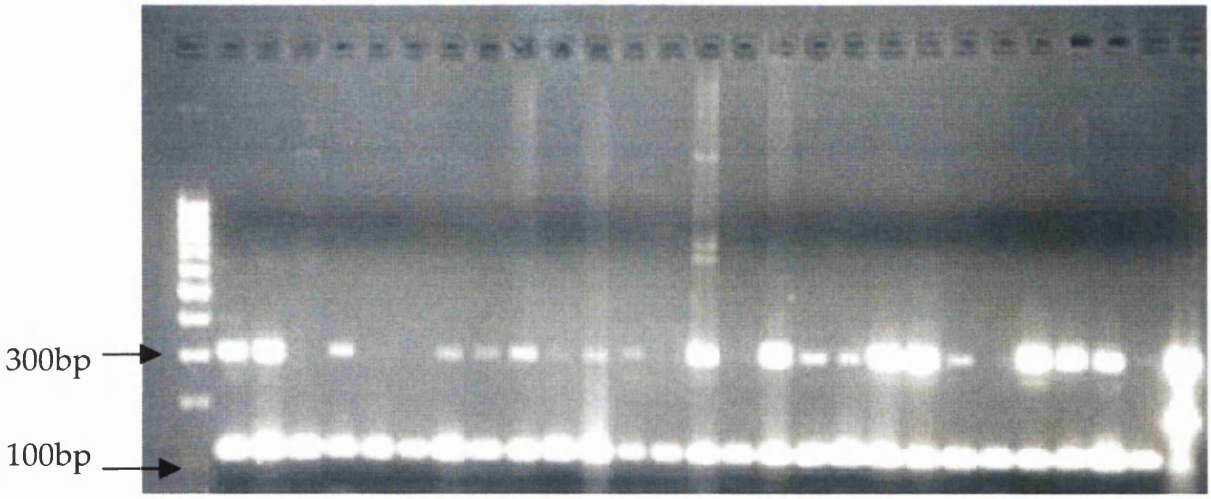


Figure 4.43: MDR1 expression in ependymoma using MDR1F primers
Lane 1 shows 100bp ladder, lane 2: normal brain, lane 3: HCT116 adenocarcinoma, Lane 3: IN772, Lane 4: IN959, Lane 5: IN1231, Lane 6: IN1258, Lane 7: IN1497, Lane 8: IN2186, Lane 9: IN2376, Lane 10: IN2511, Lane 11: IN2536, Lane 12: IN2628, Lane 13: IN2638, Lane 14: IN2682, Lane 15: IN2767, Lane 16: IN2776, Lane 17: IN2798, Lane 18: IN2923, Lane 19: IN2931, Lane 20: IN2935, Lane 21: IN2939, Lane 22: IN2941, Lane 23: IN2944, Lane 24: IN3008, Lane 25: IN3014, Lane 26: IN3029, Lane 27: IN3037. MDR1F PCR products can be seen at 308bp and the β2-MG gene product at 120bp.

IN	Source	MDR1E	MDR1F	β2MG	CGH
772	culture	0	0	1	
959	culture	0	2	1	
1231	culture	0	0	1	
1258	biopsy	0	0	1	
1497	culture	1	1	1	
2186	culture	0	1	1	
2376	biopsy	2	2	1	Gain 7q
2511	culture	0	1	1	
2536	biopsy	3	1	1	
2628	biopsy	0	1	1	Gain 7q
2638	biopsy	0	0	1	Gain 7q
2682	biopsy	3	3	1	
2767	biopsy	2	0	1	
2776	culture	3	3	1	
2798	biopsy	2	3	1	
2923	biopsy	2	3	1	
2931	biopsy	2	3	1	
2935	biopsy	3	3	1	
2939	biopsy	3	2	1	
2941	biopsy	1	1	1	
2944	biopsy	3	3	1	
3008	biopsy	3	3	1	
3014	biopsy	3	3	1	
3029	biopsy	1	1	1	
3037	biopsy	3	3	1	

Table 4.7: Expression of *MDR1* in ependymoma as determined by RT-PCR. 0 = no product detected, 1 = faint product detected, 2 = strong product detected and 3 = very strong product detected.

The effect of *MDR1* expression on survival was analysed. There was no difference in survival in those patients expressing *MDR1* and those in whom no expression could be detected (see Figure 4.44).

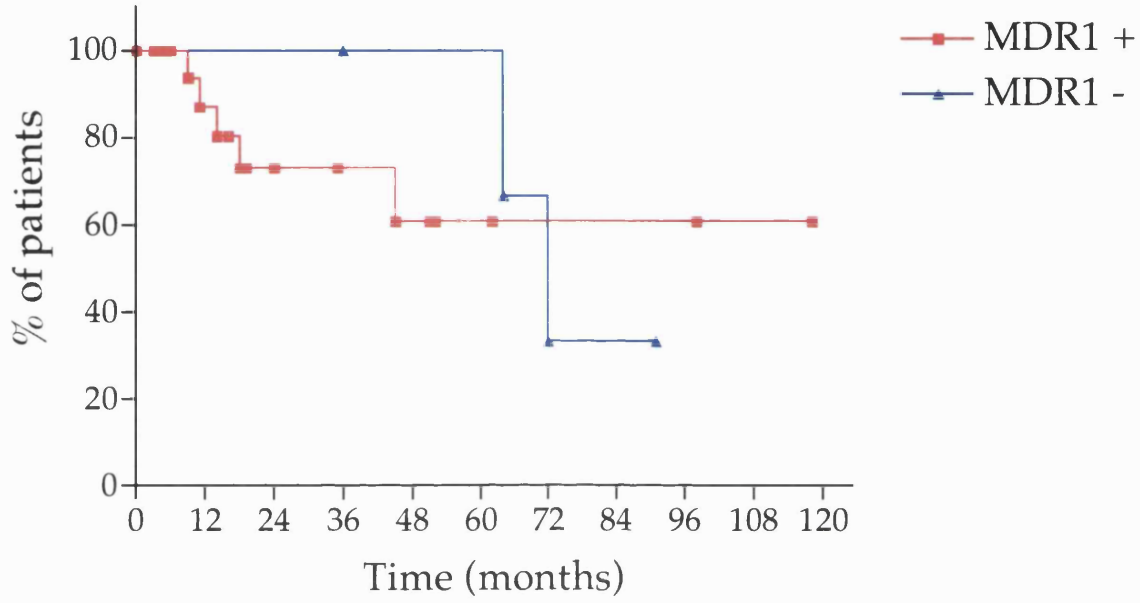


Figure 4.44: Survival of ependymoma patients with expression of MDR 1 mRNA compared to patients where no MDR1 mRNA could be detected. There was no relationship between expression and survival, $p = 0.7642$

There was no relationship between the expression of *MDR1*, patient age and survival (see Figure 4.45a and b). There was also no association between tumour histology and the expression of *MDR1*, though this may be due to the low numbers of the anaplastic variant available for analysis (n = 2).

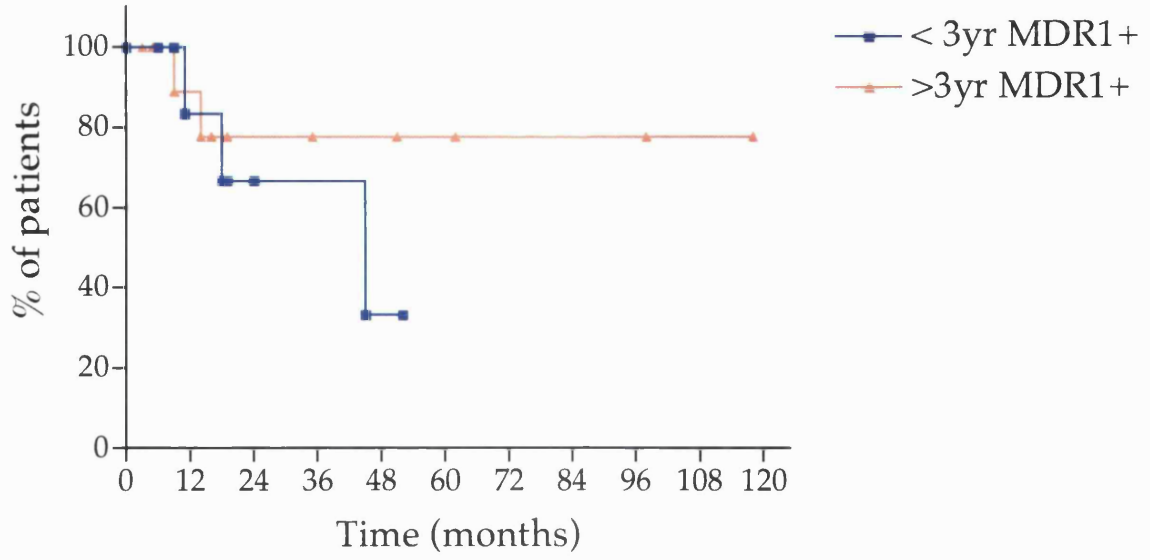


Figure 4.45a: Survival of ependymoma patients with *MDR1* expression in patients aged 3 years and younger compared to survival in older patients who expressed *MDR1*. There is no difference in survival (Log-rank test, $p = 0.7717$

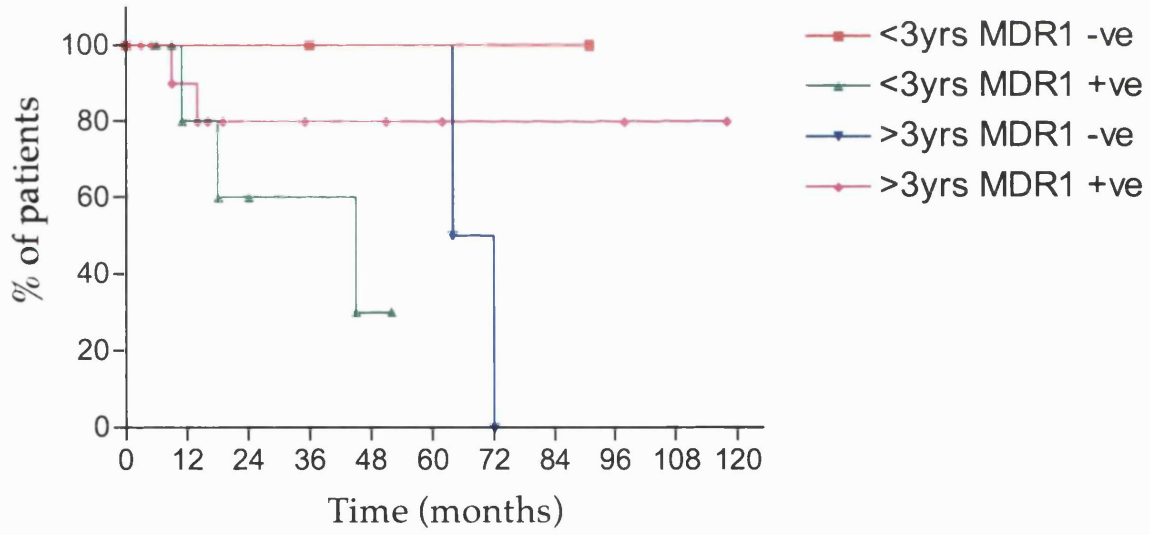


Figure 4.45b: Survival of ependymoma patients in relation to patient age and *MDR1* expression. Log-rank test, $p = 0.2500$.

DISCUSSION

Previous molecular genetic studies have indicated that ependymoma resemble other glial cell-derived tumours with respect to the chromosomal regions that show alterations, namely losses of 9p, 10, 13q, 17p and 22q (Bijlsma, Voesten et al, 1995). A summary of abnormalities previously reported in paediatric ependymoma as determined by conventional cytogenetic techniques as well as CGH can be seen in Figure 4.46. The majority of molecular genetic analyses of ependymoma have been conducted on samples derived from adult patients (Bijlsma et al, 1995; Ebert et al, 1999). As paediatric astrocytoma have been shown to be considerably different from adult astrocytoma, one might expect the same to be true of paediatric and adult ependymoma.

This study has shown copy number aberrations to be present in nearly half (49%) of ependymoma. Only four of these tumours had single abnormalities whereas the other tumours had an average of seven abnormalities each. The number of alterations did not appear to be associated with increasing malignancy in ependymoma, but this may be due to the relatively small number of anaplastic ependymoma in this study. The majority of the abnormalities seen in ependymoma in the present study have been reported previously in paediatric ependymoma, with the exception of the region of high copy number amplification on chromosome 9 seen in tumour IN2970. This is a novel finding and worthy of further investigation.

The alterations observed often occurred at a different frequency than has previously been reported. In the case of most abnormalities this may be explained by the differences in tumour populations between the different research centres. In the case of the regions of loss at 1p, 16, 19 and 22 these alterations are sometimes excluded from analysis, as they are known to be regions of CGH artefact. It was decided not to exclude these alterations from the present analysis, as these regions are known to harbour tumour suppressor genes that are associated with other malignancies. Alterations at these loci were included if there was a clear deviation from the normal central line of the CGH profile for that particular chromosome and covered a relatively large proportion

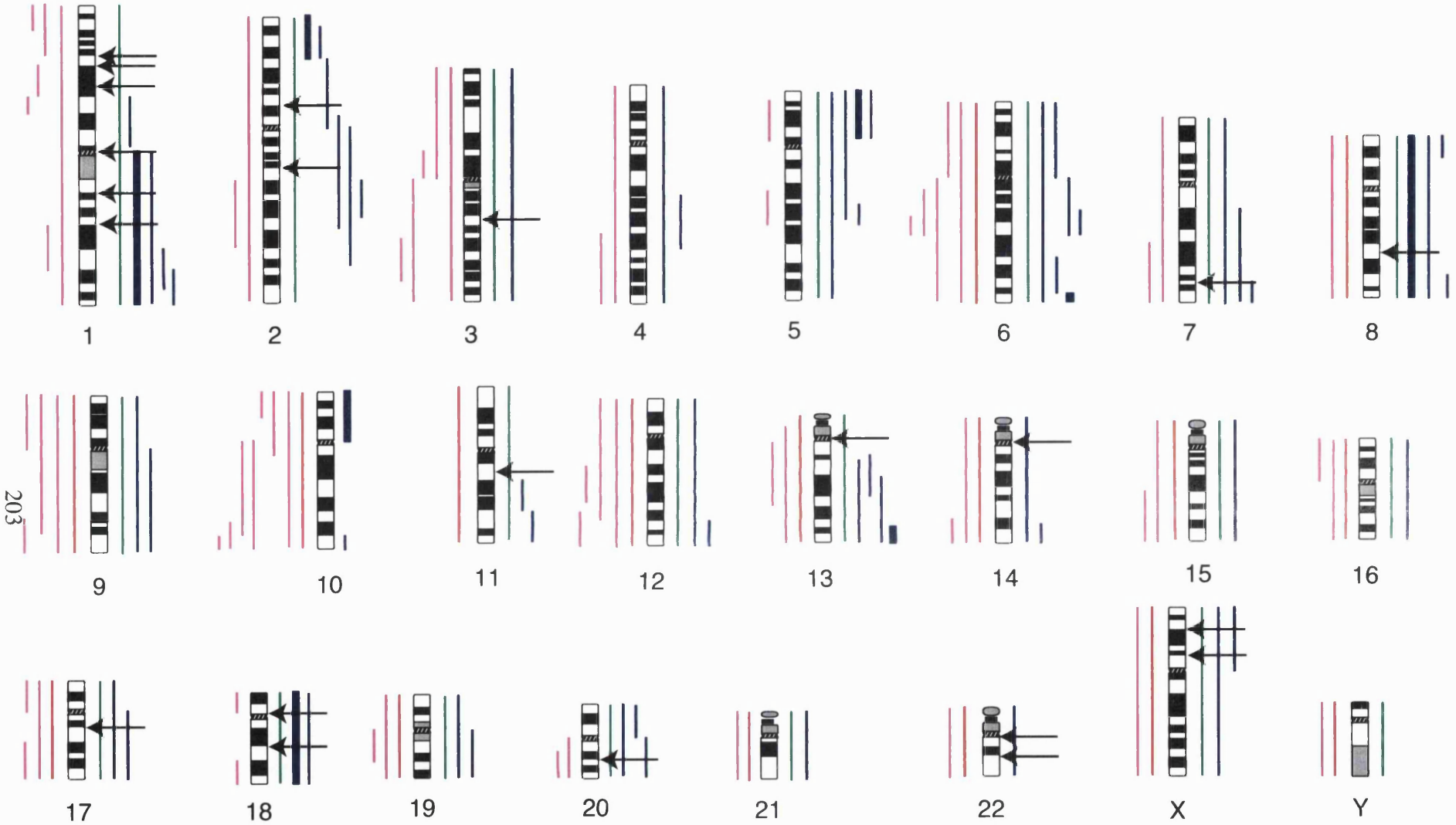


Figure 4.49: Summary of previously reported alterations in paediatric ependymoma. Red lines indicate regions of deletion identified by cytogenetics; green lines indicate regions of gain identified by cytogenetics; pink lines indicate regions of loss identified by CGH; blue lines indicate regions of gain identified by CGH; bold blue lines indicate regions of high copy number amplification identified by CGH. Arrows indicate translocation breakpoints (Stratton, 1989; Griffin, 1992; Vagner-Capodano, 1992; Weremowicz, 1992; Neumann, 1993; Rogatto, 1993; Agamanolis, 1995; Sainati, 1996; Bhattacharjee, 1997; Bigner, 1997; Mazewski, 1999; Reardon, 1999; Vagner-Capodano, 1999; Kucerova, 2000; Shlomit, 2000; Zheng, 2000; Hirose, 2001; Roberts, 2001; Scheil, 2001; Carter, 2002; Dyer, 2002; Grill, 2002; Jeuken, 2002).

of the chromosome arm, and if they were present in subsequent hybridisations. If data has been analysed cautiously in previous studies true aberrations in these regions may not have been included in the summary of alterations. One way in which the true status of these chromosomes in ependymoma can be ascertained is to verify genetic losses detected by CGH with FISH probes specific for the chromosome arm of interest. In the present study, ependymoma had a number of alterations that had previously been reported in anaplastic ependymoma only. One explanation for this discrepancy may be the difficulties experienced by neuropathologists in diagnosing the anaplastic variant. It is possible that some benign ependymoma are mis-diagnosed and may actually be anaplastic ependymoma and vice versa.

Chromosome 1 alterations

Previous cytogenetic studies have reported numerical gains of chromosome 1 in only one case (Neumann et al, 1993). In comparison, CGH analyses have shown gain of 1q to be a frequent event in ependymoma, being reported in 30% of cases (Carter et al, 2002; Dyer et al, 2002; Grill et al, 2002; Hirose et al, 2001; Reardon et al, 1999; Scheil et al, 2001; Shlomit et al, 2000; Zheng et al, 2000). Gain of 1q has been found to occur selectively in paediatric ependymoma rather than in adult ependymoma (Scheil et al, 2001). Reported structural abnormalities include t(1;20)(q21;q13), i(1q), der(1)t(1;Y)(q11.2;q12), der(1)t(1;3)(q11;p11), trp(1)(q?22q?31) and t(1;22)(q11;q13.3) (Bigner et al, 1997; Mazewski et al, 1999; Neumann et al, 1993; Slavc et al, 1995; Weremowicz et al, 1992). The present study also showed gain of 1q to be a frequent event in ependymoma, being seen in ten cases (19.6%), three of which had high copy number amplification with a minimum overlapping region of 1q22-31.

A number of CGH studies have shown gain of 1q to occur in conjunction with gain of chromosome 9 in 13/128 (10%) cases (Carter et al, 2002; Dyer et al, 2002; Hirose et al, 2001; Reardon et al, 1999; Scheil et al, 2001), a finding mirrored by the present study, which found gain of 1q and 9 in seven tumours (14%). This

may be indicative of a tumourigenic pathway that involves the increased expression of genes on these chromosomes. Another common abnormality seen with gain of 1q is loss of 16q. This pair of aberrations has been reported in 5/54 (9.25%) cases (Hirose et al, 2001; Shlomit et al, 2000). In the present study five (10%) tumours had both gain of 1q and loss of 16q, a pairing that was found to be statistically significant ($p = 0.0087$). This combination of alterations may be indicative of der(16)t(1;16), which is a well-known secondary chromosomal change in a variety of tumours. Cytogenetic studies have shown recurrent alterations of chromosome 1, including deletions, translocations, trisomies and amplifications, in a number of solid tumours, as well as in haematological malignancies. Alterations of the long arm of chromosome 1 are found in leukaemia and solid tumours, and are one of the most common chromosomal abnormalities in human cancers (Szymanska et al, 1997; Weber-Hall et al, 1996). These alterations manifest as trisomy 1q, i(1q), or a trisomy or duplication of a smaller region (Forus et al, 1998). It has been suggested that three or more copies of a gene (or genes) on 1q may provide a selective advantage to cancer cells. The finding that partial or complete trisomy 1q is more common in recurrent tumours than primary tumours may be indicative of an alteration that is associated with tumour progression than initiation (Weith et al, 1996). The present study could find no relationship between gain 1q and tumour recurrence.

Three ependymoma (IN2939, IN2904 and IN2970) had regions of high copy number amplification on chromosome 1 that mapped to 1q22-31. Two of these tumours had amplicons that exceeded the minimum overlapping regions to cover 1q44. High copy number amplification in this region has also been reported in other malignancies (see Table 4.8). High level amplification of 1q has also been reported in eleven other cases of paediatric ependymoma (Carter et al, 2002; Dyer et al, 2002). A cytogenetic analysis of ependymoma has also described a paediatric tumour with seven copies of 1q22-31 (Kramer et al, 1998).

Malignancy	No. cases	Amplicon	Reference
Pituitary adenoma	1/12	1q21-23	(Harada et al, 1999)
Lipoma-like liposarcoma	1/8	1q21-22	(Szymanska et al, 1997)
Liposarcoma	6/15	1q21-22	(Forus et al, 1998)
Fibrous histiocytoma	3/5	1q21-23	(Forus et al, 1998)
Leiomyosarcoma	3/3	1q21-q23	(Forus et al, 1998)
Osteosarcoma	3/9	1q21-23	(Forus et al, 1998)
Choriocarcinoma	1/2	1q31-32	(Rao et al, 1998)
Hepatoblastoma	1/10	1q32	(Hu et al, 2000)
Ovarian carcinoma	1/27	1q42	(Sonoda et al, 1997)

Table 4.8: Previously reported amplification of 1q in human cancers.

Genes that map to the region of gain and amplification on 1q in these ependymoma include *FLG* (1q21-22) which encodes the human epidermal profilaggrin and has been shown to be amplified in leiomyosarcoma, liposarcoma, malignant fibrous histiosarcoma and osteosarcoma (Forus et al, 1998). The *PTGS2/COX2* gene also maps within this region and has been found to be highly expressed in colon cancer, oesophageal adenocarcinoma and Barrett's metaplasia (Tsujii et al, 1998; Zimmermann et al, 1999). It has been suggested that *COX2* acts as a "landscaping tumour promotor". COX2 expression in the stromal component of solid tumours could influence growth or the expression of proangiogenic factors and may be inhibited by the action of p53 (Williams et al, 2000). In normal brain, COX2 is constitutively expressed in neurons and only faintly expressed in glial cells. In brain tumours, expression may be associated

with the formation of oedema. Constitutive expression of COX2 has been found in various types of brain tumours. Low grade astrocytoma, craniopharyngioma, medulloblastoma and schwannoma show weak positive staining for COX2 in both the cytoplasm and nucleus, whereas strong positive staining was found in the cytoplasm of malignant astrocytoma, meningioma and some ependymoma (Matsuo et al, 2001). COX2 expression has been shown to be the strongest predicator of outcome in a recent study of adult astrocytoma and is associated with more clinically aggressive glioma (Shono et al, 2001). Some melanoma cell lines show amplification (or duplication) of *CACY/S100A6* which encodes calcyclin. This gene is a member of the S100A family of calcium-binding proteins, which are located within a cluster of S100A genes at 1q21 (Schäfer and Heizmann CW, 1996). Another gene in this region is *MUC1* which codes for the epithelial tumour-associated antigen mucin 1 and has been found to be amplified in some breast cancers (Bieche and Lidereau, 1997).

Loss of 1p was a frequent finding in the present study, being observed in eleven tumours (21.5%). In this study loss of 1p occurred independently of gain of 1q in the majority of cases. The minimum region of loss in these ependymoma was 1p32-36.6. There have been no previous reports of simple deletions of 1p using conventional cytogenetic analyses. Loss of 1p has been reported in CGH analyses of ependymoma (Dyer et al, 2002; Hirose et al, 2001; Reardon et al, 1999; Scheil et al, 2001; Zheng et al, 2000). Interestingly, two of the six reported tumours with loss of 1p are myxopapillary ependymoma (Reardon et al, 1999; Scheil et al, 2001). Structural abnormalities involving 1p include del(1)(p22), del(1)(p21) and del(1)(p13) (Griffin et al, 1988; Mazewski et al, 1999). Loss of heterozygosity for markers on 1p is a frequent occurrence in malignancy, being observed in a number of different tumours including pheochromocytoma, melanoma and breast carcinoma. In brain tumours, a close association has been reported between loss at 1p and the development of neuroblastoma (Takeda et al, 1994). Loss of alleles for 1p have been reported at significant frequencies in meningioma, oligodendroglioma and all grades of astrocytoma (Bello et al, 1995). An analysis of tumours with partial 1p deletions suggests there might be two distinct regions of loss, 1p36 and 1p35-32 in which genes involved in the

development of CNS neoplasms are located (Bello et al, 1995). A study of chromosome 1 abnormalities in paediatric solid tumours have found the most frequent type of abnormality to be deletion of 1p, with the formation of i(1q) or trisomy 1q, and the most frequent area of breakage to be 1p22 (Douglass et al, 1985).

One gene that may be of some importance in ependymoma is the human *p73* gene that maps to 1p36 and encodes a product that shares significant structural homology to *p53*. This gene can trigger apoptosis and cell cycle arrest by inducing $p21^{waf}$ transcription. Hypermethylation at the 5' region upstream, and including exon 1 of the gene has been noted in 39% of oligodendroglial tumours (Dong et al, 2002). Despite sharing structural and functional similarities with *p53*, the TP73 protein has distinct biological activities. TP73 is not induced by ultraviolet irradiation and certain DNA damaging agents, it expresses multiple alternatively spliced isoforms that have different functional properties and it does not interact with viral oncoproteins that usually bind and inactivate TP53. These actions may provide clues for the role of *p73* in the carcinogenesis of some malignancies.
There are a number of other genes that map to this region that may be implicated in tumour progression. These include *MTS1* (malignant transformation suppression 1) that maps to 1pter-p22.1, *ID3* (inhibitor of DNA binding 3) that maps to 1p36, *NBS* at 1p36, *TNFR2, DAN, CDC2L1* and *FRAP1* (FKBP12-rapomycin associated protein) that map to 1p36, *MOM1* at 1p35, *HKR3* (human Kruppel-related 3 gene), and *RIZ* (retinoblastoma binding protein) (Knuutila et al, 1999; Miozzo et al, 2000).

Gain of chromosome 2

There were ten (19.6%) ependymoma with gains of chromosome 2. Four tumours had gain of the whole chromosome and the remaining six tumours had gain that was restricted to 2q, with a minimum overlapping region of 2q22-32. Previous cytogenetic analyses have shown numerical gain of chromosome 2 in only two cases (Griffin et al, 1992; Mazewski et al, 1999). Gains of chromosome 2 are

infrequent findings in CGH analyses of ependymoma, being reported in 10% cases (Carter et al, 2002; Dyer et al, 2002; Grill et al, 2002; Zheng et al, 2000). Structural abnormalities of chromosome 2 in ependymoma include t(2;17)(p11;p11), t(2;22)(p12;q13) and t(2;22)(q13;q13) (Neumann et al, 1993; Vagner-Capodano et al, 1992; Yamada et al, 1994).

High copy number amplification of 2p23-24 has been reported in two cases of adult ependymoma (Scheil et al, 2001). A gene that may be implicated in the aggressive growth of ependymoma is the *n-myc* oncogene at 2p23-24. Historically there have been few investigations of *n-myc* amplification in ependymoma. One study detected low accumulation of *n-myc* transcripts in ependymoma without an increase in gene copy number (Fujimoto et al, 1989). A further seven cases of ependymoma have been analysed with no amplification of *n-myc* being reported in these cases (James et al, 1990; Wasson et al, 1990).

The 2q22-32 region contains several genes with potential oncogenic properties. These include nine homeo box genes, a serine-threonine kinase receptor, *I-TRAF* (tumour necrosis factor receptor-associated factor) and the gene for *FRZB-1*, a secreted antagonist of WNT signalling (Leyns et al, 1997; Rothe et al, 1996). The *GRB14* gene has also been localised to 2q24 and is a member of the Grb7 family of molecular adaptors. All members of this family interact with growth factor receptors in an activation dependent manner, and this suggests they might be involved in the regulation of cell proliferation. Grb7 has been implicated as a downstream mediator of integrin-FAK signal pathways in the regulation of cell migration (Shen et al, 2002). The phosphorylation of Grb7 triggers downstream pathways that result in the simulation of cell migration (Shen et al, 2002).

Gain of 4q

Twelve (23.5%) ependymoma had gains of chromosome 4 and this was the most common region of gain in the present study. The minimum region of overlap on chromosome 4 could be localised to 4q22-28, with six cases also having gain that extended to the p arm. Simple numerical gains of chromosome 4 have only been

reported in 1 case of ependymoma using conventional cytogenetic techniques (Weremowicz et al, 1992). CGH analyses have also shown a low frequency of gain of 4 in ependymoma, with only three tumours been reported with this CNA (Dyer et al, 2002; Zheng et al, 2000). Structural abnormalities of chromosome 4 in ependymoma include add(4)(qter) (Vagner-Capodano et al, 1999).
The frequency of chromosome 4 gain seen in the tumours in this study appears to be a novel finding and worthy of further investigation.

There are a number of genes located on chromosome 4 that may have a role in ependymoma. The *ABCP* gene, mapping to 4q22, is an ATP-binding cassette transporter gene that is expressed in human placental tissue (Allikmets et al, 1998). This gene is a member of a subfamily of genes that includes several multidrug resistance transporters. Recent studies have shown *ABCP* to be overexpressed and amplified in some breast and colon cancers that are resistant to mitoxantrone and daunorubicin (Miyake et al, 1999). Data suggests that *ABCP* is a transporter for some chemotherapeutic agents and that overexpression of this transporter may play a role in drug resistance in some cancers. Chemotherapy has not prolonged survival of patients with brain tumours, mostly due to drug resistance at the cellular level. The role of genes such as *ABCP* may have a role in the failure of chemotherapy in these tumours but as yet it is unknown. Another gene that may be involved in drug resistance in cancer is the *MXR*, mapping to 4q21-22. This gene encodes a half transporter that is known to mediate mitoxantrone resistance (Knutsen et al, 2000). Evaluation of the role of these two genes in ependymoma is worthy of further investigation due to the seemingly chemoresistant nature of these tumours. Overexpression of these genes may help to elucidate the mechanism of chemoresistance in ependymoma.

The fibroblast growth factor-2 gene (*FGF-2*) has been localised to 4q25-27 and may have a role in the genesis of astrocytic tumours. *FGF-2* has a variety of biological effects in many different cells and organ systems including embryonic development, tumourigenesis and angiogenesis (Rickman et al, 2001). The gene is expressed at low levels in many tissues and cell types and reaches high

concentrations in the brain and pituitary gland. A recent study has shown that the effects of *FGF-2* on glial proliferation are controlled by cAMP and this may help to define the role of aberrant *FGF-2* signalling in the mechanisms of unbalanced glial growth which may lead to tumour formation (Bayatti and Engele, 2001; Sonoda et al, 2001).

Recently, *SMARCAD1,* a member of the DEAD/H box-containing helicase superfamily, has been localised to 4q22-23. Expression of SMARCAD1 is associated with an increased frequency of transcriptional activation events driven by the adenovirus factor, E1A. Members of the helicase superfamily are essential to some aspects of nucleic acid metabolism such as DNA replication, transcription and excision and repair of mutated bases. *SMARCAD1* gene overexpression has been shown in an E1A-expressing cell line with an increased capacity for gene reactivation events by genomic rearrangement. This suggests that *SMARCAD1* may have role in the development of genetic instability (Adra et al, 2000).

The *HPSE* (heparanase) gene has recently been mapped to 4q22. Heparanase is responsible for the degradation of heparan sulphate, the cleavage of which may regulate cell growth by releasing heparan-sulphate-bound cytokines and growth factors such as basic FGF from cell surfaces or from the extracellular matrix. These molecules are known to enhance tumour growth and migration and also induce angiogenesis (Dong et al, 2000). The expression of this gene in brain tumours has not yet been investigated.

Gain of chromosome 5

There has only been one report of gain of chromosome 5 in paediatric ependymoma, in an anaplastic tumour (Griffin et al, 1992). CGH analyses have reported gain of chromosome 5 in only ten cases of paediatric ependymoma and one case of adult anaplastic ependymoma (Carter et al, 2002; Dyer et al, 2002; Grill et al, 2002; Hirose et al, 2001; Scheil et al, 2001; Zheng et al, 2000). There are also reports of two tumours with high copy number amplification of chromosome 5 (Carter et al, 2002; Dyer et al, 2002). Gain of 5p has been reported

in myxopapillary ependymoma (Reardon et al, 1999; Scheil et al, 2001). The only reported structural abnormality of chromosome 5 is add(5p) (Neumann et al, 1993). There were nine tumours (18%) in the present study with gain of 5q, with a minimum overlapping region of 5q15-21. None of these tumours had gain that extended above the centromere. Gain of 5q was seen more frequently in primary tumours than recurrent tumours (6 versus 3), suggesting gain of 5q might be an early event in ependymoma tumourigenesis.
CGH analyses have shown gain of chromosome 5 in squamous cell carcinoma of the lung, paediatric germ cell tumours, malignant melanoma, pancreatic carcinoma, ovarian carcinoma, rhabdomyosarcoma, medulloblastoma, breast carcinoma, and oligodendroglioma (Aubele et al, 1999; Barks et al, 1997; Bridge et al, 2002; Bussey et al, 1999; Eberhart et al, 2002; Jeuken et al, 1999; Luk et al, 2001; Solinas-Toldo et al, 1996; Sonoda et al, 1997).

To date no candidate genes have been mapped to this region of gain on 5q.

Chromosome 6 abnormalities

There were two regions of alteration on chromosome 6 in the ependymoma in the present study. Two tumours (4%) had loss of 6q23-27 and ten tumours (19.5%) had gain of 6q11-16. Loss of 6q was the sole abnormality in the recurrent ependymoma IN1497. Previous cytogenetic analysis of paediatric ependymoma has reported loss of chromosome 6 in one case (Neumann et al, 1993). CGH analyses have reported loss of 6q21-qter in twenty-two cases of ependymoma (Dyer et al, 2002; Grill et al, 2002; Hirose et al, 2001; Reardon et al, 1999; Scheil et al, 2001; Zheng et al, 2000). Reported structural abnormalities include del(6)(q15) and del(6)(q25) (Neumann et al, 1993; Rogatto et al, 1993; Slavc et al, 1995).

In one study, loss of 6q and loss of chromosome 9 were mutually exclusive events (Hirose et al, 2001). This was also true for the tumours with loss of 6q in the present study. Loss of 6q was the most common abnormality in a study of twenty-three primary ependymoma (Reardon et al, 1999) and loss of 6q occurred independently of monosomy 22 in this study. This was not true in the present

study, where loss of 6q and 22 occurred in the same recurrent tumour, IN1258. A study of primary and recurrent tumours from the same patient showed a clear progression pattern involving chromosome 6 (Grill et al, 2002). In contrast to the study by Hirose. et al (2001), loss of 6q and gain of chromosome 9 were present in the first relapse of tumour, while the primary tumour had an apparently normal karyotype. This suggests that loss 6q is not an initial event but is more likely to be a promoting event that occurs early in the progression of ependymoma. In the present study, loss of 6q was only evident in recurrent tumours, in one case as a sole abnormality and in the other case as one of four regions of loss. This finding agrees with the suggestion of Grill et al. (2002) that loss 6q may be involved in ependymoma progression.

Chromosome 6q loss has been found to be a common abnormality in leukaemia and has also been reported in other brain tumours (Agamanolis and Malone, 1995; Bhattacharjee et al, 1997; Bigner et al, 1990; Griffin et al, 1988; Yamada et al, 1994), as well as ovarian, breast and prostate cancer (Rodriguez et al, 2000; Visakorpi et al, 1995). Despite numerous reports of 6q loss in a wide variety of human malignancies no candidate tumour suppressor genes have been identified.

Simple numerical gains of chromosome 6 have only been reported in one case of anaplastic ependymoma (Weremowicz et al, 1992). CGH analyses have reported high copy number amplification of 6q27 in one case of anaplastic ependymoma (Carter et al, 2002). Gain of 6q11-21 has been reported in a small number of ependymoma (Dyer et al, 2002; Shlomit et al, 2000; Zheng et al, 2000). Reported structural abnormalities of 6q include der(6)t(6;16)(q11;p11), der(6)t(6;12)(q13;q11.2), der(6)t(1;6)(q11.2;q11.2) and der(6)t(1;6)(q12;q13) (Mazewski et al, 1999; Neumann et al, 1993; Slavc et al, 1995).

Loss of 6q may be associated with progression of ependymoma and the findings of the present study suggest that gain of 6q may be an early event in ependymoma. Of the ten tumours with gain 6q, seven were primary tumours. Gain of 6q was also a common feature in the ependymoma that have to date shown no sign of clinical recurrence. It may be candidate oncogenes on 6q are involved with initiation rather than progression of ependymoma.

There are a few candidate genes on chromosome 6q that may be involved in tumourigenesis. The *ROS1* gene is located at 6q22 and is a tyrosine kinase proto-oncogene (Satoh et al, 1987). *ROS1* is expressed in glioblastoma derived cell lines but not in normal brain and one cell line has been shown to have an activating mutation at the *ROS1* locus (Birchmeier et al, 1990; Birchmeier et al, 1987). *TTK* has been localised to 6q13-21 and codes for a human protein kinase which is closely related to the SPK1 serine, threonine and tyrosine kinase, the PIM1, PBS2 and CDC2 serine/threonine kinases, and TiK kinase (Mills et al, 1992). TTK mRNA is present at high levels in tissues that contain a large number of proliferating cells, such as testis and thymus, but is not detected in other benign tissues. Freshly isolated cells from malignant tissues and rapidly proliferating cell lines express TTK mRNA (Mills et al, 1992). These findings suggest that expression of TTK is associated with cell proliferation.

Gain of chromosome 7

There were two regions of gain on chromosome 7 in the ependymoma in the present study. Three tumours had gain confined to the 7q21-31 region and four tumours had gain of almost the entire chromosome.

Conventional cytogenetic techniques have shown gain of chromosome 7 in eight cases of paediatric ependymoma (Bhattacharjee et al, 1997; Griffin et al, 1992; Rogatto et al, 1993; Vagner-Capodano et al, 1992; Vagner-Capodano et al, 1999; Weremowicz et al, 1992). CGH analyses have shown gain of chromosome 7 in nine cases of anaplastic ependymoma and four cases of ependymoma, and gain of 7q21-qter in one recurrent ependymoma (Carter et al, 2002; Dyer et al, 2002; Hirose et al, 2001; Reardon et al, 1999; Zheng et al, 2000). Trisomy 7 has been associated with the anaplastic variant of ependymoma (Vagner-Capodano et al, 1999) but no evidence for this could be found in the present study. Reported structural abnormalities of chromosome 7 in ependymoma include der(7), and add(7)(q36) (Vagner-Capodano et al, 1992; Vagner-Capodano et al, 1999).

Trisomy 7 is not restricted to tumours of the nervous system but has been found in other malignancies, such as tumours of the bladder, kidney and lung. This alteration appears to be associated with malignant tumours or with those tumours that have the potential for becoming malignant (Wernicke et al, 1997). It has been suggested that gains of chromosome 7 are a secondary feature of astrocytoma tumourigenesis and may infer some sort of growth advantage to neoplastic cells (White et al, 1995). However, trisomy 7 and loss of the sex chromosomes have also been found in cell cultures of non-neoplastic brain tissue, as well as cell cultures from kidney, bladder and lung tumours (Vagner-Capodano et al, 1992). This suggests that trisomy 7 may not be a tumour specific aberration as glioma cells and other non-neoplastic cells may have a propensity to acquire extra copies of chromosome 7 and lose sex chromosomes (Rogatto et al, 1993). One problem associated with the attribution of abnormal chromosomes to normal brain tissue is that characteristically the infiltrating edge of a primary tumour at the border between normal brain and tumour is ill defined. There is quite often an area of oedema surrounding the tumour making the differentiation between normal brain and tumour even more difficult (Neumann et al, 1993). In order to determine whether normal brain tissue exhibits gain of chromosome 7, one group used CGH to study autopsy material from eight patients who had died from non-brain tumour related disease (Schröck et al, 1996). No copy number changes of the autosomes were reported suggesting that complete or partial gain of chromosome 7 is tumour related rather than a characteristic of normal brain tissue.

In the present study gain of 7q was associated with gain of 1q and 2q and was not seen without one or other of these alterations. This abnormality was not restricted to any particular grade of ependymoma, suggesting the genes involved on chromosome 7 are not restricted to a more aggressive variant of the tumour, as may be the case with other glial cell derived tumours.

The *EGFR* gene is located at 7p12 and is a170kDa transmembrane glycoprotein involved in ligand binding (Besson and Yong, 2001). EGFR is a member of the Erb-B subfamily consisting of EGFR/ErbB-1, ErbB-2/HER/Neu, ErbB-3 and

ErbB-4. The physiological ligands of EGFR include EGF, amphiregulin, heparin binding EGF-like growth factor (HB-EGF) and the transforming growth factor α (TGFα) (Besson and Yong, 2001). Overexpression of EGFR has been reported in ependymoma but there have been no reports of gene amplification (Hall et al, 1990). The *GBAS* (glioblastoma amplified sequence) gene has been mapped to the 7p12 region and has been shown to be co-amplified with *EGFR* (Wang et al, 1998).

The *CDK6* gene at 7q21-22 is a member of the p16/CDK4/cyclinD/pRB pathway (Costello et al, 1997; Johnson et al, 1995). Amplification-associated and amplification-independent increases in CDK6 protein levels have been identified in adult gliomas. *CDK6* amplification has been reported in 2/37 malignant glioma but not in low-grade astrocytoma (Costello et al, 1997). It would appear that this gene is only one of a number of genes that are co-amplified in this region of 7q in glial cell derived tumours.
The *MDR1* gene maps to 7q21 and encodes a transmembrane protein that is an essential component of the blood brain barrier. The protein functions as a drug-transport pump that allows the passage of some drugs from the brain back into the blood. This gene is thought to have a role in the drug resistant phenotype of some ependymoma and will be discussed in detail in the section on drug resistance.

Gain of 8q

Gain of chromosome 8 was seen in six tumours in this study (12%), including two with regions of high copy number amplification. The minimum overlapping region was 8q21-22, though in one case (IN2939) high copy number amplification spanned almost the entire long arm with low-level gain of the rest of the chromosome. Conventional cytogenetic techniques have identified gain of chromosome 8 in two cases of anaplastic ependymoma and two ependymoma (Bhattacharjee et al, 1997; Griffin et al, 1992; Neumann et al, 1993; Weremowicz et al, 1992). CGH analyses have shown gain of 8p in one case of ependymoma (Reardon et al, 1999). Gain of 8q24 has been reported in one case of

myxopapillary ependymoma (Scheil et al, 2001). Gain of the entire chromosome has been reported in six primary ependymoma, along with one further tumour with amplification of the whole chromosome (Carter et al, 2002; Dyer et al, 2002). Gain of chromosome 8 has also been reported in two cases of recurrent ependymoma (Dyer et al, 2002). There do no appear to be any reports of structural abnormalities of chromosome 8 in paediatric ependymoma. In the present study, gain of 8q was only found in ependymoma and not in anaplastic ependymoma. This may be due to the relatively small number of anaplastic ependymoma available for analysis. Gain of 8 was present in three recurrent samples. Two of these samples were first and second recurrences from the same patient. The first relapse showed gain of 8q compared to amplification in the second relapse. The presence of gain of 8q in both primary and recurrent tumours suggests it may be an early event in the genesis of ependymoma.

Gains of chromosome 8 have been found in a number of malignancies including, adenocarcinoma, oligodendroglioma, breast carcinoma, fibrosarcoma, squamous cell carcinoma, ovarian carcinoma, pancreatic carcinoma and malignant germ cell tumours (Aubele et al, 1999; Bridge et al, 2002; Eberhart et al, 2002; Jeuken et al, 1999; Schmidt et al, 2002; Solinas-Toldo et al, 1996; Sonoda et al, 1997; Zielenska et al, 2001; Zitzelsberger et al, 1998).

One candidate gene on 8q is *MMP16* (matrix metalloproteinase 16). MMPs are zinc binding endopeptidases that degrade various components of the extracellular matrix and have been implicated in both normal and pathological conditions including tissue remodelling, wound healing, angiogenesis and tumour invasion. *MMP16* has been shown to induce the activation of pro-gelatinase A and is expressed as a 12Kb transcript in brain, placenta, heart and some carcinoma cell lines (http://www.ncbi.nlm.nih.gov). There have been no reports of *MMP16* alterations in human brain tumours.

E2F5 is located at 8q21.13 and encodes a predicted 345-amino acid protein that shares 69% homology with *E2F4*. Both *E2F5* and *E2F4* have conserved DNA-

binding domains and bind to p130 and p107 (www.ncbi.nlm.nih.gov). E2F4/E2F5 and p107 act as transducers of TGF-β signal upstream of *CDK*. *E2F5* has been found to be overexpressed in cDNA microarray analysis of a number of ependymoma analysed in this study (Suarez-Merino, 2002, personal communication). The *BAALC* gene (brain and acute leukaemia, cytoplasmic) is located at 8q22.3 and is almost exclusively expressed in neuroectoderm-derived tissues (Tanner et al, 2001). Overexpression of *BAALC* has been demonstrated in GBM-derived cell lines and is associated with an adverse prognosis in patients with AML (Tanner et al, 2001).

Increases in copy numbers of 8q21-22 have been seen to occur more frequently in disease free patients with ovarian clear cell adenocarcinoma than in recurrent/non-surviving patients (Suehiro et al, 2000). In the comparison between the ependymoma patients in this study with and without recurrent disease, gain (and high copy number amplification) of chromosome 8 was seen in one patient with no recurrent disease who was alive 38 months post diagnosis and in none of the patients whose tumour had recurred. This data might indicate overexpression of a gene (or genes) on 8q that confer a survival advantage in some malignancies.

Two ependymoma (IN2939 and IN2970) had regions of high copy number amplification at 8q21-23. Amplification in this region has been reported in both adult and paediatric malignant astrocytoma (Nishizaki et al, 1998; Warr et al, 2001). Other malignancies with amplification of this region include gastric cardia adenocarcinoma, adenocarcinoma of the lung, breast carcinoma, prostate carcinoma, Barrett's adenocarcinoma and oral squamous cell carcinoma (Forozan et al, 2000; Luk et al, 2001; Nupponen et al, 2000; Oga et al, 2001; van Dekken et al, 2001; Walch et al, 2000). In one tumour (IN2970) amplification was proximal to the *c-myc* locus at 8q24.1 and therefore precludes it as being the target of amplification in this tumour. IN2939 had amplification of most of the long arm of chromosome 8, so *c-myc* may be involved in this tumour. Amplification of *c-myc* has been reported in 3/3 paediatric ependymoma (Kucerova et al, 2000).

Chromosome 9 alterations.

Chromosome 9 exhibited both gains and losses in the tumours in this study. One tumour (IN2944) showed monosomy 9 as a sole abnormality. Tumours IN2638 and IN2904 showed gains of 9p and concurrent loss from 9q. There were two other tumours with loss of 9q, and four tumours with gain of 9p. One tumour showed gain of the whole chromosome and IN2970 had a region of high copy number amplification that spanned 9p24-q12. Previous cytogenetic studies have reported gain of chromosome 9 in three cases of ependymoma (Bhattacharjee et al, 1997; Griffin et al, 1992; Neumann et al, 1993). Monosomy 9 has been reported in two cases of anaplastic ependymoma (Bigner et al, 1997; Mazewski et al, 1999). CGH analyses have shown gain of chromosome 9 in twelve cases of anaplastic ependymoma, one myxopapillary ependymoma, six ependymoma and two recurrent ependymoma (Carter et al, 2002; Dyer et al, 2002; Grill et al, 2002; Hirose et al, 2001; Reardon et al, 1999; Scheil et al, 2001). Monosomy 9 has been reported in one case of ependymoma and six cases of anaplastic ependymoma (Carter et al, 2002; Dyer et al, 2002; Hirose et al, 2001; Scheil et al, 2001; Zheng et al, 2000). Loss of 9p has been reported in two of ependymoma and three cases of anaplastic ependymoma (Hirose et al, 2001; Reardon et al, 1999; Scheil et al, 2001). Structural abnormalities of chromosome 9 include t(9;?)(q34;?), inv(9)(p13;q12.3), add(9)(p?), and del(9)(q22), (Mazewski et al, 1999; Sainati et al, 1996; Stratton et al, 1989). Molecular genetic analyses of ependymoma have shown LOH for markers on chromosome 9 to occur infrequently (Bijlsma et al, 1995; Von Deimling et al, 2000).

Previous analyses show that alterations of chromosome 9 (both losses and gains) appear to occur more frequently in the anaplastic variant of ependymoma. However in the present study none of the eight anaplastic ependymoma had alterations of chromosome 9. This may be due to the low frequency of anaplastic tumours in this study group. Alterations of chromosome 9 appeared with similar frequency in both primary and recurrent tumours, suggesting such alterations are an early event in ependymoma tumourigenesis.

Deletions of 9p are common in malignant astrocytoma in adults. These deletions are interstitial in a majority of cases and often occur together with terminal deletions or monosomy (Ichimura et al, 1994). The region 9p21 contains the *CDKN2A* and *CDKN2B* tumour suppressor genes, which encode the cell cycle inhibitors p16 and p15 respectively. Inactivation of these two genes by homozygous deletion, point mutation and methylation of the 5′ promotor region may increase progression through the cell cycle in tumours. The *CDKN2A* locus also contains an alternative reading frame, which encodes $p14^{ARF}$. Expression of ARF protein suppresses growth of human glioma cells though an RB-independent pathway and can induce both G_1 and G_2- phase arrest in a variety of cell types (Cavenee et al, 2000). The role of p16, p15 and $p14^{ARF}$ in the suppression of glioma can be seen in Figure 4.47. The *CDKN2A* locus controls both the pRb and p53 pathways and indicates a close functional relationship between these two pathways. Hypermethylation of $CDKN2A^F$ and $CDKN2B^A$ has been reported in primary GBM and in low grade astrocytoma that progress to secondary GBM (Nakamura et al, 2001). Many primary malignancies have alterations of this gene, including prostate cancer, renal carcinoma, nasopharyngeal carcinoma and melanoma (Chen et al, 1999; Knuutila et al, 1999; Perinchery et al, 1999; Pollack et al, 2001; Schraml et al, 2001).

In a series of fourteen ependymoma no mutations or deletions of *CDKN2A/p16* were observed (Sato et al, 1996). Sixteen cases of ependymoma were recently studied for *CDKN2A/p16* inactivation by immunohistochemistry (Bortolotto et al, 2001). Immunohistochemistry was positive in thirteen cases, homozygous deletion was found in one tumour and none of the tumours showed methylation of the 5′ promotor region. This data suggests that *CDKN2A/p16* is not the target of 9p loss seen in ependymoma.

The low frequency of *CDKN2A* mutations in tumours such as bladder cancer and non-small cell lung cancer that have a high rate of LOH on 9p, suggests there might be other targets on 9p involved in malignancy (Wiest, 1997). A recent study has identified additional tumour suppressor loci in the 9p21-24 region in melanoma (Pollack et al, 2001). The *IFN* gene cluster (*IFNA/IFNW*, *IFNB1* and

MTAP) is interspersed within a short segment of 9p. Approximately 35% of gliomas have been shown to have deletions of the *IFN* genes or a rearrangement of the sequences around these genes (Ichimura et al, 1994; Olopade et al, 1992).

There are a number of putative tumour suppressor genes located within the region of loss on 9q that are mutated in other malignancies, such as bladder cancer (van Tilborg et al, 2001). Transforming growth factor β regulates cell cycle progression by binding to the type II receptor and activation of the type I receptor. Various tumours are insensitive to *TGF-β* mediated cell cycle arrest, suggesting that the signalling pathway is inactivated by a mutation in either of the receptors. A mutation in the *TGFBR1* gene has been observed in breast cancer metastases. This gene has alleles with five, six, nine and ten alanines, with the 9A allele being the most frequent in the population. The 6A allele is the candidate tumour susceptibility allele as it is less active in signal transaction (van Tilborg et al, 2001). The*ZNF189* gene encodes a Krüppel-like zinc finger protein, with a KRAB A domain, a spacer region and 16 Cys_2His_2 type zinc fingers. These regions are frequently found in proteins involved in repression of transcription (van Tilborg et al, 2001). The *DBCCR1* gene (deleted in bladder cancer chromosome region candidate 1) is located at 9q32-33 and the *TSC1* gene (tuberous sclerosis complex 1) is located at 9q34 (Knuutila et al, 1999). LOH for this gene has been reported in three ependymoma and does not appear to be associated with any one grade of tumour (Parry et al, 2000). The human *PATCHED* (*PTCH*) gene maps to 9q22.3 and is expressed in target tissues of *sonic hedgehog* (*SHH*) pathway (Hahn et al, 1996).

The *PTCH* gene product functions as a receptor for the *SHH* gene product. In *Drosophila, SHH* has been implicated in a congenital brain malformation known as holoprosencephaly that results from abnormal migration of CNS cells (Zurawel et al, 2000). *PTCH* also interacts with *SMO*, the mammalian homolog of *Drosophila's smoothened (smo).* Alterations in the *PTCH* tumour suppressor gene are involved in the development of some sporadic desmoplastic medulloblastoma associated with Gorlins syndrome (Raffel et al, 1997). There are no reports of *PTCH* alterations in ependymoma.

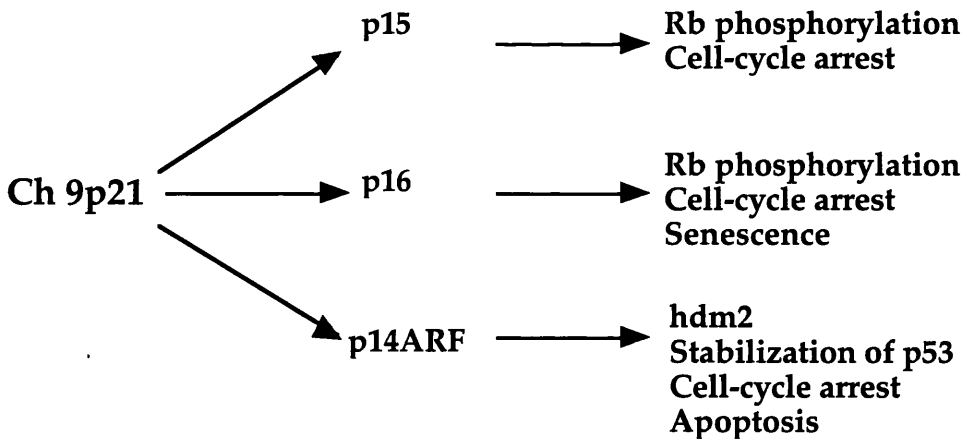


Figure 4.47: The role of INK4a locus in glioma suppression.
Transfer of any of the products of the p15 and p16 genes induces growth arrest in G1. In addition, p14ARF induces arrest in G2. The protein p14ARF represents a 'functional bridge' between Rb and p53 pathways and when overexpressed induces p53-related apoptosis. The expression of p14ARF is regulated among other genes by the transcription factors E2F-1 and E2F-2, and therefore upregulation of p14ARF may explain the co-operation between p53 and E2F-1 in the induction of apoptosis. Co-transfer of p53 and p14ARF could be important in those gliomas overexpressing hmd2 and resistant to the p53 apoptotic effect (Fueyo et al, 2001).

High copy number amplification of chromosome 9 has not been reported previously in paediatric ependymoma and the region of amplification seen in IN2970 is therefore a novel finding. Amplification of the region 9p22-pter has been reported in oral squamous cell carcinoma, medulloblastoma and anaplastic astrocytoma (Eberhart et al, 2002; Nishizaki et al, 1998; Oga et al, 2001). Amplification spanning the whole of chromosome 9 has been reported in two cases of paediatric anaplastic astrocytoma (Rickert et al, 2001). A novel gene, *GASC1*, lies within the region of amplification at 9p. This gene contains a PX domain and two PHD fingers. PX domains participate in protein-protein interactions and the PHD finger is found in nuclear proteins involved in chromatin-mediated transcriptional regulation. Both of these motifs are potentially oncogenic suggesting *GASC1* may have an important role in

tumourigenesis and *GASC1* amplification and overexpression has been observed in oesophageal cancer (Yang et al, 2000).

Gain of chromosome 11

Three (6%) tumours in this study showed gain of chromosome 11. IN2939 had two separate regions of gain at 11p12-pter and 11q14-23. IN1759 had gain of the entire chromosome and IN2376 had gain of 11p only.
Conventional cytogenetic techniques have shown gain of chromosome 11 in one case each of ependymoma and anaplastic ependymoma (Griffin et al, 1992; Sawyer, 1994). Previous CGH analyses have reported gain of chromosome 11 in three cases of myxopapillary ependymoma, one ependymoma and four cases of anaplastic ependymoma (Carter et al, 2002; Dyer et al, 2002; Reardon et al, 1999; Scheil et al, 2001). Structural abnormalities involving chromosome 11 have been reported including t(11;18)(q13;q21) in ependymoma, t(11;17)(q13;q21), add (11)(q23) and add(11)(p15) in anaplastic ependymoma (Kucerova et al, 2000; Neumann et al, 1993; Rogatto et al, 1993; Sainati et al, 1996).

The 11q13 breakpoint is a frequent chromosomal abnormality in malignant brain tumours (Griffin et al, 1988). The oncogenes *BCL1*, *HST* and *INT2*, which map to 11q13 are frequently amplified in some human tumours. There have been five reports of chromosome 11 gain as a sole abnormality in ependymoma, three of which had the 11q13 breakpoint (Dal Cin et al, 1999; Jenkins et al, 1989; Sainati et al, 1992). Only one tumour in the present study showed gain in the 11q13 breakpoint region. Gain of 11p seems to be a more common abnormality in the ependymoma in this study.

Previous data suggests gain of chromosome 11 is more common in the anaplastic or myxopapillary variants of ependymoma. This was not apparent in the ependymoma in the present study due to the low numbers of anaplastic tumours and the absence of myxopapillary tumours. Two of the tumours with chromosome 11 gain were a primary and recurrent pair. The primary tumour showed gain of the whole chromosome compared to 11p gain in the recurrence.

Loss of 11q was also seen in one case of recurrent ependymoma suggesting genes on 11q may be involved in progression rather than initiation of ependymoma.

Chromosome 12 abnormalities

There were two regions of alteration on chromosome 12 in the ependymoma in this study. Three tumours (6%) had gain of 12q15-21 and eight tumours (16%) had loss of 12q23-qter. Two cases (IN2923 and IN2638) had both gain and loss of 12q. The results point towards a breakpoint in the 12q22 region.

Gain of chromosome 12 has been reported in one case of anaplastic ependymoma (Weremowicz et al, 1992). There appear to be no reports of monosomy 12 in the literature. CGH analyses have shown chromosome 12 abnormalities to occur infrequently. Loss of 12pter-q23 has been reported in one ependymoma and monosomy 12 in one anaplastic ependymoma (Dyer et al, 2002; Hirose et al, 2001). Loss of 12q23-qter has been reported in three ependymoma (Zheng et al, 2000). A myxopapillary ependymoma has also been reported with loss of 12q15-21 and one with monosomy 12 (Reardon et al, 1999; Scheil et al, 2001). Gain of 12 has been reported in two ependymoma and one anaplastic ependymoma (Dyer et al, 2002; Grill et al, 2002; Zheng et al, 2000). Gain of 12q24 has been reported in a case of myxopapillary ependymoma, along with gain of 12q23-qter in two cases of intracranial ependymoma (Scheil et al, 2001). Reported structural abnormalities of chromosome 12 in ependymoma include der(12)t(12;?)(q21.3;?) and del(12)(q22) (Mazewski et al, 1999; Rogatto et al, 1993).

In the present study loss of 12q occurred more frequently in the recurrent tumours than the primary tumours suggesting the gene(s) lost from this region are likely to be involved in progression.

LOH on 12q accompanied by amplification of genes from 12q13-14 has been reported in 50% of adult malignant glioma yet the molecular basis between amplification and loss on the same chromosome is not yet known (Reifenberger et al, 1995). An amplification cluster has been described at 12q13-14 (Reifenberger et al, 1994a). This region contains a number of genes with oncogenic potential, including *MDM2*, *SAS*, *GADD*, *GLI* and *CDK4*. Amplification of these genes have been reported in adult astrocytoma but not in

paediatric ependymoma (Mao and Hamoudi, 2000; Reifenberger et al, 1996; Reifenberger et al, 1994a; Rollbrocker et al, 1996). Amplification of genes in the 12q13-15 region has also been reported in neuroblastoma and osteosarcoma (Gisselsson et al, 2002; Van Gele et al, 1997). *BAZ2A*, a gene that encodes a bromodomain protein, has been localised to 12q24.3-qter (Jones et al, 2000). There are four genes in the *BAZ* family that may have tumour suppressor activity although the function of *BAZ2A* is still unknown.

Gain of chromosome 13

In the present study twelve (23.5%) tumours had gain of chromosome 13. In ten cases the region of gain was confined to the 13q21-31 region. One tumour showed gain of the entire chromosome and one tumour showed gain of 13q14-qter.

Conventional cytogenetic techniques have shown gain of chromosome 13 in four ependymoma (two ependymoma, one anaplastic ependymoma and one recurrent anaplastic ependymoma (Neumann et al, 1993; Vagner-Capodano et al, 1999; Weremowicz et al, 1992). Gain of 13q has been reported by CGH in four ependymoma and three anaplastic ependymoma (Carter et al, 2002; Dyer et al, 2002; Grill et al, 2002; Zheng et al, 2000). In one study, gain of 13q21-31 occurred more frequently in recurrent tumours than primary tumours (Dyer et al, 2002). Amplification of 13q33-34 has been reported in one case of anaplastic ependymoma (Carter et al, 2002). There has only been one reported structural abnormality of 13q in ependymoma: t(13;14)(q11;p11) (Neumann et al, 1993).

Incidental gains of 13q have been reported in a number of other human cancers, including non-small cell lung carcinoma, testicular germ cell tumours, malignant melanoma, ovarian carcinoma, squamous cell carcinoma, fibrosarcoma, medulloblastoma, breast carcinoma and high grade oligodendroglioma (Barks et al, 1997; Daigo et al, 2001; Eberhart et al, 2002; Jeuken et al, 1999; Luk et al, 2001; Mostert et al, 1996; Schmidt et al, 2002; Sonoda et al, 1997; Speicher et al, 1995).

The analysis of chromosome 13 as part of the human genome project has shown this region on the long arm to be a gene-poor region (International Human Genome Dequencing Consortium, 2001). Within the region of 13q21-22 there are a number of genes with biological functions that may have relevance in cancer. *PIBF1* inhibits natural killer activity and prostaglandin synthesis. This gene has been found to be expressed prominently in the testis and spleen (Rozenblum et al, 2002). *KIAA1008* is a gene with sequence homology to the dis3+ gene. It encodes a mitotic-control protein with a PilT amino (PIN) terminus domain and has been found to be overexpressed in colorectal cancers (Lim et al, 1997). *KLF12* and *KLF5* are members of the mammalian Kruppel-like transcription factor family (Rozenblum et al, 2002). *KLF12* is a zinc finger protein that binds to the AP-2α gene promotor and represses its expression. *KLF5* may be an activator of *EGF*. *LMO7* is a member of a group of proteins that are thought to have a transcription regulatory function through protein-protein interactions (Rozenblum et al, 2002).

Loss of chromosome 16

In the present study two tumours had loss of 16q, seven had loss of 16p and three tumours had monosomy 16. Conventional cytogenetic techniques have shown monosomy 16 in 2/14 (14%) of ependymoma (Griffin et al, 1988; Neumann et al, 1993). CGH analyses have shown loss of 16 in ten ependymoma and two anaplastic ependymoma (Carter et al, 2002; Dyer et al, 2002; Hirose et al, 2001; Shlomit et al, 2000; Zheng et al, 2000). In one CGH study, loss of 16q was only seen in conjunction with gain of 1q (Shlomit et al, 2000). This finding was also true in a number of other CGH analyses of ependymoma (Carter et al, 2002; Dyer et al, 2002). In the present study, loss of 16 was seen along with gain of 1q in six cases, a relationship that was found to be statistically significant (p = 0.0087). A small number of structural abnormalities of chromosome 16 have been reported in ependymoma including der(16)t(1;16)(q11.2;q11.2) and del(16)(q13) (Mazewski et al, 1999; Stratton et al, 1989).

Loss of chromosome 16 in the present study was seen in association with a number of CNAs in addition to gain 1q, namely gain of 4q, 7q and 13, and loss of

1p. This may be indicative of a pathway of alterations that are required in order for ependymoma to form. Chromosome 16 alterations occurred in both primary and recurrent ependymoma suggesting that loss from this chromosome is an early event in ependymoma tumourigenesis.

The region of loss on 16p in the present study includes the location of the *TSC2* gene (tuberous sclerosis 2) at 16p13.3. The tuberous sclerosis complex is a genetic disorder which predisposes sufferers to mental retardation, developmental brain defects, seizures and increased risk of developing tumours of the brain, kidney and heart (Gutmann et al, 2000). The *TSC2* gene product is tuberin, which has an amino acid domain with sequence similarity to GTPase-activating proteins for rap1. Active rap1 can transform mouse fibroblast cells resulting in tumour formation in immunocompromised mice. Tuberin may regulate cell growth by decreasing rap1-induced cell proliferation. *TSC2* mRNA and protein expression is reduced or absent in 30% of sporadic astrocytoma suggesting that alterations in the rap1 signalling pathway may be important in the development of astrocytoma (Gutmann et al, 2000). LOH at the *TSC2* locus has been observed in 20% of ependymoma but no intragenic mutations of the gene were found in these tumours (Parry et al, 2000). The lack of mutation of the *TSC2* gene in these tumours suggests it may not have a role in the development of ependymoma.

The *CYLD1* gene is located at 16q12-13 and is frequently associated with LOH 16q in patients with hereditary cylindromatosis (Thomson et al, 1999). Hereditary cylindromatosis is a rare autosomal dominant disease characterised by the development of benign tumours of skin appendages. There have been no reports of alterations of this gene in brain tumours.

Loss of chromosome 17

Monosomy 17 was seen in four cases in the present study and one further tumour showed loss of 17q. Previous cytogenetic analyses have shown monosomy 17 in six cases of ependymoma (Rogatto et al, 1993). CGH analyses have shown monosomy 17 or loss of individual arms in seven ependymoma and

six anaplastic ependymoma (Dyer et al, 2002; Grill et al, 2002; Reardon et al, 1999; Zheng et al, 2000). Structural abnormalities of chromosome 17 reported in paediatric ependymoma include der(17)t(17;?) and der(17)t(17;?)(p13;?) (Neumann et al, 1993; Stratton et al, 1989). Isochromosome 17q has also been detected in two cases (Kramer et al, 1998). Loss of heterozygosity at 17p13.3 has been detected in 50% of tumours studied by von Haken et al. (1996), yet other studies have shown much lower rates of LOH at 17p (Bijlsma et al, 1995; Blaeker et al, 1996; James et al, 1990; Kramer et al, 1998). There is a general consensus that *p53* is not the target of 17p loss in ependymoma, since mutations of this gene are rare in both adult and paediatric ependymoma (Fink et al, 1996; Tominaga et al, 1995; Tong et al, 1999; Von Haken et al, 1996).

Loss of chromosome 17 was associated with a number of other CNAs in the ependymoma in the present study, including loss of 1p and gain of 4q. Loss of chromosome 17 occurred with equal frequency in both primary and recurrent tumours, suggesting it is an early event in the genesis of ependymoma.

Gain of 18q

Gain of 18q, spanning the entire long arm, was seen in three cases of ependymoma. There was also one tumour with loss of 18q. Previous CGH studies have reported both gains and losses of 18q in paediatric ependymoma (Reardon et al, 1999; Zheng et al, 2000). Gain of chromosome 18 was only seen in primary tumours, suggesting it is an early event in the genesis of ependymoma.
cDNA microarray analysis of a number of the ependymoma in this study have shown overexpression of the *NCAD* gene at 18q11.2 (Suarez-Merino, 2002, personal communication). *NCAD* is a member of the cadherin gene family that encode proteins that mediate calcium-ion-dependent adhesion (Takeichi, 1987).

Loss of chromosome 19

Loss of chromosome 19 was the most frequent abnormality in the ependymoma in the present study. Eleven tumours (21.5%) had monosomy 19 and three other tumours had loss of 19q13.1-qter. Previous cytogenetic analyses have shown

monosomy 19 in four ependymoma (Rogatto et al, 1993; Stratton et al, 1989). CGH analyses have shown monosomy 19 in four cases of ependymoma and loss of 19q in one anaplastic ependymoma (Dyer et al, 2002; Reardon et al, 1999; Zheng et al, 2000). There are no reports of structural alterations involving chromosome 19 in paediatric ependymoma.

LOH for loci on 19q occur more frequently in anaplastic astrocytoma than diffuse astrocytoma suggesting that potential tumour suppressor genes on 19q are more likely to be involved in tumour progression than initiation (Von Deimling et al, 1994). The frequency of 19q loss in tumours such as oligodendroglioma, neuroblastoma and other glioma suggests the presence of a tumour suppressor gene. The minimum region of deletion on 19q seen in oligodendroglioma and neuroblastoma is in the 19q13 region, a region of loss shared by fourteen of the ependymoma in the present study. A number of candidates in this region, such as *GMFγ* (glia maturation factor γ), *ANOVA* (astrocytic NOVA1-like gene) have shown no mutations in glioma, and have therefore been excluded as the gene involved in these tumours (Ueki et al, 1997; Yong et al, 1995).

There are many candidate tumour suppressor genes on chromosome 19 including *STK11/LKB1* located at 19p13.3, which encodes a widely expressed serine/threonine kinase of 433 amino acids. This gene is mutated in Peutz-Jeghers syndrome, a autosomal dominantly inherited condition that is characterised by benign intestinal hamartoma and melanin spots of the lips, buccal mucosa and digits, as well as increased risk of gastrointestinal, ovarian, pancreatic and breast cancers (Bignell et al, 1998; Hemminki et al, 1998). The *BAX* gene at 19q13.3 is a primary response gene for *p53*, involved in the *p53*-regulated pathway for the induction of apoptosis. *BAX* acts to reduce the death-repressing activity of *BCL2*. Also located at 19q13.3 is the gene that codes for ZIP kinase. When ZIP kinase is overexpressed, it induces morphological changes in cells, suggesting it has an important role in the induction of apoptosis. There are three functionally related genes located at 19q13.2-q13.3, known as *XRCC1*, *ERCC1* and *ERCC2*, and there is some evidence that these genes have a role in both UV cross-link repair and nucleotide excision repair (Knuutila et al, 1999; Thompson et al, 1990; Von Deimling et al, 1992). Mutations of *ERCC1* have been reported in ovarian cancer (Yu et al, 1997). The *p190-A* gene has recently been localised to

19q13.3 and is one of two genes that code for Ras GAP-binding phosphoproteins of 190kDa (Tikoo et al, 2000). This gene is located in the region that is commonly deleted in astrocytoma and may be the candidate tumour suppressor gene involved in these tumours. The *PLA2G4C* gene, that encodes cytosolic phospholipase A_2-γ (cPLA_2-γ), is also located within the putative glioma tumour suppressor gene location. This gene has a role in inflammatory cascades and may also be involved in the *p53* regulated apoptotic pathway. This latter role raises the possibility that *PLA2G4C* may be the tumour suppressor gene involved in glial tumours (Hartmann et al, 2002).

Loss of chromosome 20

Monosomy 20 was seen in two tumours in the present study and loss of 20q in five (10%) other tumours. Losses of chromosome 20 have been reported in both intracranial and myxopapillary ependymoma (Griffin et al, 1992; Zheng et al, 2000) and structural abnormalities of chromosome 20 have been reported in previous cytogenetic analyses, including t(1;20)(q21;q13) and der(20)t(20;9)(q13.2;p22) (Griffin et al, 1992; Neumann et al, 1993). In one recent CGH study, gain of 20q was one of the most frequent abnormalities in twelve ependymoma, and was seen in only benign tumours and not the anaplastic variant (Scheil et al, 2001).

The *TP53TG5* (TP53-target gene 5) has been localised to 20q13.1 and has been shown to be a negative regulator of cell growth. The gene product is likely to have an important role in the checkpoints along the *TP53* signalling pathway (Isaka et al, 2000). A number of potential tumour suppressor genes on 20q have been implicated in myeloid leukaemia patients, including *PLCG1* (phospholipase C γ1), *HNF4* (hepatocyte nuclear factor 4), *TOP1* (topoisomerase 1), *MYBB* (myeloblastosis viral oncogene homologue like 2), *ADA* (adenosine deaminase) and *CD40* (Knuutila et al, 1999). The role of these potential target genes in glial tumours is as yet unknown.

Loss of chromosome 22

Loss of chromosome 22 was present in twelve cases (23.5%) in the present study. The region of loss could not be confined to any one band but spanned the entire chromosome. Monosomy 22 has been reported in 8-40% of ependymoma analysed cytogenetically (Griffin et al, 1992; Hirose et al, 2001; Kramer et al, 1998; Neumann et al, 1993; Ransom et al, 1992; Reardon et al, 1999; Vagner-Capodano et al, 1992; Vagner-Capodano et al, 1999) and CGH analyses have shown loss of 22q in 44% of paediatric ependymoma (Carter et al, 2002; Zheng et al, 2000). It has been suggested that this alteration occurs twice as frequently in adult ependymoma than in paediatric tumours (Mazewski et al, 1999). In the present study, loss of chromosome 22 was a frequent finding and was often seen in conjunction with a number of other CNAs including loss of 1p and 16p and gain of 4q and 5q. Loss of chromosome 22 was seen as frequently in primary tumours as in recurrent tumours and appeared to be more common in benign tumours than in anaplastic tumours, though the latter may be due to the small number of anaplastic tumours included in this study. This data is indicative of loss of 22 being an early event in the genesis of ependymoma.

Chromosome 22 is a rich source of genes associated with a variety of medical conditions. The genes for neurofibromatosis type 2 (NF2) and familial schizophrenia are located in 22q11-12 and genes causing the developmental abnormalities DiGeorge and Cat-Eye syndromes are located at 22q11 (Dumanski, 1996) (see Figure 4.48). Segregation analysis carried out in a family with four cousins affected by ependymoma has indicated that an ependymoma susceptibility gene may be located at 22q11.2 (Hulsebos et al, 1999). Translocations and interstitial deletions involving 22q11 have been reported in ependymoma supporting the presence of an ependymoma locus proximal to the *NF2* locus (Park et al, 1996; Roberts et al, 2001; Stratton et al, 1989; Vagner-Capodano et al, 1999; Von Haken et al, 1996). The *hSNF5/INI1* gene that is involved in malignant rhabdoid tumours, maps to 22q11.2 and was at one time thought to have a role in the progression of astrocytoma. Deletions of loci in this

region were observed in 22/120 cases but in 20 of these cases the loss extended to the entire long arm of the chromosome (Ino et al, 1999). Mutations of *hSNF5/INI1* have been found in choroid plexus carcinoma and in a subset of central primitive neuroectodermal tumours, but not in astrocytoma or ependymoma even though the majority of these tumours have shown LOH at 22q11.2 loci (Kraus et al, 2001; Sevenet et al, 1999).

Deletions have also been reported at 22q12-13, a region encompassing the *NF2* gene at 22q12 (Vagner-Capodano et al, 1999). NF2 is a dominantly inherited disease with a predisposition to development of multiple slow-growing central and peripheral nervous system tumours such as schwannoma, meningioma, and to a lesser extent ependymoma (Slavc et al, 1995). The status of the *NF2* gene in ependymoma has been widely investigated. Mutations of *NF2* appear to be a rare event in intracranial ependymoma and are more strongly associated with spinal ependymoma (Ebert et al, 1999; Singh et al, 2002). Sporadic ependymoma have not been shown to have mutations of the *NF2* gene even when loss of heterozygosity for markers in the 22q11-12 region has been present (Lamszus et al, 2001; Rubio et al, 1994; Slavc et al, 1995; Von Haken et al, 1996). A report of an ependymoma patient with a t(1;22)(p22;q11) suggests the breakpoint is proximal to the *NF2* locus and maps to 22q11 (Park et al, 1996).

Translocations with a breakpoint in q13 have also been reported (Oskam et al, 2000; Vagner-Capodano et al, 1992; Vagner-Capodano et al, 1999; Weremowicz et al, 1992). A recent study of LOH of chromosome 22 in ependymoma showed two common regions of allelic deletions, at 22q11.21-q12.2 and 22q13.1-13.3 (Huang et al, 2002). In one study, a deletion of 22q13 was found in 11% of astrocytoma, 11% of oligodendroglioma and 18% of ependymoma (Koschny et al, 2002). A novel putative tumour suppressor gene, *SCN6*, has recently been identified by subtractive hybridisation in colorectal cancer. This gene has been localised to chromosome 22q13.2 and could therefore be a candidate for the postulated tumour suppressor gene at the deleted 22q13 locus in gliomas. Additional studies are still needed to narrow these regions on 22q and identify potential tumour suppressor genes.

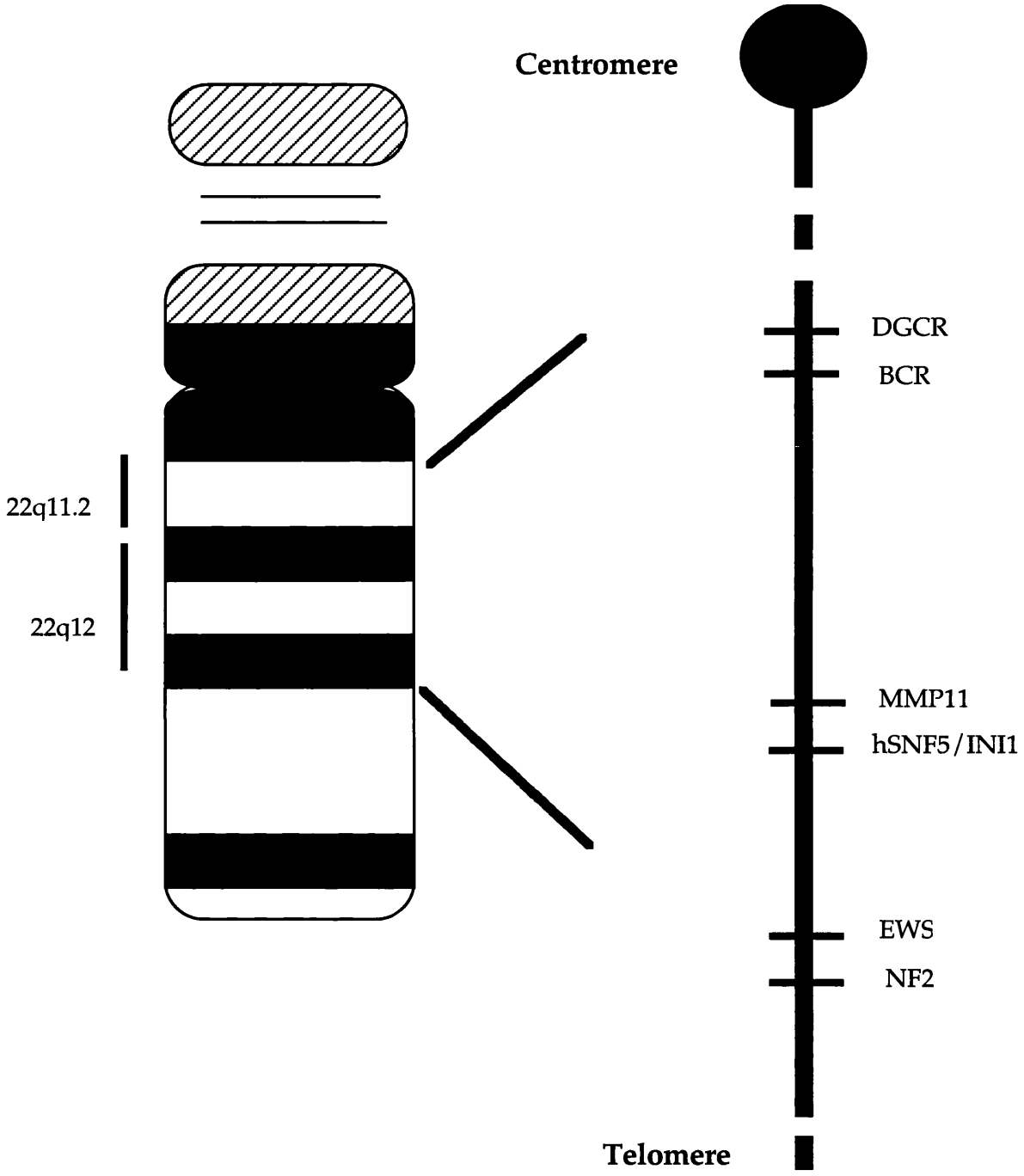


Figure 4.48: Schematic diagram of human chromosome 22
Chromosome bands 22q11 and 22q12 are shown with the location of candidate tumour suppressor genes (Rousseau-Merck et al, 2000).

Alterations of the sex chromosomes

CGH analyses identified one ependymoma (IN1932) with gain of chromosome X. Gains of X have been reported in cytogenetic analyses, as well as in CGH analyses of ependymoma (Bhattacharjee et al, 1997; Grill et al, 2002; Kucerova et al, 2000; Neumann et al, 1993; Sainati et al, 1996; Scheil et al, 2001; Weremowicz et al, 1992). Structural abnormalities of chromosome X include t(X;22)(p22.1;q11) and t(X;18)(p11.4;q11.2) (Neumann et al, 1993; Roberts et al, 2001).

CGH could not be used to identify alterations of the Y chromosome, and hence a Y chromosome specific PCR was used to determine the presence of Y-specific sequences in tumour samples. The Y-PCR identified three ependymoma (IN1638, IN1932 and IN1759 which appeared to have loss from the Y chromosome. Loss of Y has been reported previously in paediatric ependymoma (Mazewski et al, 1999; Reardon et al, 1999; Rogatto et al, 1993; Vagner-Capodano et al, 1992; Vagner-Capodano et al, 1999). Translocations involving the Y chromosome have also been reported in ependymoma (Mazewski et al, 1999).

Sex chromosome loss as a sole abnormality or accompanied by autosome loss has been reported in 46% of malignant gliomas (Hecht et al, 1995). It has been suggested that loss of the sex chromosomes is associated with increasing age, but there is no evidence as to why this occurs. Loss of the Y chromosome could occur as a result of Y chromosome rearrangements. Males with malignant glioma have been reported with an XX karyotype. This apparent sex reversal is a result of two events: Y loss and X isodisomy. It has been hypothesised that X loss in females could also be followed by X isodisomy. Tumourigenesis could relate to loss of the inactive X followed by duplication of the active X, resulting in X isodisomy for the active X chromosome (Hecht et al, 1995).

Genetic losses involving the sex chromosomes are among the most frequent events found in solid tumours. CGH analyses have shown loss of chromosome X in 25% of lung and ovarian tumours (El-Naggar et al, 1999; Mertens et al, 1997) and FISH analysis has revealed loss of Y in 36% of pancreatic endocrine tumours

(Missiaglia et al, 2002). A recent study has suggested that loss of sex chromosomes may be associated with tumour aggressiveness (Missiaglia et al, 2002). Of the tumours with loss of the sex chromosomes in this investigation, three of the patients had died as a result of disease (mean survival = 48 months) and the other patients is still alive 98 months post diagnosis.

A recent study has identified *MAGE-E1* (melanoma-associated antigen) at Xp11 (Kawano et al, 2001). There are a number of other *MAGE* genes located on the X chromosome, including *MAGE-A* (Xq28), *MAGE-B* (Xp21) and *MAGE-C* (Xq26-27). Some *MAGE* genes encode antigens that are localised on the cell surface of a number of human tumours and are recognised by autologous cytolytic T-lymphocytes (Traversari et al, 1992). *MAGE-E1* gene expression may be regulated by CpG island methylation in keeping with other members of the *MAGE* family. *MAGE-E1* has been found to be highly expressed in GBM relative to normal astrocytes and also to be predominately expressed in histologically undifferentiated rapidly growing tumours (Sasaki et al, 2001). Early data suggests that *MAGE-E1* has a function in cell cycle regulation.

Given the propensity for loss of the sex chromosomes during the ageing process, the significance of these gonosomal losses in paediatric tumours is worthy of further investigation.

Associations between copy number aberrations

From the CGH results it is obvious that certain chromosome abnormalities occurred in conjunction with one another. These associations between CNAs allowed the separation of the tumours into three distinct groups based on their aberrations. The first group of tumours had seventeen CNAs that occurred together and were statistically significant at the 95% confidence level. The alterations found predominantly in this group of tumours were loss of 1p seen in association with gain of 4q, 13, 5q, 6q, 2q, 9p, 8q and 18q, and loss of 22, 19, 16p, 12q, 17 and 20. Within this group of tumours there were also a number of pairs of alterations that occurred together (detailed in Table 4.3). The second group of

tumours shared ten CNAs that were statistically significant at the 95% confidence level, including gain of 1q, 2q, 7q, 8q, 9p, 13, 6q and 11p, and loss 12q and 16q. There were also a number of pairs of alterations that occurred together within this second group of tumours. The third group contained those tumours that had no detectable CNAs and those with sole abnormalities that meant they could not be assigned to one of the other two groups.

Loss of 1p and loss of 1q occurred almost independently of one another allowing the tumours to be separated into two groups based on their chromosome 1 status. Alterations of this chromosome appear to be an important factor in ependymoma development and further investigation of the gene(s) involved is required. Previous CGH analyses have reported CNAs that occur together or independently of one another. In all instances this has been pairs of abnormalities and no previous associations have been made between multiple CNAs. In one study, loss of 6q was seen to occur independently of monosomy 22 in a series of twenty-three primary paediatric ependymoma (Reardon et al, 1999). In the present study, loss of 6q only occurred in two cases, once as a sole abnormality and once with monosomy 22 and two other regions of loss. In a second study of twenty-four primary ependymoma (14 paediatric), loss of 6q and loss of 9 (either p or q arm) were seen to be mutually exclusive (Hirose et al, 2001). This was also true in the present study but could not be said to be a significant finding due to the low number of tumours with loss of 6q in the study.

The fact that several CNAs occur together suggests a pathway (or pathways) which require the increased expression of some genes and the deletion of other genes in ependymoma development. The CGH data presented here suggests the possible involvement of at least three different pathways that lead to the formation of ependymoma. There were a number of CNAs that were common to both of the groups of tumours with abnormalities, including gain 2q, 6q, 8q, 9p and 13. This may be indicative of genes common to both pathways but altered at different frequencies between the two groups. The regions of alteration identified by this study contain genes that are involved in a number of regulatory pathways including, control of the cell cycle, the MAPK signalling pathway, the p53 pathway, PDGF pathway, pRB pathway and the PTEN pathway. Further

investigation is required to determine if disruption of these pathways actually occurs in ependymoma.

Potential prognostic markers

Prognostic markers in ependymoma have not yet been established. A number of clinicopathological features have been proposed as prognostic indicators but evidence remains controversial. One aim of this study was to determine whether any chromosome abnormalities could be used as prognostic markers or could be associated with clinicopathological features such as tumour grade or recurrence, in order to predict the outcome of children with ependymoma.

Extent of resection is considered to be the most important prognostic factor in ependymoma patients. Gross total resection has been shown to increase PFS compared to sub-total excision in a number of studies (Fouladi et al, 1998; Healey et al, 1991; Mohri et al, 2000; Nitiss and Beck, 1996; Robertson et al, 1998; Sutton et al, 1990-1991). In the present study, there was no difference in survival between those patients who had undergone total excision or those who had partial excision (see Figure 4.49).

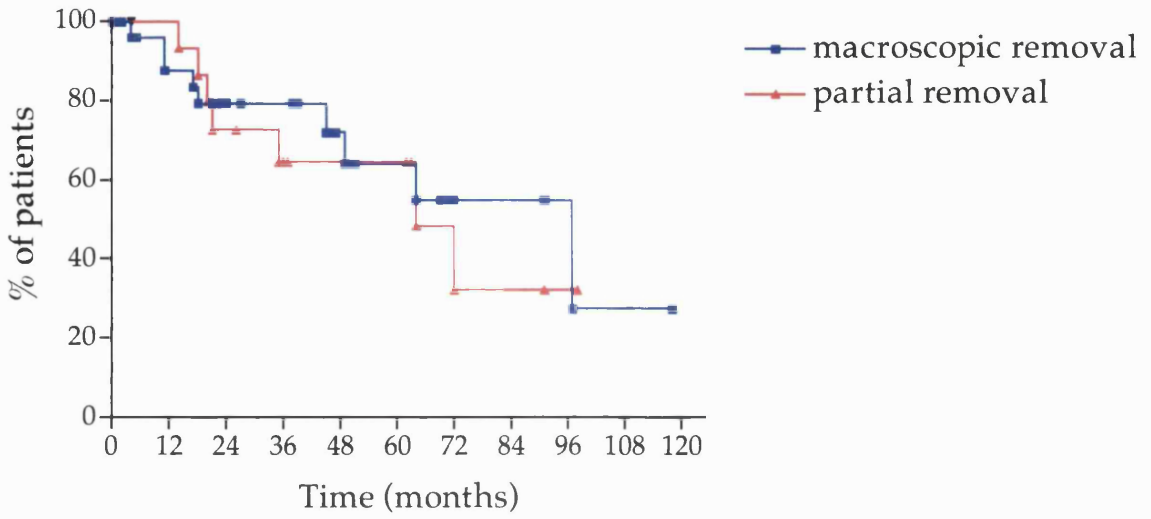


Figure 4.49: The effect of surgical resection on survival in paediatric ependymoma patients. Survival is measured in months from the date of diagnosis. Log-rank test, $p = 0.695$.

The use of radiotherapy in the treatment of ependymoma has also been seen to improved survival (Dohrmann et al, 1976; Goldwein et al, 1990; Lyons and Kelly, 1991; Pierre-Khan et al, 1983; Salazar, 1983; Salazar et al, 1983; Wallner et al, 1988). The use of whole brain irradiation results in prolonged survival compared to partial brain irradiation (Salazar et al, 1983). In the present study, treatment regimen had an impact on patient survival. The patients in the study could be separated into four treatment groups; those who had undergone surgery alone, those who had surgery followed by radiotherapy, those who had surgery followed by chemotherapy and those who had surgery followed by radiotherapy and chemotherapy. Survival was better for those patients who had received post-operative therapy compared to those who had just undergone surgery. The best survival was seen in the group of patients who received post-operative radiotherapy (90% at 5 years), followed by those who had received post-operative chemotherapy (62% at 5 years). Survival in those patients who had received post-operative chemotherapy and radiotherapy had a 5-year survival of 60%. However, while survival remained stable for the first two groups of patients, by 8 years post diagnosis all patients who had received both chemotherapy and radiotherapy had died. Patients who had received no post-

operative therapy had a very poor survival (0% at 2 years). This data suggests that surgery alone is not the best treatment option for patients with intracranial ependymoma and that it should be used in conjunction with either radiotherapy or chemotherapy to improve survival.

Tumour location has also been reported as an important prognostic marker. Survival is often better for patients with infratentorial tumours compared to those with supratentorial tumours (Lyons and Kelly, 1991). In the present study, there was no difference in survival between those patients with supratentorial tumour and those with infratentorial tumour (see Figure 4.50). This is a finding that has been reported by other groups (Salazar et al, 1983).

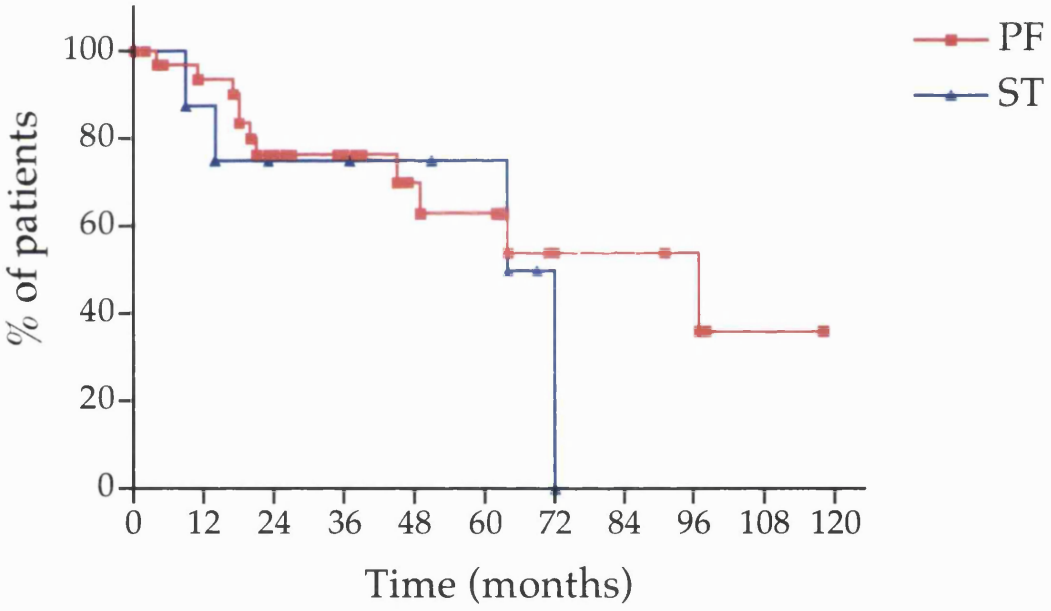


Figure 4.50: Effect of tumour location on survival in paediatric ependymoma patients. Survival is measured in months from the date of diagnosis. Log-rank test, $p = 0.5397$. PF = posterior fossa, ST = supratentorial.

The influence of age on outcome in patients with ependymoma is controversial. Some studies have reported no difference in outcome between young children and older children (McLaughlin et al, 1998; Paulino and Wen, 2000; Robertson et al, 1998; Salazar et al, 1983). In contrast, age has been shown to be a significant

prognostic factor in other studies, with older children having better survival than younger children (Chiu et al, 1992; Goldwein et al, 1990; Nazar et al, 1990; Paulino et al, 2002; Pierre-Khan et al, 1983). There was no significant difference in survival between the older and younger patients in the present study.

There is also some controversy regarding the prognostic significance of histological grade in ependymoma. This is due in part to the disagreement between pathologists as to how ependymoma should be graded (Chiu et al, 1992; Goldwein et al, 1990; Healey et al, 1991). There are occasions where assigning a grade to a tumour is difficult because of heterogeneous histological patterns within the tumour (Chiu et al, 1992). Several studies have shown survival differences between patients with ependymoma and anaplastic ependymoma (Kovalic et al, 1993; Liu et al, 1976; McLaughlin et al, 1998; Nazar et al, 1990; Salazar et al, 1983; Schiffer and Giordana, 1998). The present study could find no difference in survival between patients with benign and those with anaplastic ependymoma. This finding has also been reported by other groups (Chiu et al, 1992; Goldwein et al, 1990; Healey et al, 1991; Sutton et al, 1990-1991). In the present study, the lack of a difference in survival between the two histological variants of ependymoma may be due to the small number of anaplastic tumours compared to the benign tumours.

The reports of chromosome abnormalities as prognostic markers in ependymoma are limited. Gain of 1q has been found to be associated with a number of clinicopathological features including patient survival, tumour histology and tumour recurrence (Carter et al, 2002; Dyer et al, 2002; Sainati et al, 1996). Despite gain of 1q being a frequent finding in the ependymoma in the present study, it was not associated with any clinicopathological feature.

Only one CNA could be associated with patient survival in the present study and that was monosomy 19. Monosomy 19 gave a survival advantage to the patients with this aberration (90% at 5 years) compared to those with no alteration of chromosome 19 (50% at 5 years). No other CNA could be associated with any of the clinicopathological criteria examined (patient age or sex, tumour histology or

location). Loss of 19q in conjunction with loss of 1p has seen to be associated with longer PFS in adults with oligodendroglioma (Cairncross et al, 1998; Hoang-Xuan et al, 2001). Virtually all oligodendroglioma with loss of 1p also have loss of 19q, a finding that suggests a synergistic effect of both alterations that confers a selective growth advantage (Kraus et al, 1995; Reifenberger et al, 1994b). However, in the present study patients with 1p/19q had no survival advantage over those patients who did not have this alteration (see Figure 4.51). The presence of 19q is also associated with an increased chance of chemotherapeutic response in oligodendroglioma (Cairncross et al, 1998). However, in neuroblastoma loss of 19q is associated with local treatment failure and poor prognosis (Mora et al, 2001).

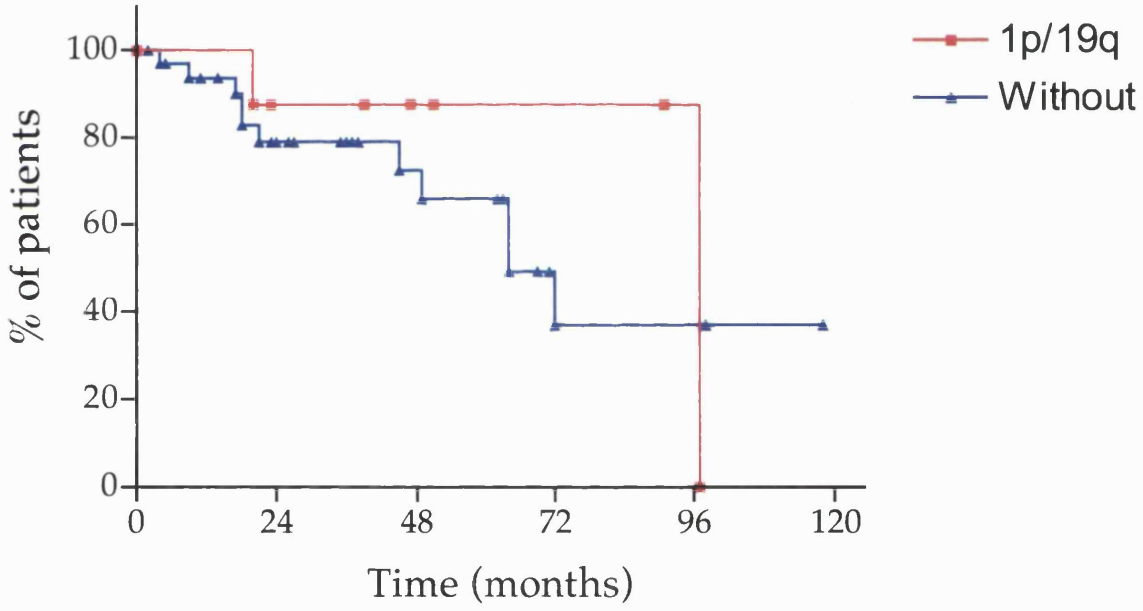


Figure 4.51: Influence of 1p/19q loss on survival in ependymoma patients. There is no difference in survival (Log-rank test, $p = 0.4503$). Survival is measured in months from the date of diagnosis.

Tumour recurrence

The most common site of recurrence in ependymoma is at the primary tumour site (Salazar, 1983). Local recurrence is the most significant cause for progression of neurological deficit in ependymoma patients (Cobbs et al, 1996). In light of the

importance of tumour recurrence in ependymoma, the association between CNAs and tumour recurrence was examined in two different ways in the present study. In the first instance, the CNAs present in primary ependymoma were compared to those in the recurrent tumours. There was a significant association between the presence of gain of 7q and loss of 12q and recurrent tumours. Loss of 6q and 8p were also seen at higher frequencies in the recurrent tumours compared to the primary tumours, though this association was not statistically significant. High copy number amplification was also associated with recurrent tumours. This data suggests that different CNAs are present in primary tumours compared to recurrent tumours. There is further evidence for this when primary and recurrent tumours from the same patient are examined. In the literature there are cytogenetic data available for fifteen primary and recurrent pairs of ependymoma (Dyer et al, 2002; Scheil et al, 2001; Vagner-Capodano et al, 1992; Vagner-Capodano et al, 1999). Only one of these pairs had no abnormalities in the primary or the recurrent tumour (Dyer et al, 2002). In two cases, both the primary and recurrent tumours had the same abnormality, both involving structural abnormalities of chromosome 22 (Vagner-Capodano et al, 1999). In the remaining twelve cases, the primary and recurrent tumours showed different abnormalities. In most cases there was an acquisition of abnormalities with tumour recurrence. In two cases the primary tumour had more abnormalities than the recurrent tumour (Dyer et al, 2002). In two cases the recurrent tumour had gained a region of high copy number amplification that was not present in the primary tumour (Dyer et al, 2002; Scheil et al, 2001). In the present study, there were six pairs of primary and recurrent ependymoma and two recurrent samples from the same patient where no primary sample was available for analysis. Three of the paired samples had no CNAs in either the primary or recurrent sample. In one pair, the primary sample had three CNAs and the recurrent sample had no aberrations. In the remaining instances, the recurrent sample had gained aberrations that were not present in the primary tumour. In the pair of tumours that were both recurrences from the same patient, there was acquisition of regions of high copy number amplification on 8q and 9p in the second recurrence that had previously been low level gains in the first recurrence. There are several reasons why tumour cells acquire alterations as

they progress or recur. One explanation is the growth of a different clone of cells to the original tumour. Tumour cells on the rim of the tumour edge may remain after incomplete tumour resection. These cells will continue to divide and produce a new tumour that may have slightly different alterations to those cells that were present in the main bulk of the tumour. Some tumour cells may have alterations that confer a survival advantage. For example, if a cell is able to express genes that allow it to expel cytotoxic drugs it will have a growth advantage over those cells that cannot expel cytotoxic agents and will therefore be killed by expose to such agents. Treatment with chemotherapy and radiotherapy can cause damage to cells but tumour cells are able to repair sublethal or potentially lethal DNA damage and can re-populate in between treatment doses (Cavenee et al, 2000). Cell death following radiation-induced DNA damage occurs via apoptosis, usually mediated by TP53. Cells with mutated TP53 can avoid apoptosis and can continue to grow. It is also possible that treatment with radiation may actually promote the acquisition of new aberrations (Dirks et al, 1994).

In the second analysis, the tumours in the study were separated into primary tumours with no reported clinical recurrence and those primary tumours that had recurred. The CNAs were compared in the primary tumours only. There was a significant difference in overall survival between these two groups of tumours, with those patients whose tumour recurred having a worse outcome than those who had shown no recurrence. There was one aberration that occurred more frequently in the tumours that showed recurrence than in the tumours with no recurrence, and this was loss of 16q. It is possible that there are genes on chromosome 16q which, when altered in ependymoma, allow a growth advantage to the tumour cells with this abnormality. Any residual tumour remaining after resection would be able to continue dividing, resulting in recurrence of the tumour. There have been no reports in the literature of a single chromosome abnormality being used as a marker for tumour recurrence and further analysis is required in order to determine whether the presence of loss of 16q really can be used to predict the recurrence of ependymoma in children.

MDR1 expression in ependymoma

Although, there is some controversy in the literature regarding the value of current prognostic markers in ependymoma, most series agree that a worse prognosis is seen in children less than 3 years of age. Even though gross total resection may prolong survival, surgery alone is rarely curative. Ependymoma are sensitive to treatment with radiotherapy but as most of these tumours occur in children oncologists prefer not to use radiotherapy in order to avoid treatment-related neurotoxic effects. The increased use of chemotherapy to treat ependymoma has lead to the identification of multidrug resistance in these tumours. The ineffectiveness of chemotherapy as treatment for brain tumours may be due, in part, to the low efficiency of the penetration of chemotherapeutic agents through the BBB. The presence of P-GP in the capillaries of normal brain and in some tumour capillaries is consistent with a role for P-GP in restricting drug entry into normal brain and some areas of brain tumours. Several studies have identified expression of P-GP in human brain tumours (Becker et al, 1991; Henson et al, 1992; Nabors et al, 1991; Toth et al, 1996). As many as 60% of astrocytomas have been shown to have P-GP in tumour blood vessels, although expression does not appear to be related to grade (von Bossanyi et al, 1997).

The activation of *MDR1* as a mechanism for resistance may only be relevant in cells with low or undetectable levels of *MDR1* (Knutsen, 1998). It has been suggested that random chromosomal rearrangements involving 7q may be responsible for the overexpression of *MDR1* when cells with low levels of *MDR1* are exposed to drugs. Figure 4.52 shows a schematic diagram of the known or putative sites of rearrangement that might lead to drug resistance. The study by Knutsen indicated that the gene juxtaposed to MDR1 that controls its expression after gene rearrangement has occurred, is chosen randomly. The only requirement is that the partner gene is constitutively active.

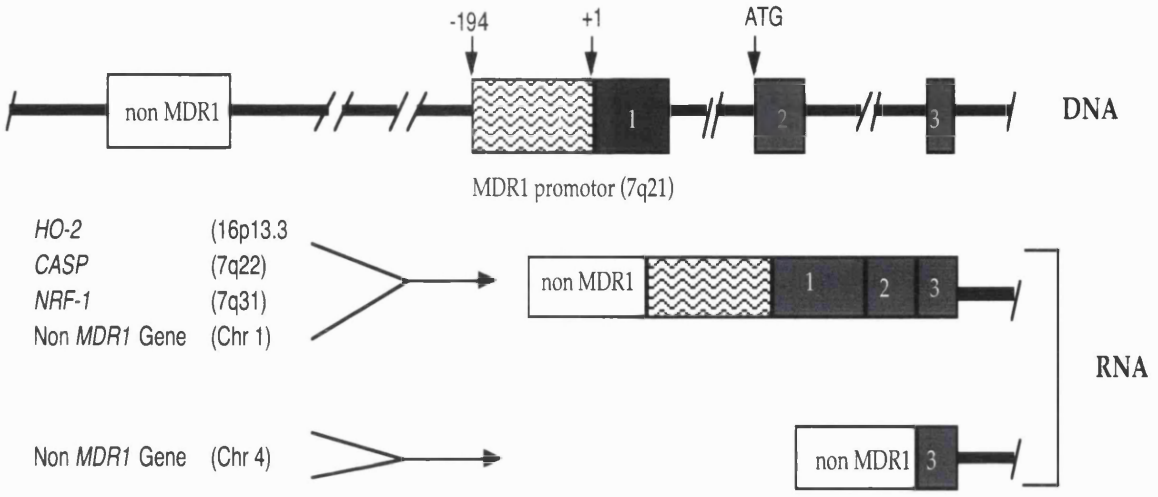


Figure 4.52: Summary of molecular data obtained from cell lines (drug resistant adenocarcinoma) and patient samples (ALL) that shows known or putative sites of MDR1 gene rearrangements (Knutsen et al, 1998).

In vitro studies of human tumour cell lines have shown that high levels of P-GP expression alone can lead to the development of MDR. Tumours arising in organs that normally express P-GP will overexpress P-GP, will be generally resistant to treatment with chemotherapy and may have even higher levels of P-GP after treatment (Becker et al, 1991; Weinstein et al, 1990). Studies of expression of P-GP in normal adult brain have shown only the capillary endothelium expresses P-GP. Neurons and glia do not express P-GP (Cordon-Cardo et al, 1989; Thiebaut et al, 1989).

The first immunohistochemical evidence of P-GP expression in ependymoma was reported in a study by Geddes et al. (1994). Expression was observed in both adult and paediatric ependymoma from all sites. This study included 10 paediatric ependymoma, of which 60% showed positive immunostaining with the antibodies JSB-1 and C219. JSB1 recognises the extracellular epitope of P-GP and C219 recognises a conserved cytoplasmic domain in the COOH- terminal region of P-GP. Tumour blood vessel staining using MRK16, which binds to the extracellular epitope of P-GP, was found to be positive in 8/8 cases of paediatric ependymoma in a study by Billson et al. (1994). This study also demonstrated an increase in the proportion of vessels expressing P-GP following treatment with

chemotherapy, but not radiotherapy. Chou et al. (1996) showed 83% of paediatric ependymoma had positive immunostaining for P-GP. The resistance in these tumours was shown to be intrinsic as most of the tumours were studied before treatment with chemotherapy. Progression free survival has been shown to be greatest in patients with benign ependymoma, whereas a poor outcome is associated with anaplastic tumours that had no P-GP immunoreactivity (Korshunov et al, 1999). The fact that normal ependymal cells do not express P-GP suggests that ependymoma fall into a category of tumours derived from tissues that do not normally express P-GP but in which P-GP expression is accompanied by cellular transformation. This raises the possibility of simultaneous activation of an oncogene and *MDR1* expression that results in progression of the tumour.

Antibodies that have been used to study P-GP expression have been seen to show cross reactivity with other proteins such as mitochondrial pyruvate carboxylase (JSB1) and muscle protein (C219) (Kirches et al, 1997). Reliable detection of *MDR1* gene expression is also complicated by the existence of an homologous gene, known as MDR2, whose gene products cross react with some *MDR1*- reactive antibodies and nucleic acid probes (Noonan et al, 1990). Therefore many people have made the decision to check for the presence of specific gene transcripts in tumour samples using RT-PCR. This technique allows even low levels of mRNA, undetectable by conventional assays, to be detected. It is 1,000 to 10,000 times more sensitive than RNA blot techniques (O'Driscoll et al, 1993). By careful choice of sequence specific primers, it is possible to amplify selectively only the *MDR1* gene and not its *MDR2* homologue. Expression of *MDR1* alone is sufficient to confer the multidrug resistance phenotype to drug sensitive cells. Even very low levels of *MDR1* expression can result in a severalfold increase in the level of drug resistance, which may be clinically significant.

This study used RT-PCR to show that 65% of ependymoma had detectable levels of MDR1 mRNA. This level of expression was slightly lower than that previously reported in paediatric ependymoma, though this may be due to the small

number of tumours analysed in the earlier investigation. (Chou et al, 1996) Expression was seen in both benign and anaplastic tumours and there was no association between expression and survival as observed by Korshunov et al. (1999). *In vitro* chemosensitivity data was available for four of the samples in this study. These tumours all showed resistance to cisplatin and vincristine. Surprisingly, only two of these tumours had detectable levels of MDR1 mRNA. As only three tumours in the study showed gain of 7q using CGH and low level expression of MDR1, this would suggest that a mechanism other than amplification of *MDR1* maybe responsible for the MDR phenotype in some ependymoma.

Increased expression of MDR1 is only one mechanism of drug resistance in tumours. Other mechanisms of resistance include low efficiency of drug penetration through the BBB, reduction of intracellular accumulation of drugs, reduction in drug-activated enzymes and the increase in detoxification enzymes and proteins that may be responsible for the inactivation of chemotherapeutic agents. There are a number of chemoresistance related proteins (ChRPs) that may have a role in chemoresistance in brain tumours in general. A recent study showed a significant preponderance of expression of these ChRPs in low grade ependymoma, with PFS time being shorter for the tumours found to be immunonegative (Korshunov et al, 1999).

The *MRP* gene was isolated by Cole et al. in 1992 from a doxorubicin-resistant lung cancer cell line. The *MRP* gene encodes a 190 kDa membrane bound glycoprotein with minor sequence homology to P-GP. Their work demonstrated that non- P-GP mediated MDR may be conferred by MRP. *MRP* is a member of the ATP binding cassette (ABC) superfamily and is thought to act as a transporter protein. MRP expression has been observed in 70% of untreated glioma. P-GP expression was only observed in 17% of the same samples, suggesting intrinsic drug resistance in gliomas may be mediated primarily through *MRP* rather than P-GP (Abe et al, 1998; Mohri et al, 2000). MRP expression was also seen to increase in samples after chemotherapy, indicating that acquired drug resistance may also be associated with *MRP*. MRP localisation

exists primarily in the endoplasmic reticulum of resistant cells, suggesting a function of MRP in the distribution of a drug into specific organelles (Mohri et al, 2000). MRP mRNA and protein are undetectable in normal brain. Despite MRP mRNA being expressed in most glioma cell lines tested, the expression levels did not correlate with resistance to any one particular drug. MRP expression was undetectable in surgical specimens of glioma suggesting that amplification-induced activation of *MRP* might occur during the establishment of a cell line, or there may be an alteration in promotor activity of the gene. The fact that *MRP* is not expressed in capillary endothelial cells of the brain suggests it might not play a role in the construction of the BBB (Nagane et al, 1999). MRP mRNA has been detected in some childhood solid tumours, including neuroblastoma and nephroblastoma (Oda et al, 1997).

Repair of cytotoxic damage by O^6- methylguanine-DNA methyltransferase (*MGMT*) is an important factor in resistance to chloroethylnitrosoureas (Esteller et al, 2000). *MGMT* inhibits the killing of tumour cells by alkylating agents that would usually bind to the DNA causing cell death. The *MGMT* gene is located on chromosome 10q26. A possible relationship between *p53* and *MGMT* has been suggested. In a subset of cancer cells the *MGMT* gene has been silenced, maybe due to hypermethylation of the 5′ flanking region of the promotor or cytoplasmic sequestration of the enhancer-binding protein. Glioma cell lines that are deficient in *MGMT* show an increased sensitivity to O^6 alkylation by nitrosureas (Rolhion et al, 1999). Methylation of the promotor has been shown to be associated with sensitivity to carmustine and an increase in survival in 40% of gliomas (Esteller et al, 2000). Wild type p53 acts as an inhibitor of *MGMT* gene expression. *MGMT* gene expression has been seen to be lower in *p53* altered GBM, suggesting a low level of *MGMT* expression might promote *p53* alterations via a gene mutation (Rolhion et al, 1999). Evidence suggests that *MGMT* plays a central role in the resistance of glioma cells to ACNU (Nagane et al, 1999). MGMT mRNA has been detected in all grades of astrocytoma and in anaplastic ependymoma including some paediatric tumours. (Tanaka et al, 2000). In paediatric brain tumours activity of MGMT is greater in high-grade tumours than low grade. MGMT expression has also been seen to be age dependent, with expression being greater

in infants than adolescents. Elevated expression of *MGMT* may have clinical implications for the treatment of paediatric brain tumours. Neoplasms with high MGMT expression may show enhanced resistance to alkylating agent based chemotherapy (Bobola et al, 2001).

Another mechanism of chemoresistance is intracellular drug inactivation or transformation as a result of increased concentrations or activity of detoxifying enzymes. Glutathione S-transferases (GST) are a major class of drug-detoxifying enzymes, that catalyse the conjugation of glutathione with a great number of compounds with an electrophilic centre, including chemotherapeutic agents (von Bossanyi et al, 1997). The placental form, GSTττ, is the predominant form in normal brain. Its level of expression has been seen to be related to grade of malignancy. Due to their detoxifying properties, GSTs are thought to play an important role in chemoresistance. A recent study showed that there was no evidence for GST- mediated protection from chemotherapeutic agents or an increase in expression caused by exposure to drug, as seen with other MDR related genes (Winter et al, 2000). Expression of GSTττ mRNA has been reported in a range of brain tumours including paediatric anaplastic ependymoma, medulloblastoma and all grades of astrocytoma (Tanaka et al, 2000). GSTττ expression is extremely high in brain tumours and glioma cell lines compared to that in normal brain (Nagane et al, 1999). Western blot analysis of GSTττ in paediatric glioma and other brain tumours revealed an increase in expression with progression of malignancy. Endothelial cells of tumour blood vessels were found to be immunoreactive for GSTττ and to a lesser extent GSTα and GST-mu. Drug resistance may result not only from the expression of GSTs in neoplastic cells but also from the protein function in the endothelial cells of the tumour blood vessels that form a blood-tumour barrier (von Bossanyi et al, 1997).

Metallothioneins (MTs) are metal binding proteins that are overexpressed in a range of drug resistant human neoplasms. MT is a low molecular weight protein that can chelate seven bivalent heavy metal ions through mercaptide bonds (Korshunov et al, 1999). The MTI and MTII isoforms are present in the cytoplasm

and nuclei of many normal cell types, including nervous tissue. The MTIII isoform is expressed in neurons and is absent from glial components. MT expression correlates with a poor outcome. MT immunoreactivity has been observed in a range of human brain tumours, including childhood neoplasms such as pilocytic astrocytoma, GBM, anaplastic ependymoma, ganglioglioma and craniopharyngioma (Maier et al, 1997). The gene location for the negative regulation of MT transcription is as yet unknown, but maybe related to the long arm of chromosome 16, possibly at q13, where the MT locus can be found (Maier et al, 1997). In breast cancer chromosome 16q has been shown to be a frequent site of LOH as determined by microsatellite instability studies. It is possible that the high association of malignancy and MT overexpression in breast tumours is sue to the loss of the negative regulator mechanism for MT expression (Maier et al, 1997; Stratton et al, 1995) The expression level of MTIIA is associated with the extent of CDDP resistance in resected glioma tissue – tumours (medulloblastoma and ependymoma) having a high MTIIA expression level showed recurrence following CDDP-based chemotherapy (Nagane et al, 1999).

DNA topoisomerase IIα (Topo IIα) is a nuclear enzyme essential for many aspects of DNA functioning and is highly associated with tumour cell proliferation. It has also been implicated in the drug resistance of tumour cells. There is some evidence that Topo IIα determines the response of some tumours to a number of anticancer agents, including etoposide, teniposide, doxorubicin, mitoxantrone and amsacrine. Alteration of Topo IIα reduced expression/activity) has been associated with a form of drug resistance known as atypical MDR. The increased expression of Topo IIα in children <3 years with optic pathway gliomas suggests that Topo IIα interfering drugs may be of benefit in the treatment of these tumours (Bredel et al, 2002).

It has been suggested that acquired resistance and cross resistance to cisplatin is associated with substantial genomic instability (Wasenius et al, 1997). In this

study 12/22 ependymoma showed varying degrees of instability by CGH analysis. The drug sensitivity of these tumours has not yet been established.

CHAPTER 5
GENETIC ANALYSIS OF ASTROCYTOMA

Sample details

CGH analysis was performed on sixty-four tumours from sixty-three patients. The age at diagnosis ranged from nine months to thirty-three years (mean = 9.37 years), with a male: female ratio of 32:31. Eight samples came from young adults aged 21-33 years. These samples were included in the analysis to investigate the different alterations seen in children and young adults, and also to determine if it was possible to determine at what age the alterations that are commonly associated with adult astrocytoma could be detected. Twenty-nine samples were pilocytic astrocytoma, twenty were diffuse astrocytoma, five were anaplastic astrocytoma and the remaining ten samples were GBM. In thirty three cases analysis was performed using DNA extracted from biopsy material whilst in thirty one cases analysis was carried out using DNA extracted from short term cell cultures (passage 3-14). In eight cases tumour DNA was labelled using the DOP-PCR method, whilst the majority of the tumours were labelled using nick translation.

Summary of copy number aberrations observed

Table 5.1 and Figure 5.1 give a summary of sample details and copy number aberrations. Regions of genomic imbalance were detected in thirteen of sixty-four tumours (20%). Gains were seen more frequently than losses. Multiple copy number changes, ranging from 1 to 17 per tumour (mean 5.15), were observed. Copy number aberrations were seen on all chromosomes with the exception of chromosomes 11 and 21. A number of common areas of genomic imbalance were seen in the astrocytomas as a whole group. The most common changes were gain of 4q and 7, each seen in five cases (8%), gain 6q in four cases (6.25%) and loss 16p in three cases (4.7%). High copy number amplifications were observed at 1p32-34 (IN699), 1q21 (IN699), 2q24-32 (IN2240), 7p11.1-14 (IN3032), 7q22-31 (IN2017), 7q31-33 (IN2240), 9q32-34 (IN2240), 12p11.2 (IN3032), 13q21-31 (IN1524), 17p (IN699), 19q (IN699) and 20 (IN699).

IN	AGE	SEX	GRADE	P/R	LOCATION	SURVIVAL	SOURCE	LABELLING	AMPLICONS	GAINS	LOSSES
17/81	14	F	PA	P	supratentorial	unknown	CC	NT			
168	15	M	PA	P	unknown	unknown	CC	NT			
324	15	M	PA	P	supratentorial	190 (D)	CC	NT		7q11.2-pter	
1133	5.5	M	PA	P	infratentorial	1 (A)	CC	NT			
1533	1.75	F	PA	P	infratentorial	143 (A)	CC	NT			
1950	4	M	PA	P	infratentorial	26 (A)	FF	NT			
1953	2.5	F	PA	R	supratentorial	159 (A)	FF	NT			
2017	7	M	PA	P	infratentorial	24 (A)	FF	NT	7q22-31	7q	
2110	2.5	M	PA	P	infratentorial	124 (A)	FF	NT			
2356	4	M	PA	P	infratentorial	106 (A)	FF	DOP-PCR			
2368	8	F	PA	P	infratentorial	87 (A)	FF	NT			
2466	3.5	F	PA	P	infratentorial	103 (A)	FF	NT			
2576	5	M	PA	P	infratentorial	68 (A)	CC	NT			
2596	4.5	F	GCA	P	infratentorial	45 (A)	FF	NT			
2775	9.5	F	PA	P	infratentorial	75 (A)	FF	NT			
2788	9	M	PA	P	infratentorial	39 (A)	FF	NT		4q11-26	
2797	6.5	M	PA	P	supratentorial	59 (A)	FF	DOP-PCR			
2825	2.75	F	PA	P	infratentorial	67 (A)	FF	NT			
2826	7	M	PA	P	infratentorial	54 (A)	FF	DOP-PCR			
2893	11.6	M	PA	P	infratentorial	47 (A)	FF	NT			
2921	3	M	PA	P	infratentorial	2 (A)	FF	NT			
2940	3	M	PA	P	infratentorial	39 (A)	FF	NT			
2946	9	F	PA	P	infratentorial	28 (A)	FF	NT			
2969	2.5	F	PA	P	infratentorial	34 (A)	FF	NT			
2977	5	F	PA	P	infratentorial	1 (A)	CC	NT			
3002	6	F	PA	P	infratentorial	13 (A)	FF	NT			
3017	8.25	F	PA	P	infratentorial	25 (A)	CC	DOP-PCR			
3066	0.8	M	PA	P	infratentorial	12 (A)	CC	NT			
3085	9	F	PA	P	infratentorial	8 (A)	CC	NT			
18	33	M	DA	P	unknown	12 (D)	CC	NT			
276	4.5	F	DA	P	thalamic	120 (A)	CC	NT		1p 19p 20q	Xq
380	1.8	F	DA	P	infrantentorial	92 (D)	CC	NT			10q 16p 19 Xp
699	15	M	DA	P	supratentorial	unknown	CC	NT	1p32-34 1q21 9q32-34 17p 19q 20	1p 2p 7 8 9q 12q	4 9p 13 15 18
1145	11	M	DA	P	supratentorial	60 (A)	FF	NT			
1382	10	F	DA	P	infrantentorial	unknown	CC	NT			
1520	7	M	DA	P	supratentorial	110 (A)	FF	NT			
1524	13	M	DA	P	infrantentorial	13 (A)	FF	NT	13q21-31	2q 4q 5q 6q 9p 13q	1p 16p 19 22q
1805	3	F	DA	P	thalamic	2 (A)	CC	NT			
1859	3	F	DA	P	brainstem	139 (A)	FF	NT			
1930	13	F	DA	P	supratentorial	60 (A)	FF	NT			
2003	9	F	DA	P	supratentorial	117 (A)	CC	NT			
2102	8	M	DA	P	brainstem	67 (A)	CC	NT			
2122	10	F	DA	P	optic chiasm	78 (A)	CC	NT			
2355	19	M	DA	P	unknown	unknown	CC	NT			
2587	13	F	PXA	P	supratentorial	44 (A)	FF	NT			6q 9p
2591	12	F	DA	R	supratentorial	117 (A)	CC	NT			
2698	9.33	M	DA	P	brainstem	80 (A)	FF	NT			
2988	26	F	DA	P	supratentorial	14 (A)	FF	NT			
3032	9.5	F	DA	P	supratentorial	12 (D)	FF	NT	7p11.1-14 12p11.2	2q 5p 7q 12p	14q 17
1651	2.5	M	AA	P	infrantentorial	unknown	CC	NT			
2538	21	M	AA	P	supratentorial	3 (A)	CC	NT			20q
2563	24	F	AA	P	unknown	unknown	CC	DOP-PCR			
2758	21	F	AA	P	unknown	unknown	FF	DOP-PCR			
2774	30	M	AA	P	supratentorial	unknown	FF	DOP-PCR		4q 6q 13q	
146/81	27	M	GBM	P	unknown	100 (D)	FF	NT			
178	13	M	GBM	P	unknown	3 (D)	CC	NT			
1180	8	M	GBM	P	right parieteal occipital	4 (D)	CC	NT			
1262	14	M	GBM	P	supratentorial	12 (A)	CC	NT			
1419	8.5	F	GBM	P	supratentorial	12 (D)	CC	NT			
1495	6	F	GBM	R	supratentorial	80 (A)	CC	NT			
1566	12	F	GBM	P	infratentorial	7 (A)	CC	DOP-PCR		4q 6q	1p
1786	27	M	GBM	P	left temporal lobe	120 (A)	CC	NT			
2240	14	F	GBM	P	unknown	4 (D)	FF	NT	2q24-32 7q31-33	1 2 3q 4q 5q 6q 7q 12q 18q X	16
3046	15.9	M	GBM	P	left frontal	14 (D)	CC	NT			
P/491	4	F	GBM	P	supratentorial		PEM	NT		5q	7q 16p
P/584	11.7	F	GBM	P	infratentorial	17 (A)	PEM	NT		1q 2q 4q	7q 16p 17
P/2043	5	F	GBM	P	supratentorial	112 (A)	PEM	NT		4q 5q 20p	10q 12q 16 17p 19p
2087	7	M	GBM	P	infratentorial	(D)	CC	NT		2q 4q 5q 6q 8q 11q 12q 13q	1p 19 22
2675	15	M	GBM	P	infratentorial	16 (D)	FF	NT		2q 4q 5q 6q 11q 12q 13q 18q	16p 17p 20 22
2726	8	F	GBM	P	infratentorial	9 (D)	CC	NT			
2809	11	F	GBM	P	infratentorial	3 (D)	FF	NT	2q22 7q22-ter 8q13-23 12q13-ter	1p 2 7 8q 20	1q 3p 8p 9q 12q 15 17 19 22
2846	10	F	GBM	P	infratentorial	7 (D)	FF	NT	8q21-22 12q15-21	1q 2p 8q 9p 12p 12q 13q	
2914	6	M	GBM	P	supratentorial	66 (A)	CC	NT			

Table 5.1: Summary of Copy number aberrations in astrocytoma samples

Key to table Age in years

M = male; F = female

PA = pilocytic astrocytoma, DA = diffuse astrocytoma, AA = anaplastic astrocytoma, GBM = glioblastoma multiforme, GCA = giant cell astrocytoma,

PXA = pleomorphic xanthoastrocytoma

P = primary sample; R = recurrent sample

Survival in months from date of initial diagnosis

A = alive; D = deceased

FF = fresh frozen material, CC = short term cell culture; NT = nick translation, DOP-PCR = degenerate oligonucleotide primed PCR

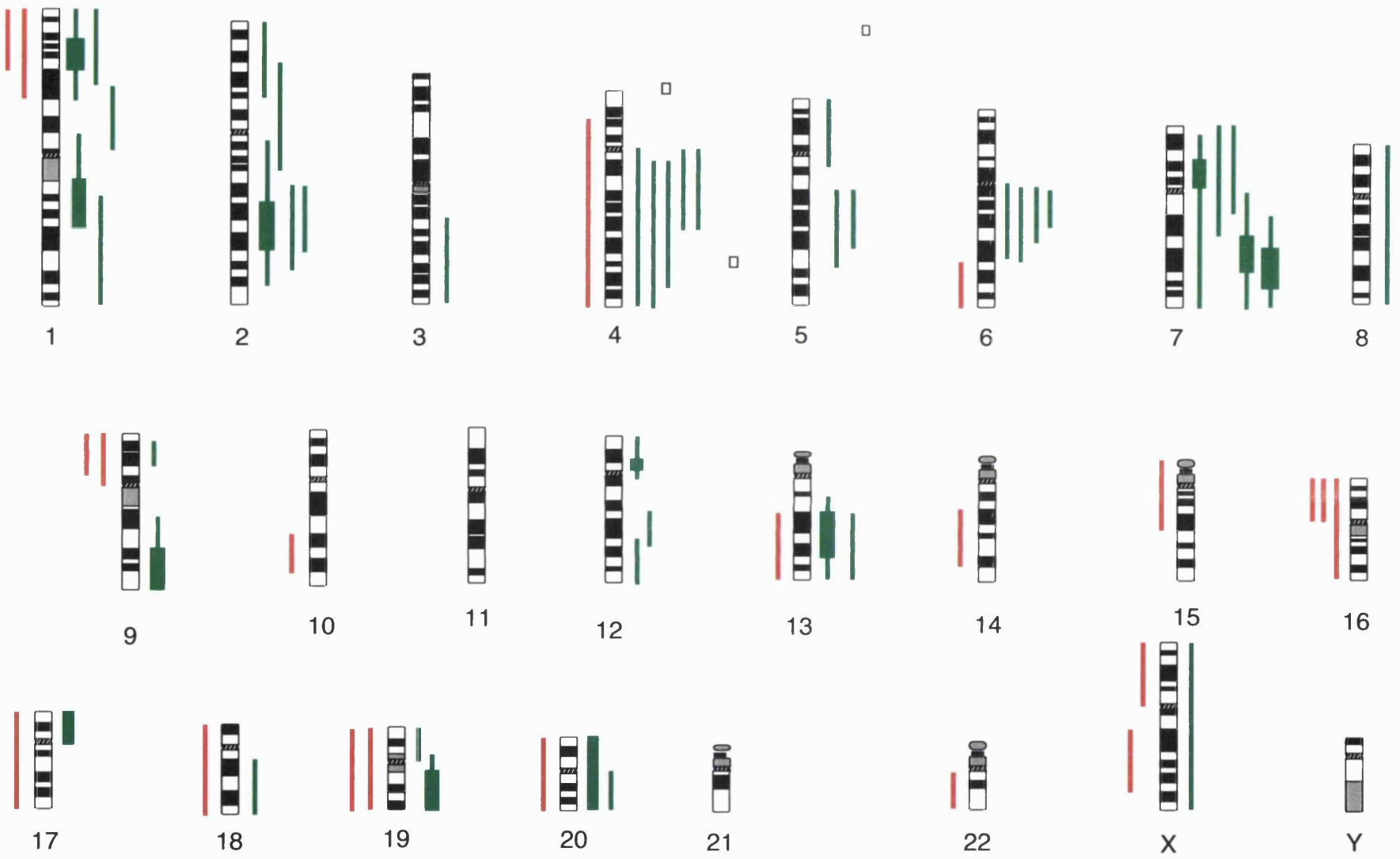


Figure 5.1: Chromosomal imbalances detected in 64 paediatric astrocytoma . Vertical lines to right of chromosomes indicate regions of gain; vertical lines to the left of the chromosome indicate regions of loss. Bold lines indicate regions of high copy number amplification.

Pilocytic astrocytoma

Figure 5.2 gives a summary of the aberrations seen in the pilocytic astrocytoma, and Figures 5.3 – 5.5 show the composite profiles of the tumours with detectable copy number aberrations. CNAs were infrequent events in this grade of tumour, with only three (10%) tumours having aberrations. All three of these tumours had sole abnormalities. IN324 and IN2017 both had alterations of chromosome 7 (see Figure 5.6). IN324 showed gain of 7q11-pter and IN2017 had gain of 7q, including a region of high copy number amplification at 7q22-31. IN2788 had gain of 4q11-26.

Diffuse astrocytoma

Figure 5.7 gives a summary of the aberrations seen in diffuse astrocytoma, and Figures 5.8 – 5.13 show the composite profiles of the tumours with detectable copy number aberrations. Six (30%) diffuse astrocytoma had detectable CNAs ranging from 2 to 17 per tumour (mean 7.6). The most common alterations in this grade of tumour were gain of 1p, 2q, 7q, loss of 9p, 16p and monosomy 19, each being seen in two cases (11%). There were nine regions of high copy number amplification at 1p32-34 (IN699), 1q21 (IN699), 7p11-14 (IN3032), 9q32-34 (IN699), 12p11.2 (IN3032), 13q21-31 (IN1524), 17p (IN699), 19q (IN699), and 20 (IN699). Figures 5.14 and 5.15 give examples of the alterations seen in IN699 and the pleomorphic xanthoastrocytoma, IN2587.

Anaplastic astrocytoma

Two of five (40%) anaplastic astrocytoma had copy number aberrations. A summary of the aberrations seen in these tumours can be seen in Figure 5.16 and the composite profiles of the tumours with aberrations can be seen in Figures 5.17 and 5.18. One tumour (IN2538) had a sole aberration involving monosomy 20. IN2774 had three CNAs, which were gain of 4q, 6q and 13q. There were no high copy number amplifications in this grade of tumour. No alteration was seen in more than one tumour.

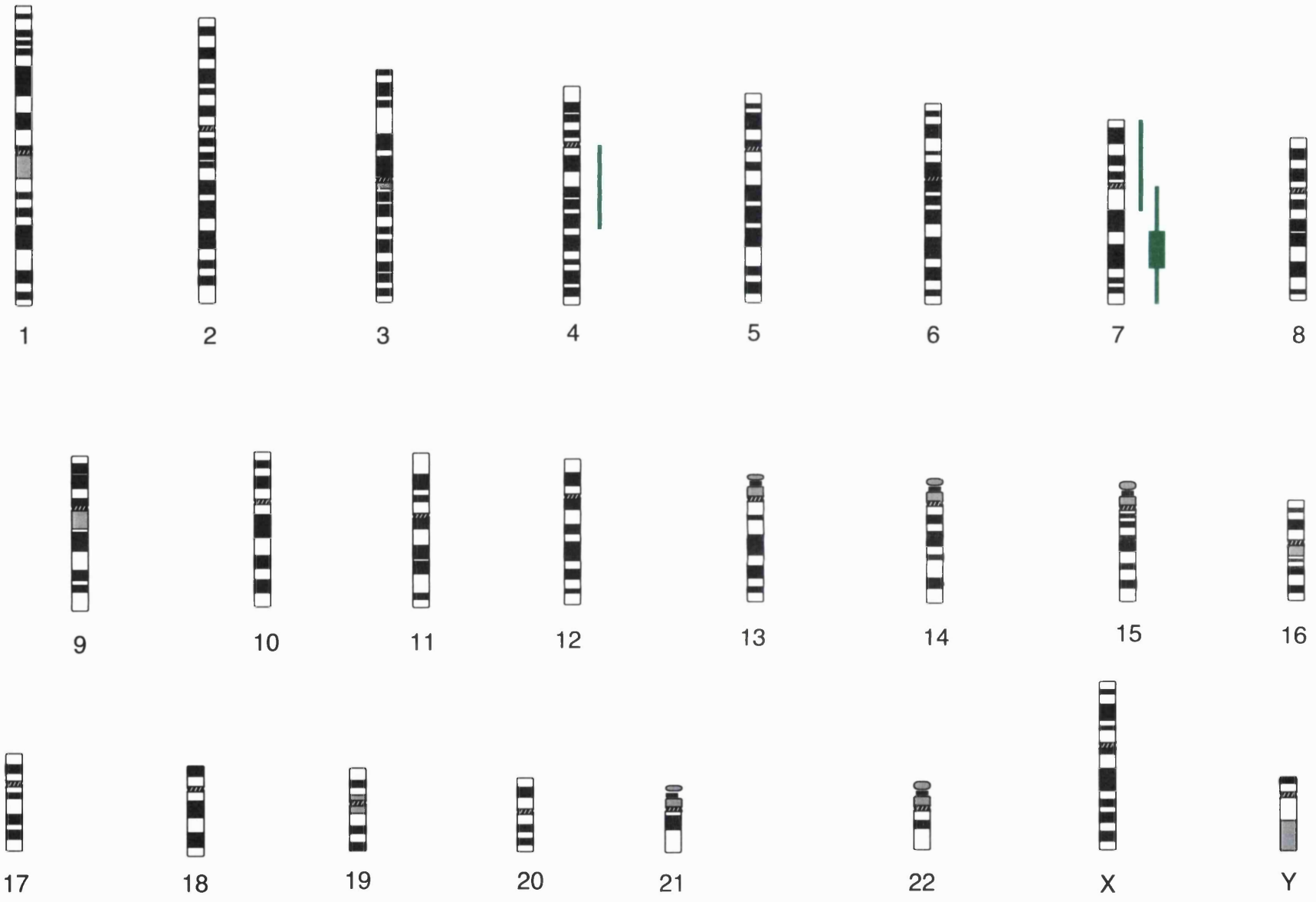


Figure 5.2: Chromosomal imbalances detected in 29 pilocytic astrocytoma . Vertical lines to right of chromosomes indicate regions of gain. Bold lines indicate regions of high copy number amplification. There were no regions of loss in this grade of astrocytoma.

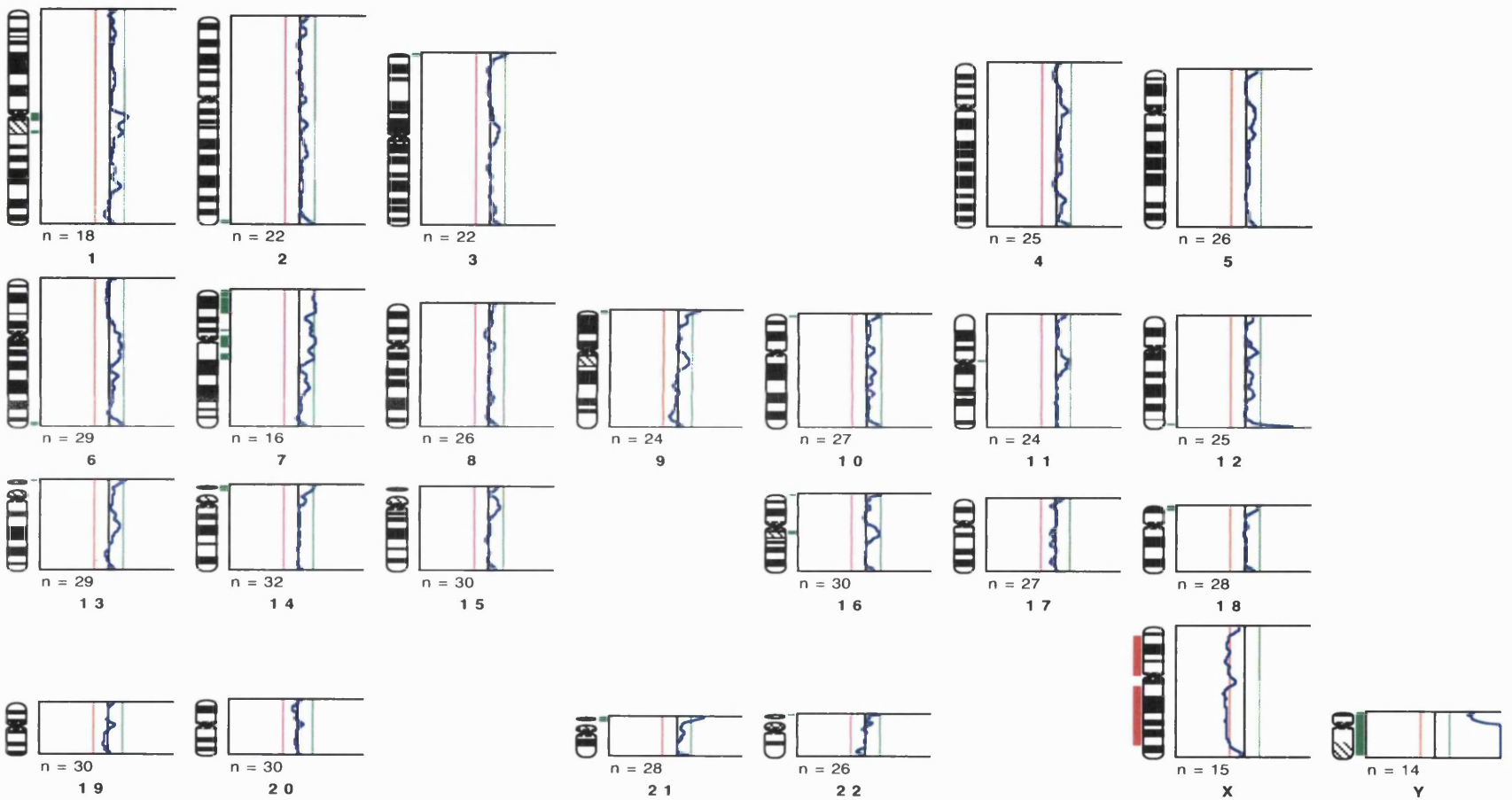


Figure 5.3: CGH composite profile of IN324, a pilocytic astrocytoma. This composite was compiled from the average of the individual ratio profiles from fourteen metaphase spreads. This tumour had a single copy number aberration, which was gain of 7q11.2-pter.

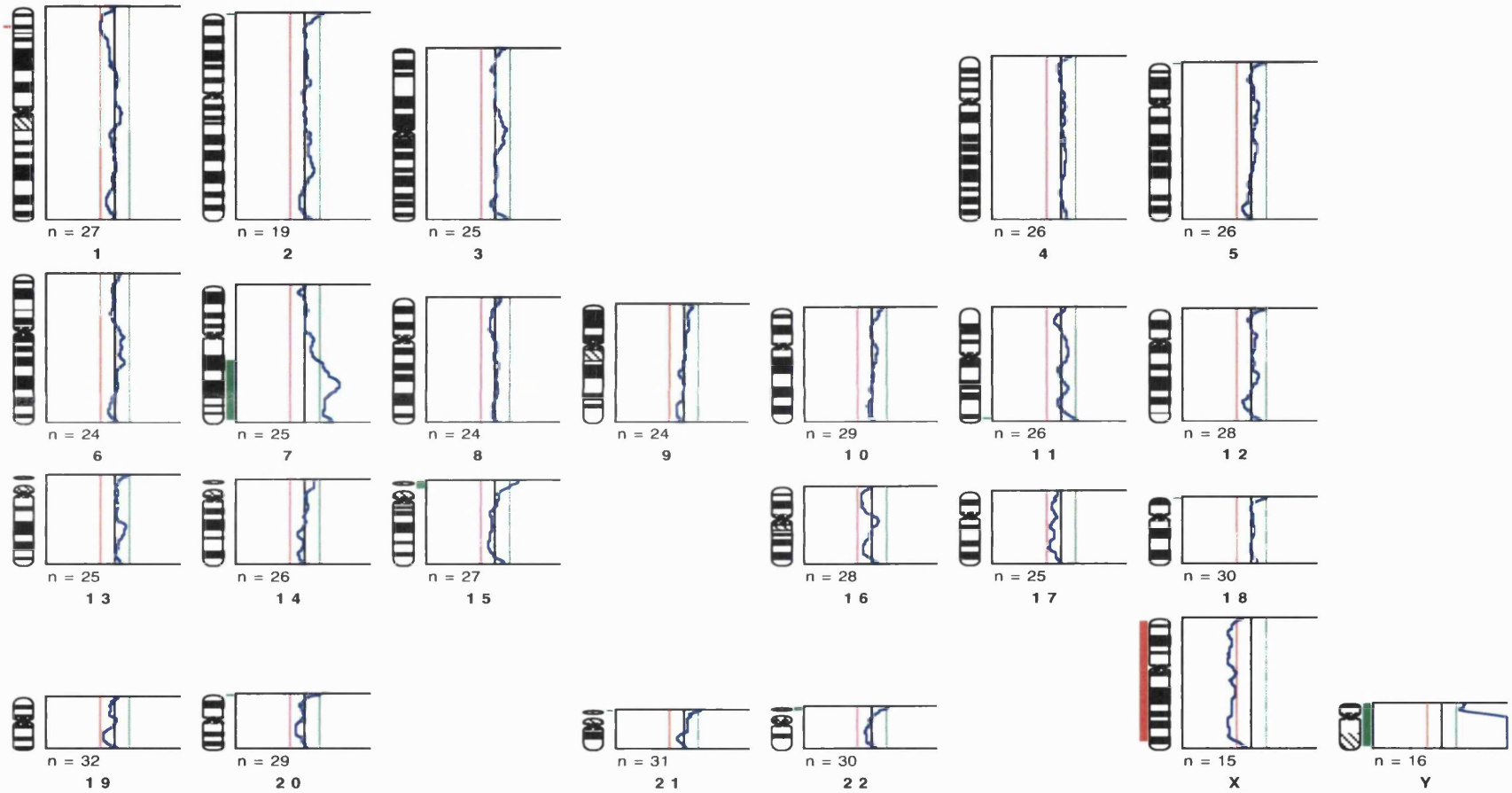


Figure 5.4: CGH composite of IN2017, a pilocytic astrocytoma. The composite was compiled from the average of the individual ratio profiles of sixteen metaphase spreads. This tumour showed a sole aberration on chromosome 7. There was a region of high copy number amplification at 7q22-31, as well as gain of the rest of the q arm.

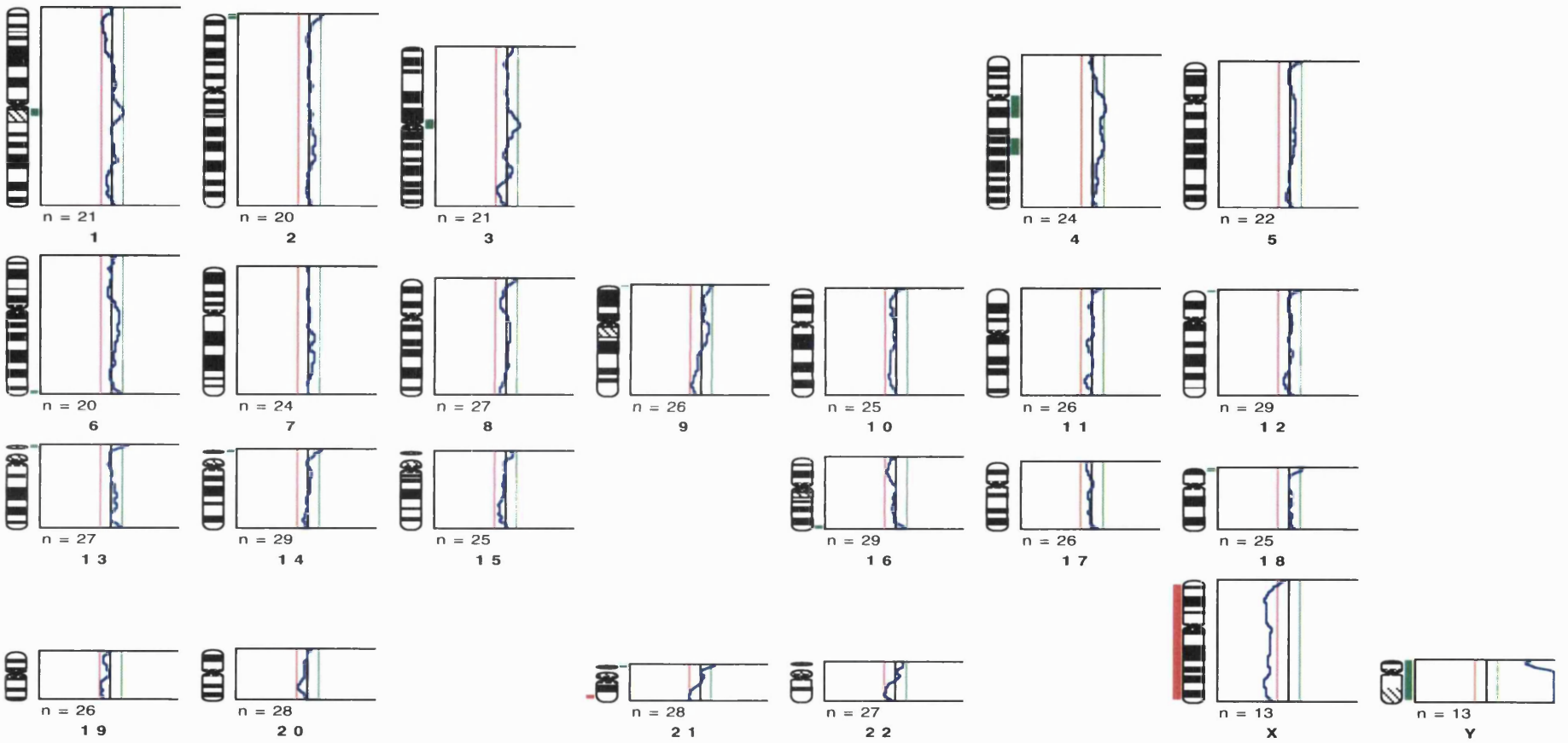
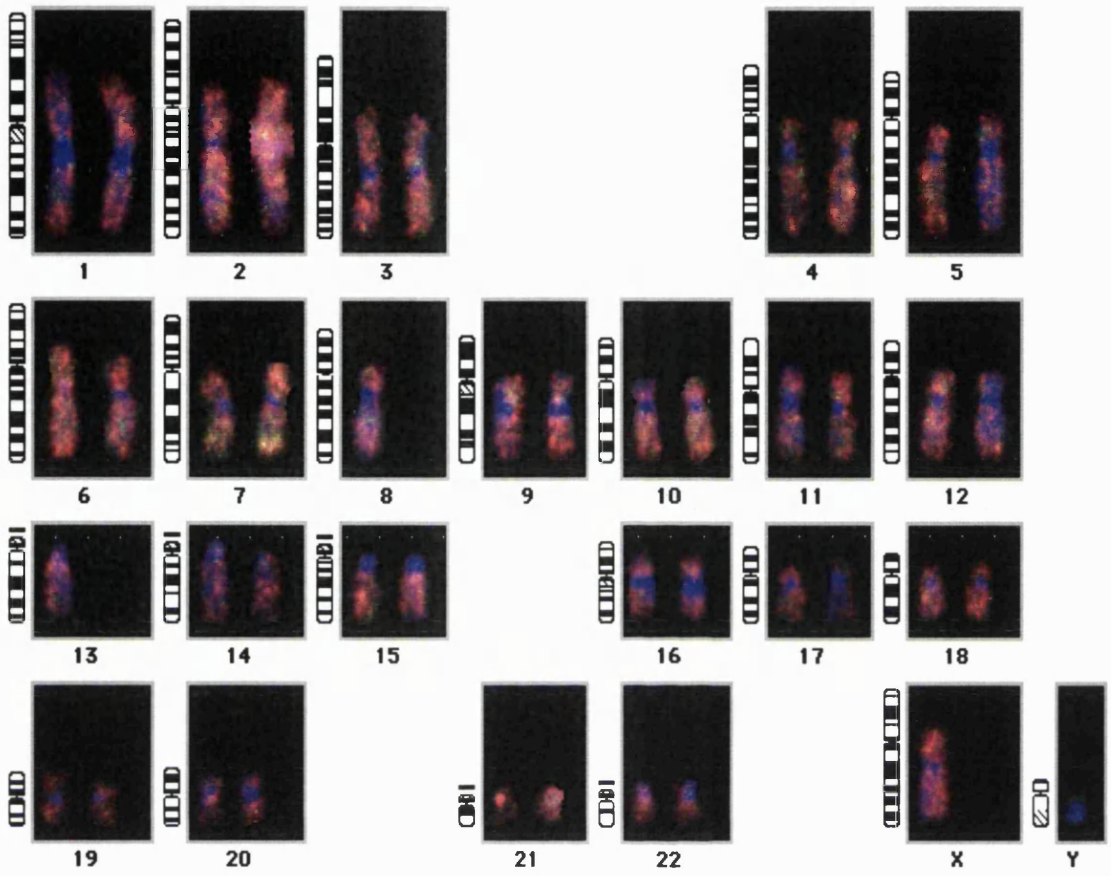


Figure 5.5: CGH composite of IN2788, a pilocytic astrocytoma. The composite was compiled from the average of the individual ratio profiles from thirteen metaphase spreads. This tumour had a sole aberration, which involved gain of 4q11-26.



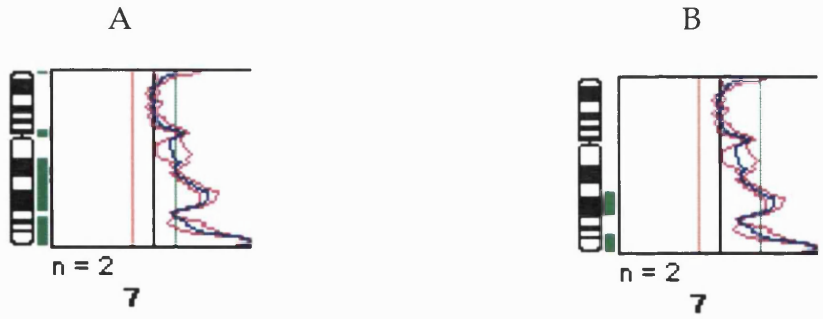


Figure 5.6: Colour karyotype of pilocytic astrocytoma IN2017, with the sole abnormality of gain of 7q (A) and amplification of 7q22-31 (B) being shown below. Threshold is set at 1+ 0.2 for gain and 1+0.4 for high copy number amplification.

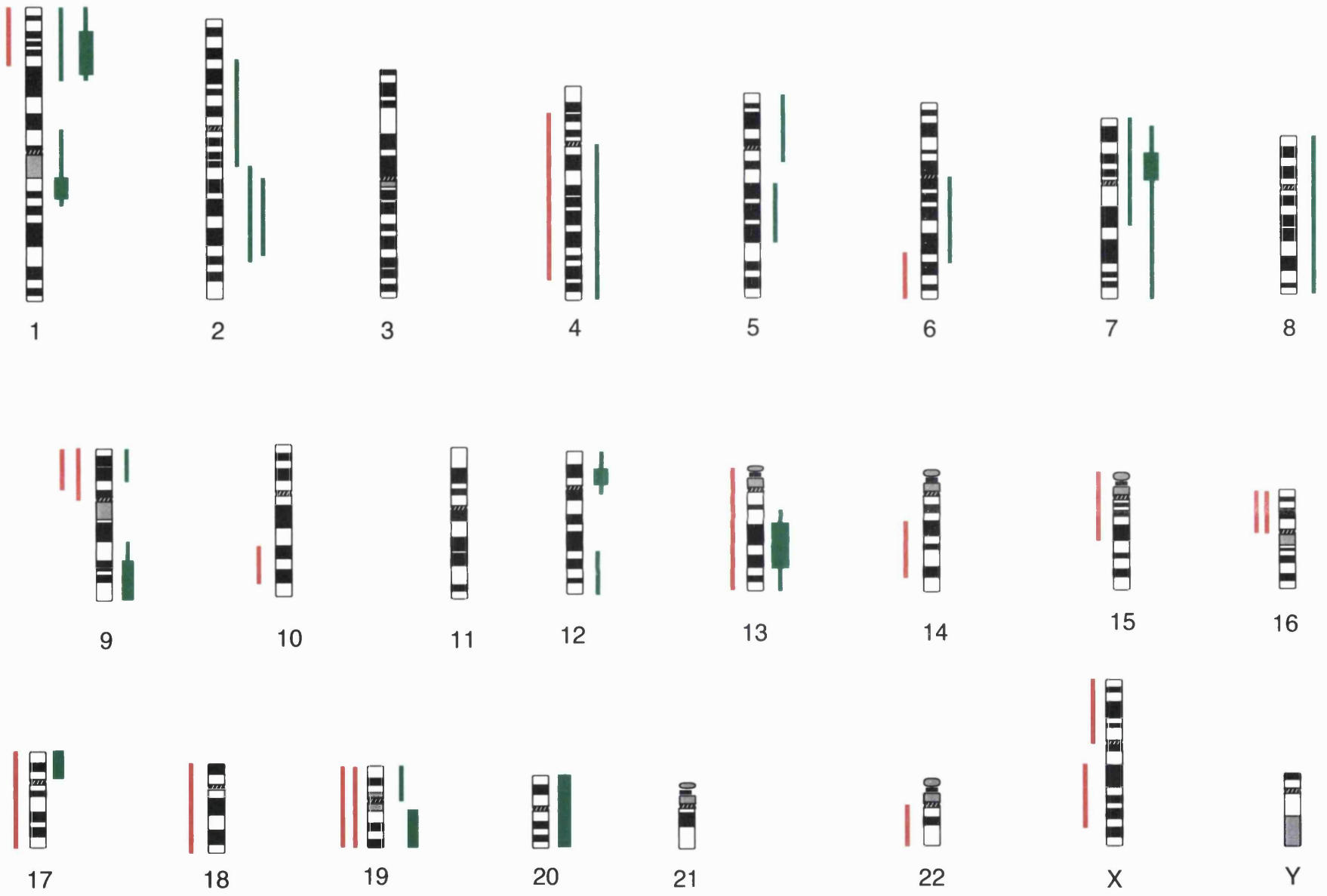


Figure 5.7: Chromosomal imbalances detected in 20 diffuse astrocytoma . Vertical lines to right of chromosomes indicate regions of gain; vertical lines to the left of the chromosome indicate regions of loss. Bold lines indicate regions of high copy number amplification.

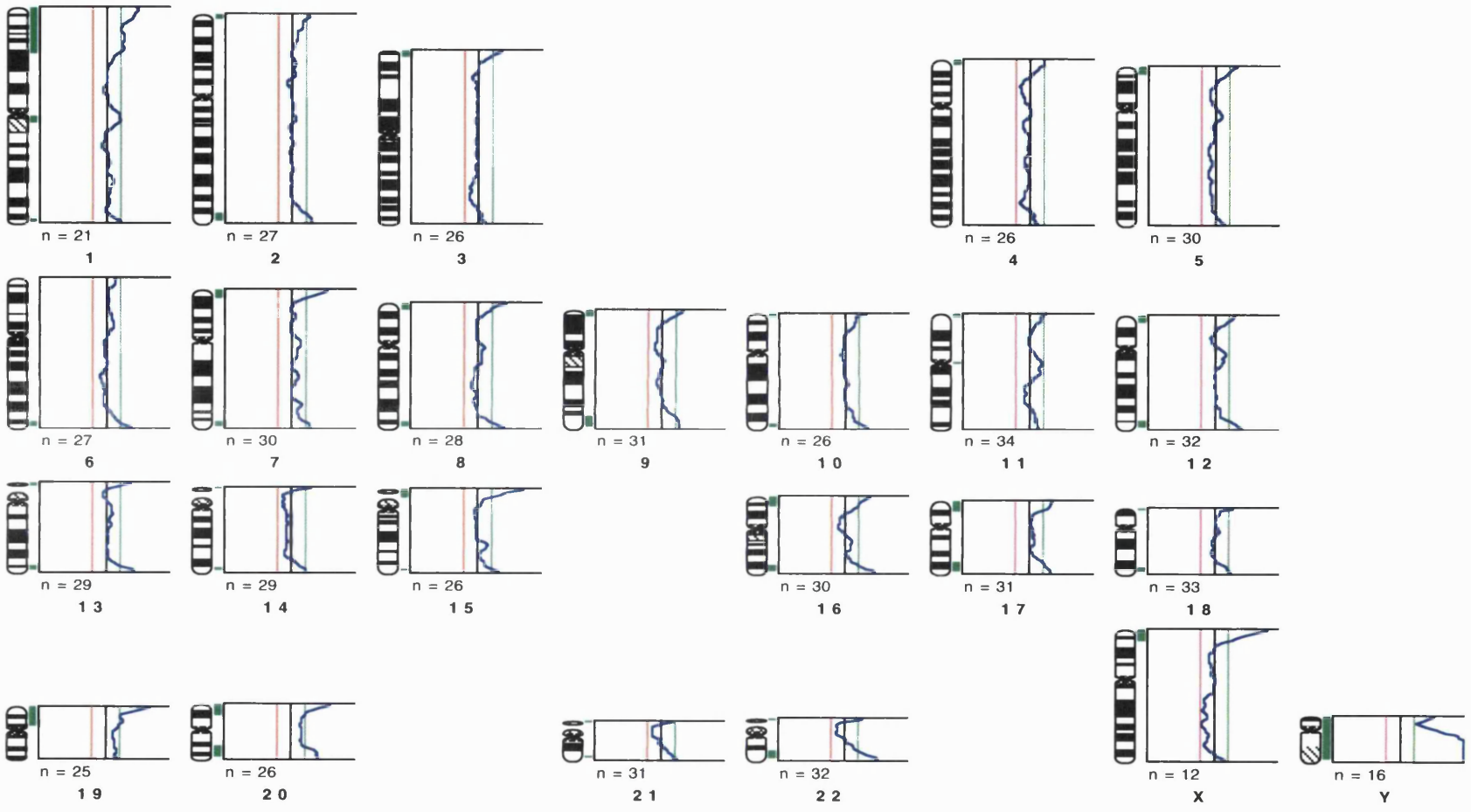


Figure 5.8: CGH composite of IN276, a diffuse astrocytoma. The composite was compiled from the average of the individual ratio profiles from nineteen metaphase spreads. This tumour had four CNAs. These were gain of 1p31-36, gain of 19p, gain of 20q and loss of Xq21-27.

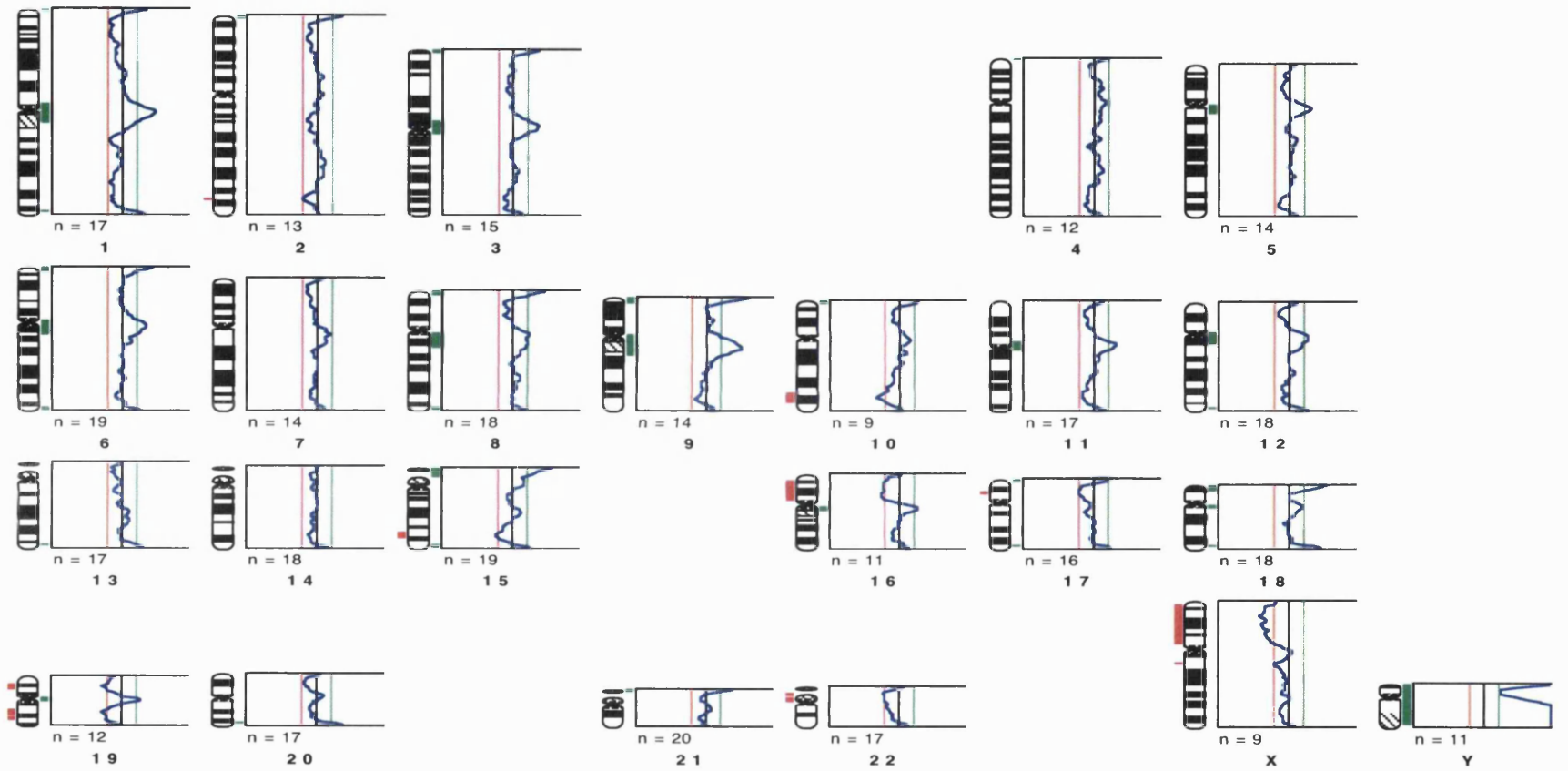


Figure 5.9: CGH composite of IN380, a diffuse astrocytoma. The composite was compiled from the average of the individual ratio profiles from eleven metaphase spreads. There were four CNAs, all involving regions of loss. These were loss of 10q24-25, loss of 16p, monosomy 19 and loss Xp.

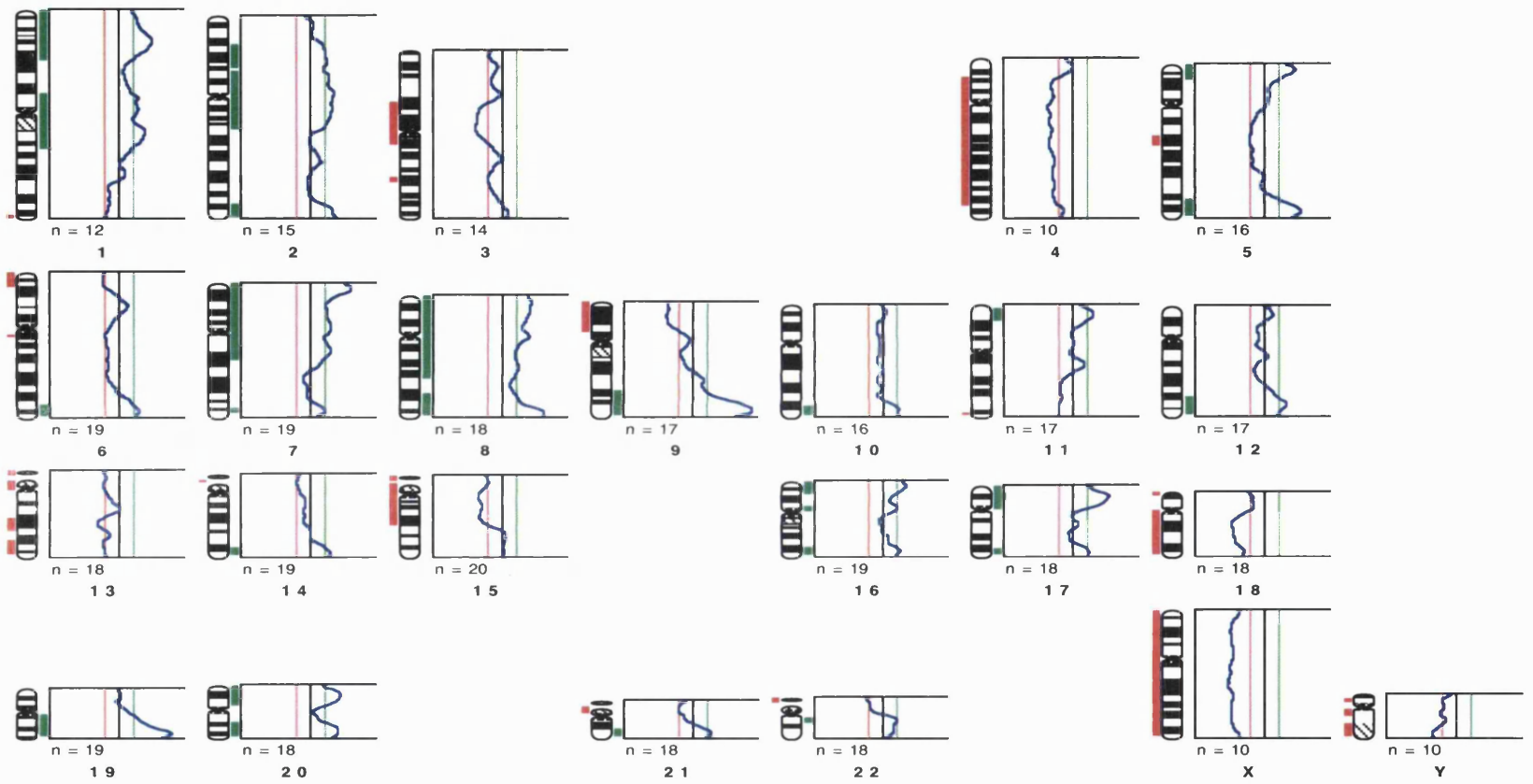


Figure 5.10: CGH composite of IN699, a diffuse astrocytoma. The profile is compiled from the average of the individual ratio profiles from ten metaphase spreads. There are five regions of high copy number amplification at 1p32-34, 1q21-23, 9q32-34, 17p and 20 and fifteen other CNAs. All the regions of alteration were quite large. There is gain of 1p31-36.3, 2p22-q21, 7p22-q21, 9q22-34, 12q23-24.3, 17p, 19q and 22q. There were also gains of chromosomes 8 and 20. Chromosomes 13 and 18 were monosomic. There was loss of 9p and large regions of deletion at 4p15-qter and 15pter-q21.

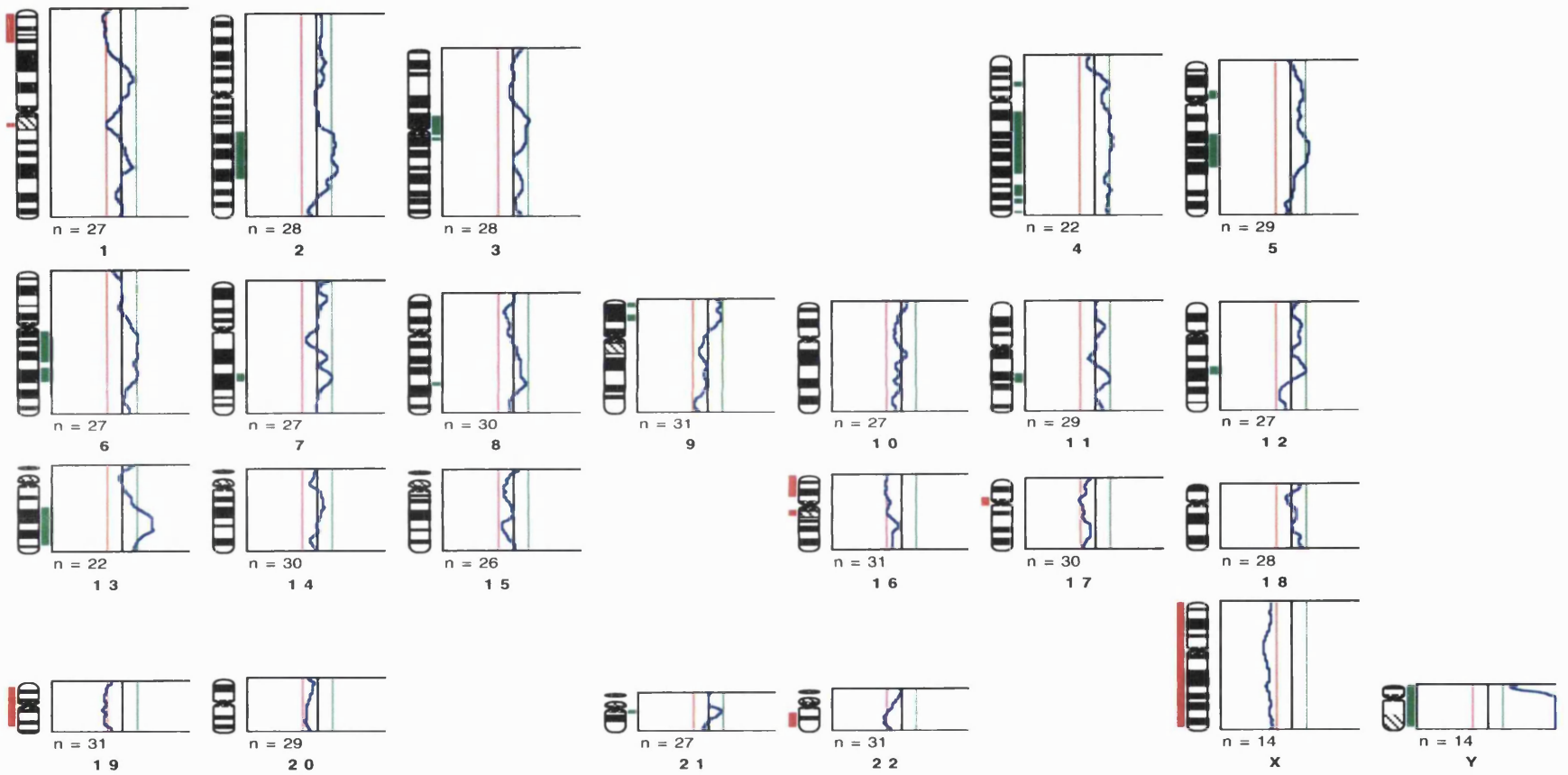


Figure 5.11: CGH composite of IN1524, a diffuse astrocytoma. The composite was compiled from the average of the individual ratio profiles from sixteen metaphase spreads. There were ten CNAs, mostly involving large regions of gain. There was gain of the whole of 4q, as well as smaller regions of gain at 2q21-32, 5q14-23, 6q12-22, 9p21-23 and 13q14-34. There was also a region of high copy number amplification at 13q21-31. There was also monosomy 19 and large regions of loss at 1p31-36, 16p and 22. The apparent small regions of gain at 7q, 8q, 11q and 12q were not included as aberrations as they were such small regions of change and were not seen in subsequent hybridisations.

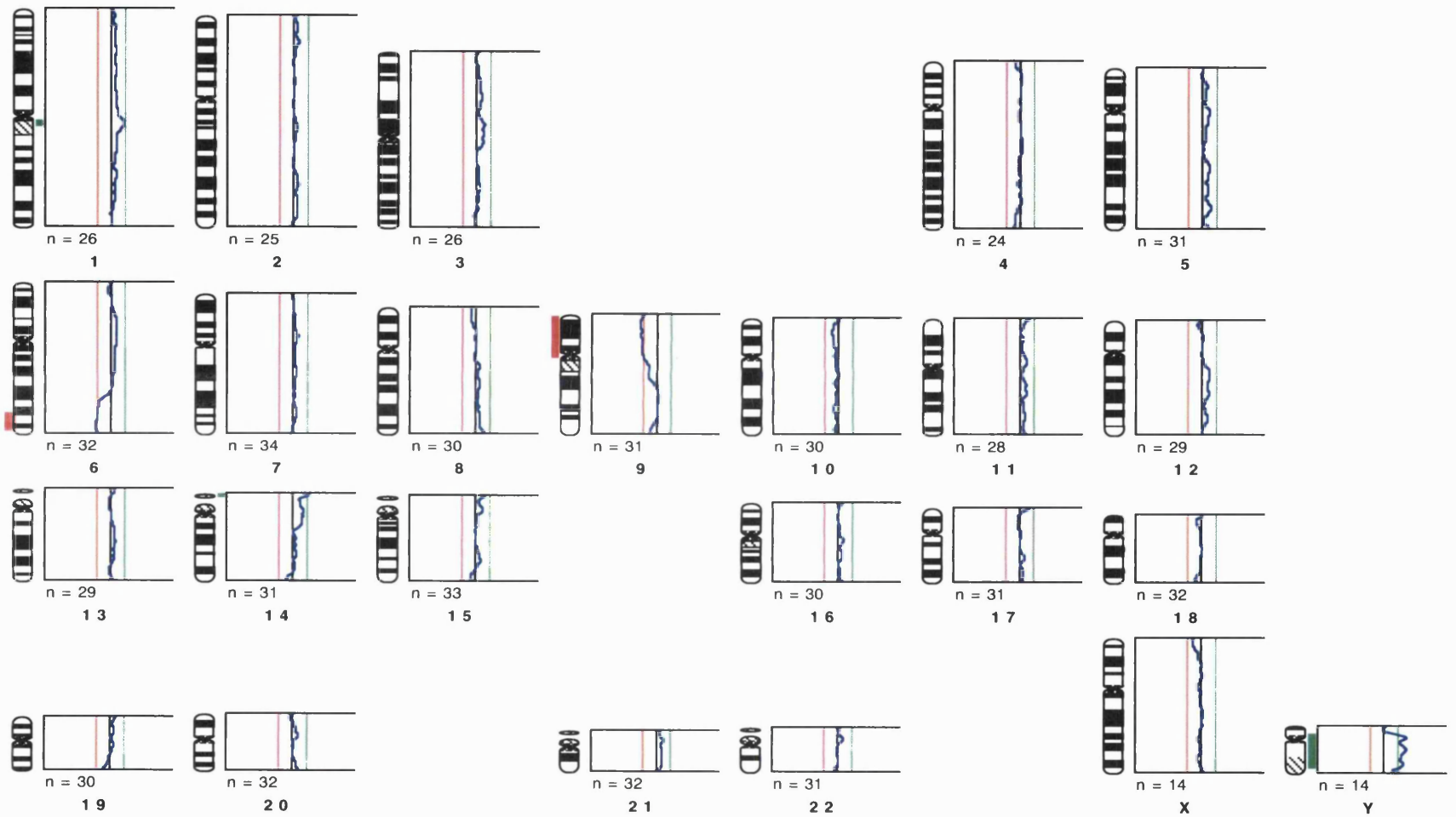


Figure 5.12: CGH composite of IN2587, a grade II pleomorphic xanthoastrocytoma. This tumour had two CNAs and the profile was compiled from the average of the individual ratio profiles of seventeen metaphase spreads. There is loss of the p arm of chromosome 9 and a region of loss at 6q23-27.

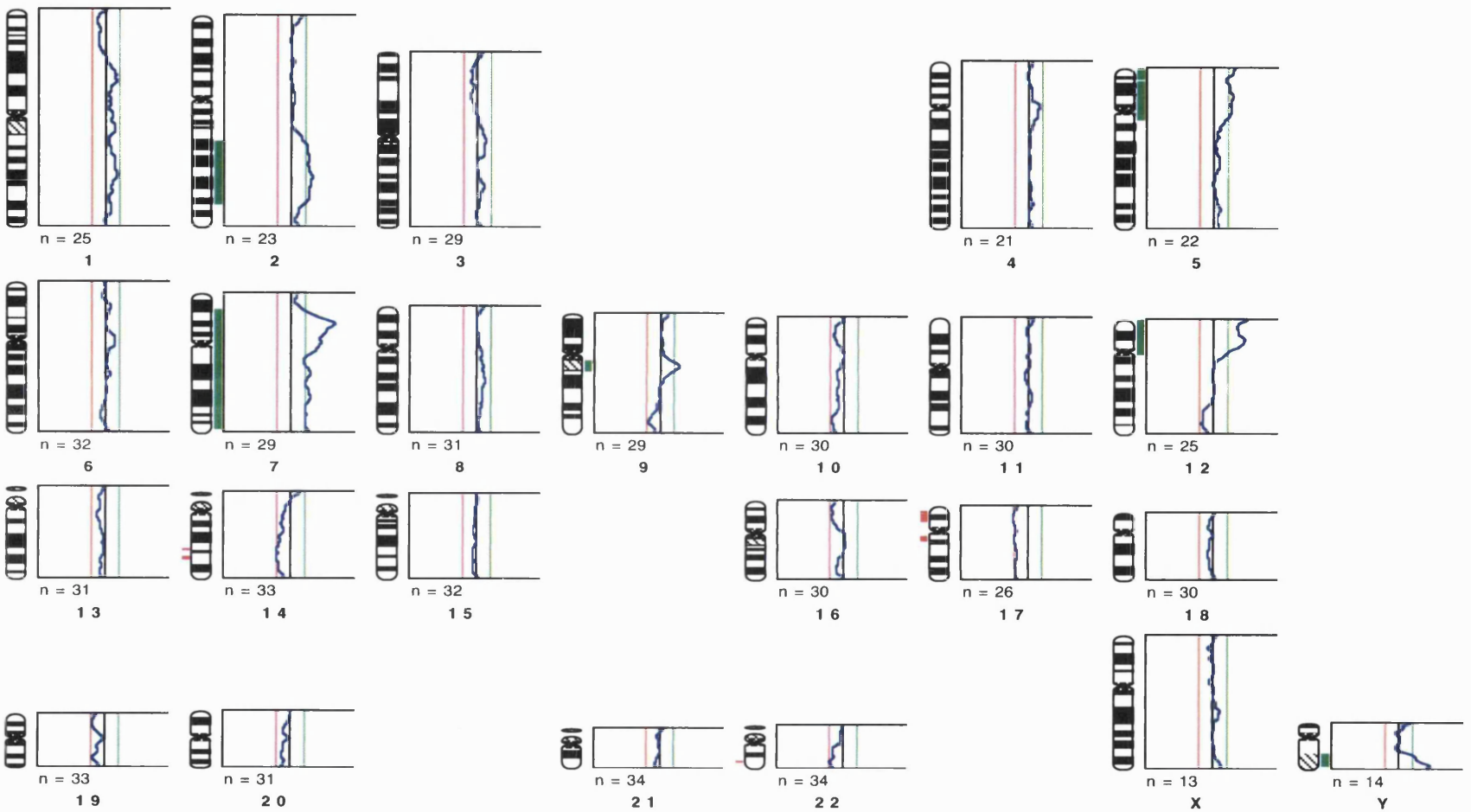


Figure 5.13: CGH composite of IN3032, a diffuse astrocytoma. The composite is compiled from the average of the individual ratio profiles of eighteen metaphase spreads. There are six CNAs as well as two regions of high copy number amplification at 7p11.1-14 and 12p11.2. There are gains of 2q22-34, 5p, 7q and 12p, and monosomy 17 and loss of 14q21-31.

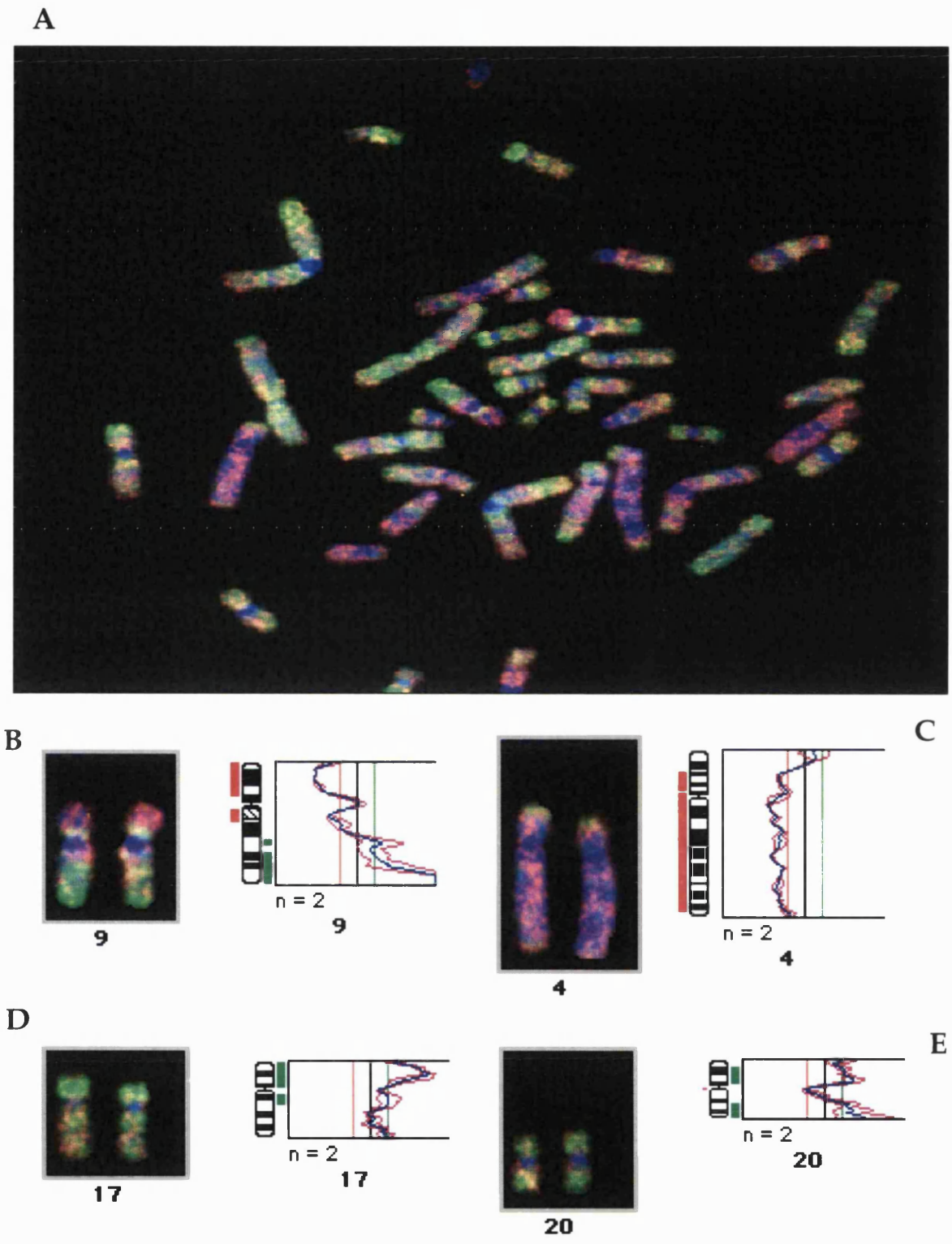


Figure 5.14: Diffuse astrocytoma IN699.
A) shows colour composite of metaphase. Red chromosomes are lost and green regions have been gained or amplified. B) shows chromosome 9 with loss of the P arm and amplification of 9q32-34. C) shows monosomy of chromosome 4. D) shows amplification of the short arm of chromosome 17 and E) shows amplification of chromosome 20.

A

B

Figure 5.15 IN2587, a pleomorphic xanthoastrocytoma. This tumour had two abnormalities, a region of loss at 6q23-qter (A) and loss of the short arm of chromosome 9 (B).

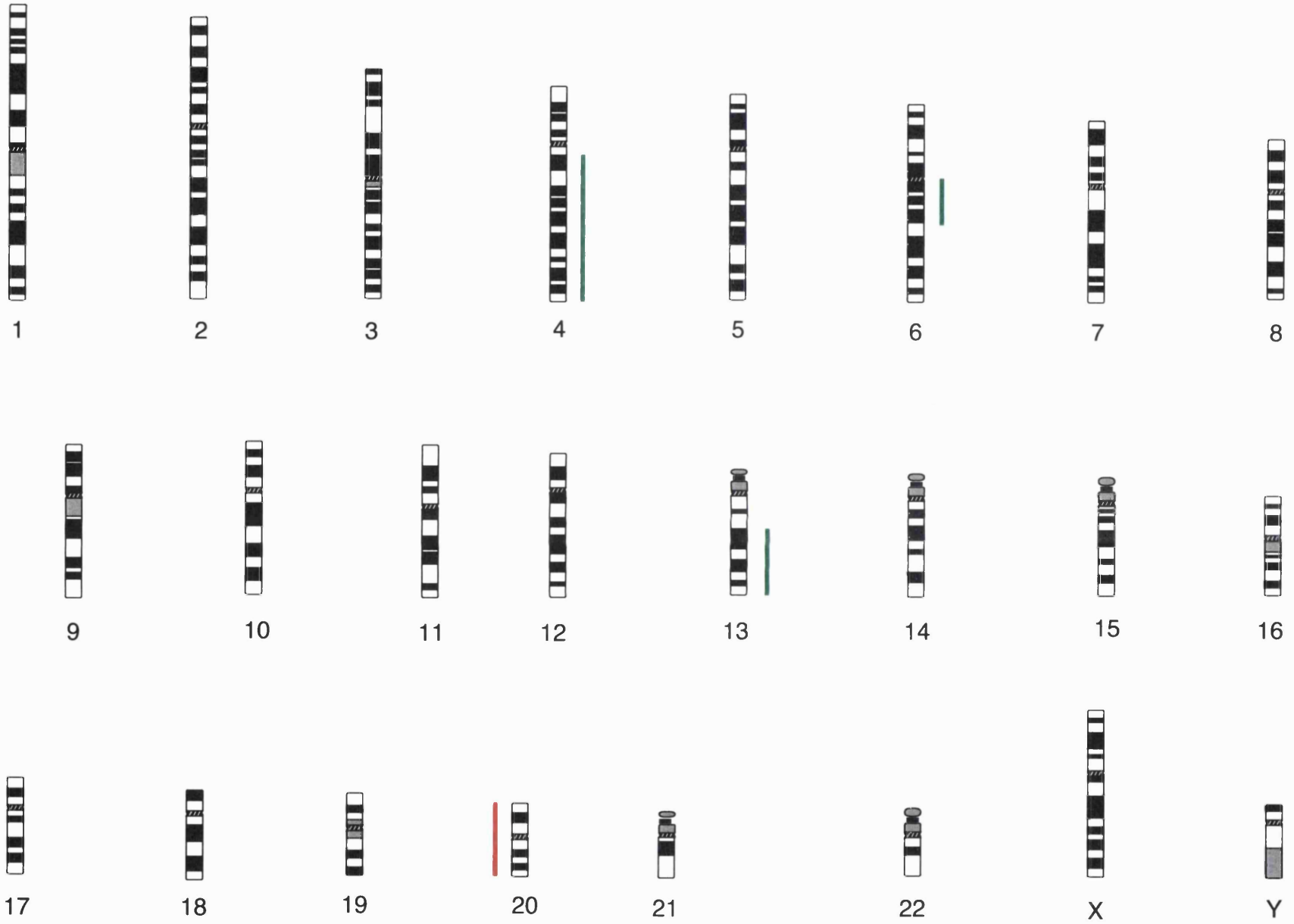


Figure 5.16: Chromosomal imbalances detected in 5 anaplastic astrocytoma . Vertical lines to right of chromosomes indicate regions of gain; vertical lines to the left of the chromosome indicate regions of loss.

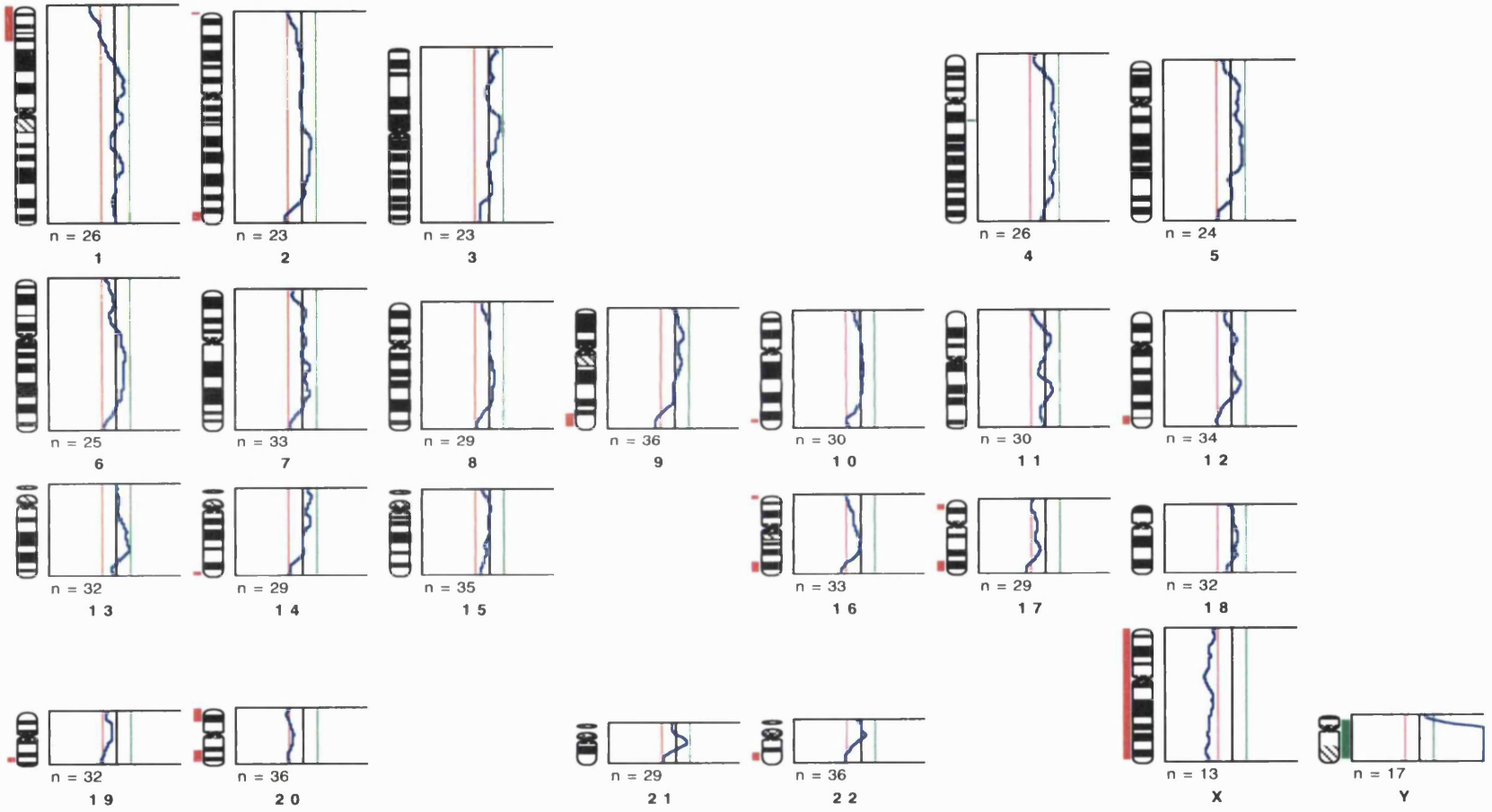


Figure 5.17: CGH composite of IN2538, an anaplastic astrocytoma. The profile is composed from the average of the individual ratio profiles from eighteen metaphase spreads and shows one alteration, monosomy of chromosome 20.

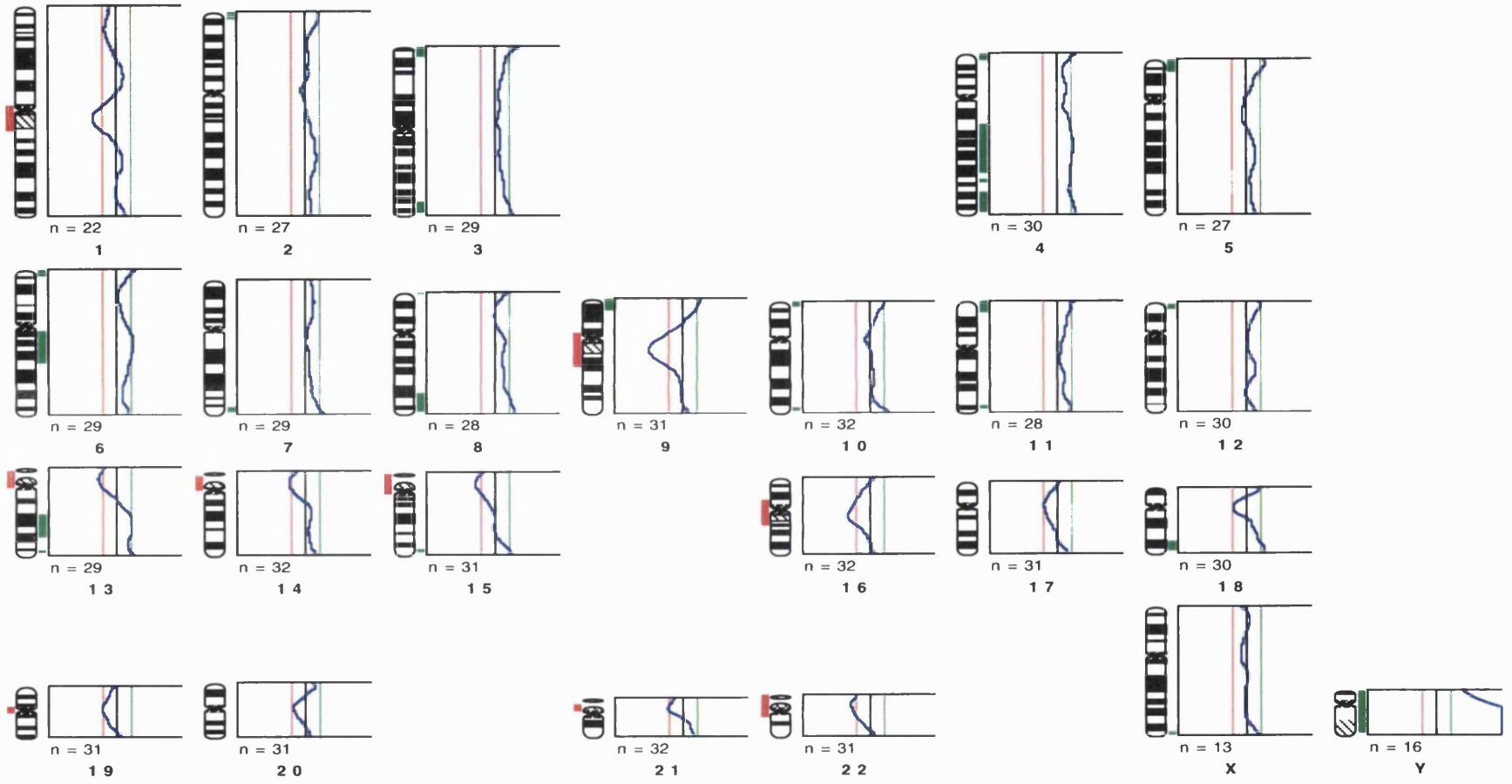


Figure 5.18: CGH composite of IN2774, an anaplastic astrocytoma. This tumour has three CNAs and the profile is compiled from the average of the individual ratio profiles from 16 metaphase spreads. The alterations all involve large regions of gain, localised to 4q 21-35, 6q12-21 and 13q21-qter. Regions of apparent loss around the centromeres of chromosomes 1, 9, 13, 14, 15, 16, 19, 21 and 22 are not included in the aberrations for this tumour as they are likely to be artifacts from the DOP-PCR labelling of the tumour DNA.

Glioblastoma multiforme

Two (20%) GBM had detectable CNAs. A summary of the abnormalities can be seen in Figure 5.19 and the composite profiles of the tumours with aberrations can be seen in Figures 5.20 and 5.21. There were no single abnormalities in any tumour. Regions of alteration ranged from 3 to 10 per tumour (mean 6.5). The most common abnormalities were gain of 4q and 6q, which were present in both of the tumours with CNAs. IN2240 had two regions of high copy number amplification at 2q24-32 and 7q31-33.

Estimation of minimum overlapping regions

The minimum overlapping regions of alteration, estimated from the composites of all the alterations seen in the astrocytoma in this study, were as follows:

Chromosome 1: The minimum region of gain on 1p was 1p31-36, which included the region of high copy number amplification seen in IN699. The minimum overlapping region of gain on 1q spanned 1q21-25 and included the region of high copy number amplification seen in IN699.

Chromosome 2: The minimum region of gain on 2p could be localised to 2p14-21 and that on 2q could be localised to 2q21-32.1 and included the region of high copy number amplification seen in IN2240.

Chromosome 4: The minimum region of gain on 4q could be localised to 4q13-26. This region of gain was the most frequent alteration observed, seen in five cases.

Chromosome 5: Two tumours showed gain on 5q. This region could be minimised to 5q14-23.

Chromosome 6: Four tumours showed gain of 6q and the region could be minimised to 6q12-16.

Chromosome 7: There were two separate regions of gain on chromosome 7. These could be localised to 7p12-21 and 7q21-36. These regions included the areas of high copy number amplification seen in IN2017, IN3032 and IN2240.

Chromosome 9: Two tumours showed loss from 9p. The minimum region of overlap was 9p13-24.

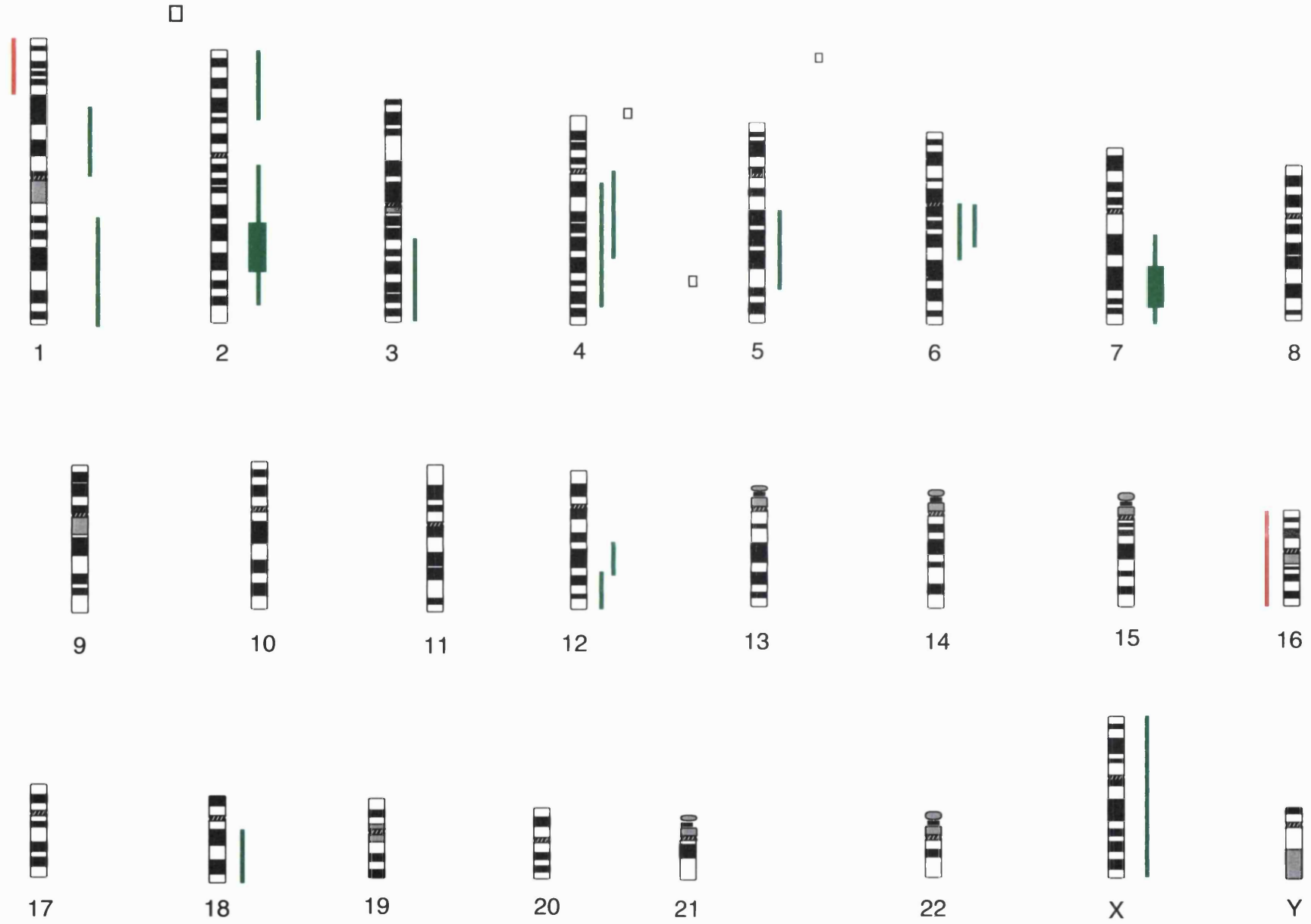


Figure 5.19: Chromosomal imbalances detected in 10 glioblastoma multiforme . Vertical lines to right of chromosomes indicate regions of gain; vertical lines to the left of the chromosome indicate regions of loss. Bold lines represent regions of high copy number amplification.

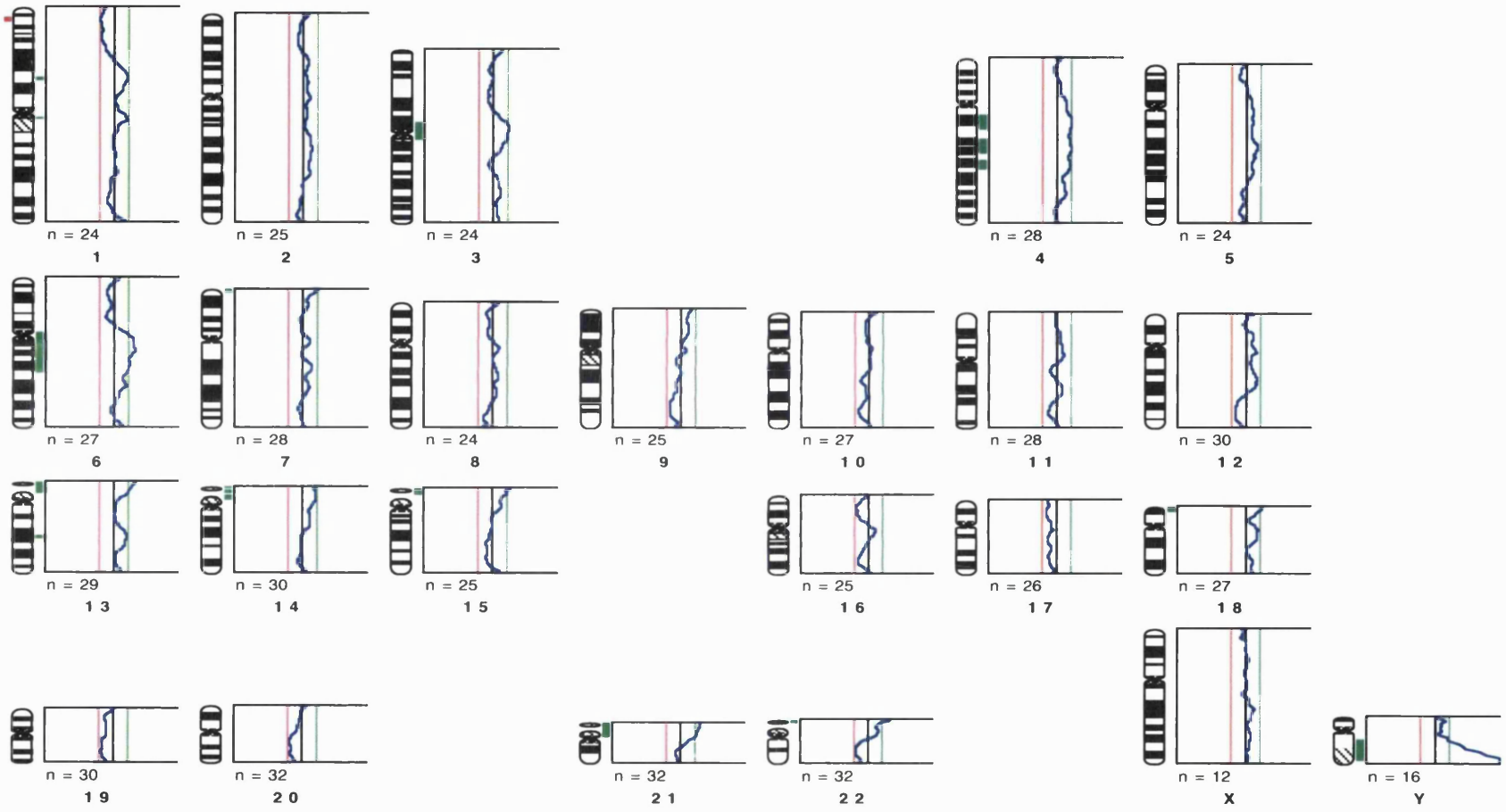


Figure 5.20: CGH composite of IN1566, a glioblastoma multiforme. The profile was compiled from the average of the individual ratio profiles from seventeen metaphase spreads. There were three CNAs. There were regions of gain at 4q13-28 and 6q11.1-21 and a region of deletion at 1p32-36.3.

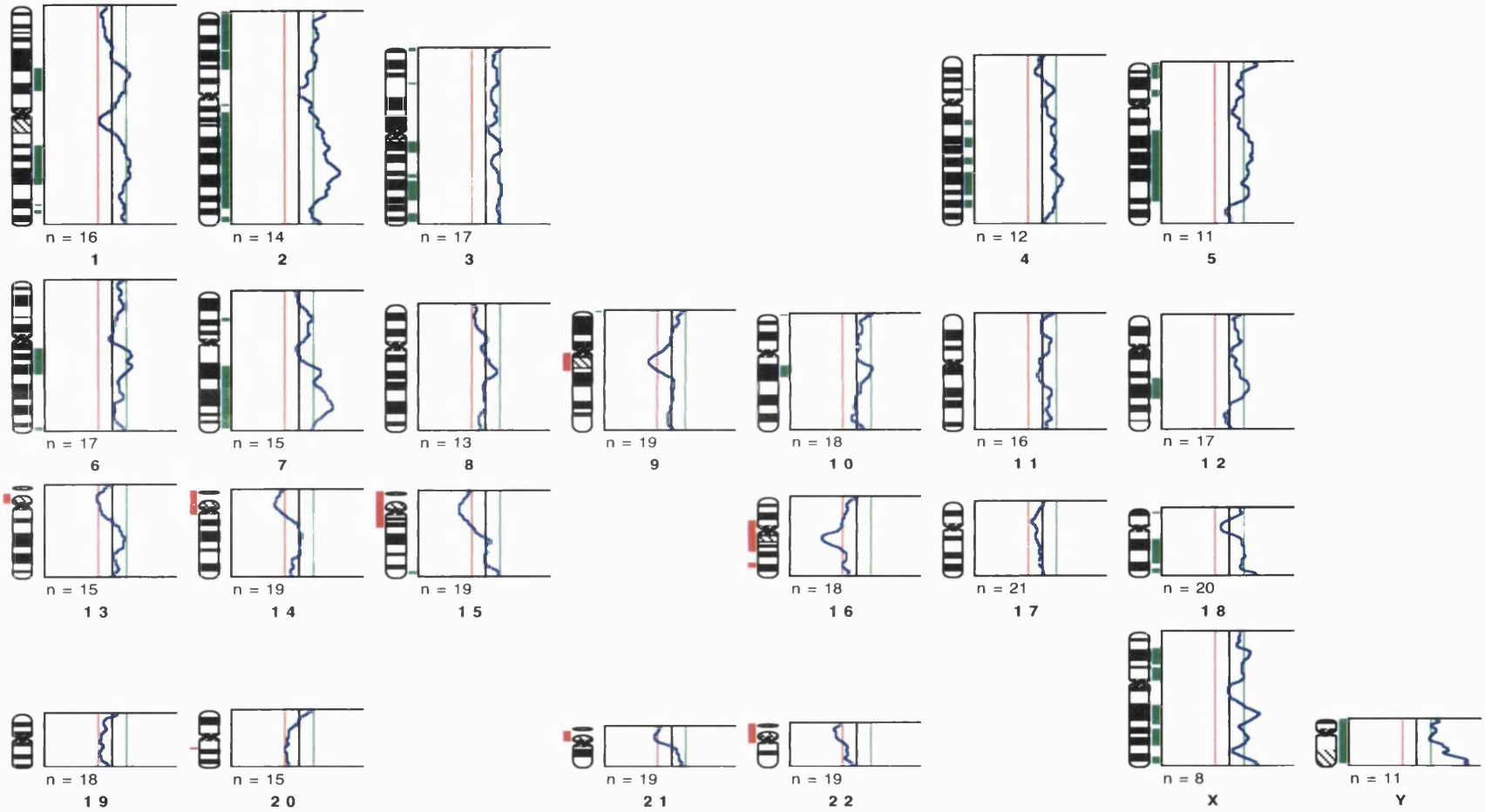


Figure 5.21: CGH composite of IN2240, a glioblastoma multiforme. The composite is compiled from the average of the individual ratio profiles of eleven metaphase spreads. There are two regions of high copy number amplification at 2q24-32 and 7q31-33. There are also thirteen other CNAs. These are gain of 1p13-31, 1q22-qter, 2p13-pter, 2q13-qter, 3q21-qter, 4q13-q33, 5q13-33, 6q11-21, 7q21-qter, 12q14-23, 18q12-22 and X, and monosomy 16.

Chromosome 12: Two tumours showed gain of 12q, with the minimum region of overlap being 12q22.

Chromosome 13: Two tumours showed gain of 13q. The minimum region of gain was 13q21-24 and this included the region of high copy number amplification seen in IN1524.

Chromosome 16: Two tumours showed loss of 16p. The loss spanned the entire p arm and a minimum region could not be estimated.

Chromosome 19: Two tumours showed loss of chromosome 19, as the loss spanned the whole chromosome no minimum region of deletion could be estimated.

Chromosome 20: Three tumours showed gain of chromosome 20. The minimum region of overlap was 20q11.1-13.3. This region included the area of high copy number amplification seen in IN699.

Correlation of CNAs with clinicopathological criteria

The presence of CNAs was not associated with any particular grade of astrocytoma (p=0.2470) despite the anaplastic astrocytoma having a higher percentage of tumours with alterations than the other grades of tumours, though this may be due to the low number of anaplastic astrocytoma in the study. The presence of high copy number amplifications could not be associated with tumour grade (p = 0.4416). Loss of 20q was seen only in the anaplastic astrocytoma and this was significant at the 95% confidence level (p = 0.0074). The presence of CNAs were not associated with patient age (p=0.941), sex (p=0.100) or survival (p = 0.418). Full details of the statistical analysis can be seen in Appendix III.

Associations between CNAs

Associations between CNAs were analysed using GraphPad prism. p values of less than 0.0500 at the 95% confidence level were considered to be statistically significant. Gain of 4q was seen in association with gain of 6q in four cases (p = 0.0070). Loss of 19 was only seen in association with loss of 16p in two cases (p =0.0385).

Survival analysis

Survival data was available for fifty-four of the patients in this study. The overall survival for the astrocytoma patients was 88% at 5 years and 76% at 10 years (see Figure 5.22). There was a clear relationship between grade of tumour and patient survival (see Figure 5.23). With increasing tumour grade the outcome was poorer (log rank test, p =0.0002). The 5 and 10 year survival for each grade of tumour can be seen in Table 5.2. Survival was not influenced by patient age at diagnosis (log-rank test, p = 0.568), sex (log-rank test, p = 0.700) or the extent of surgical resection (log-rank test, p=0.3413).

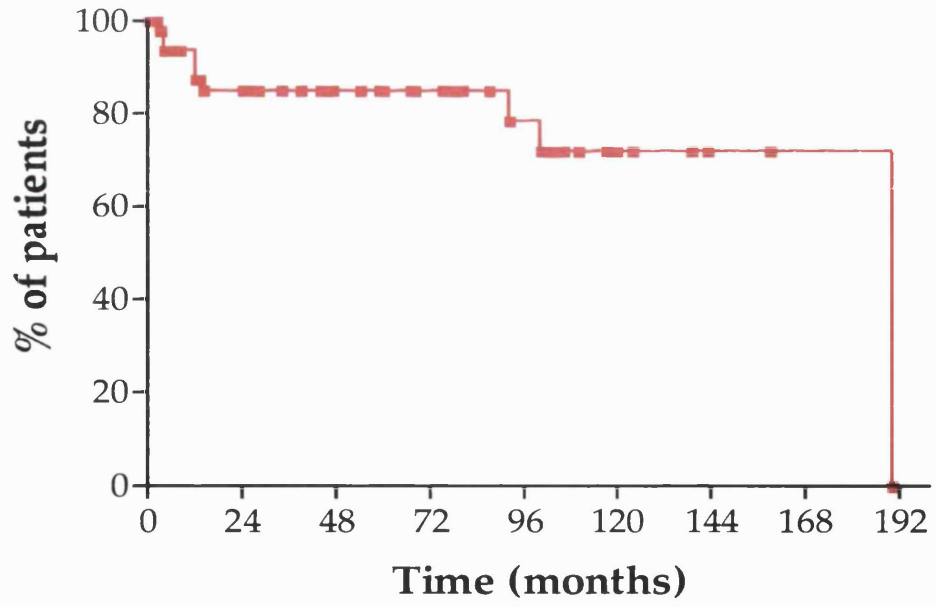


Figure 5.22: Overall survival of astrocytoma patients. Survival is measured in months from the date of initial diagnosis.

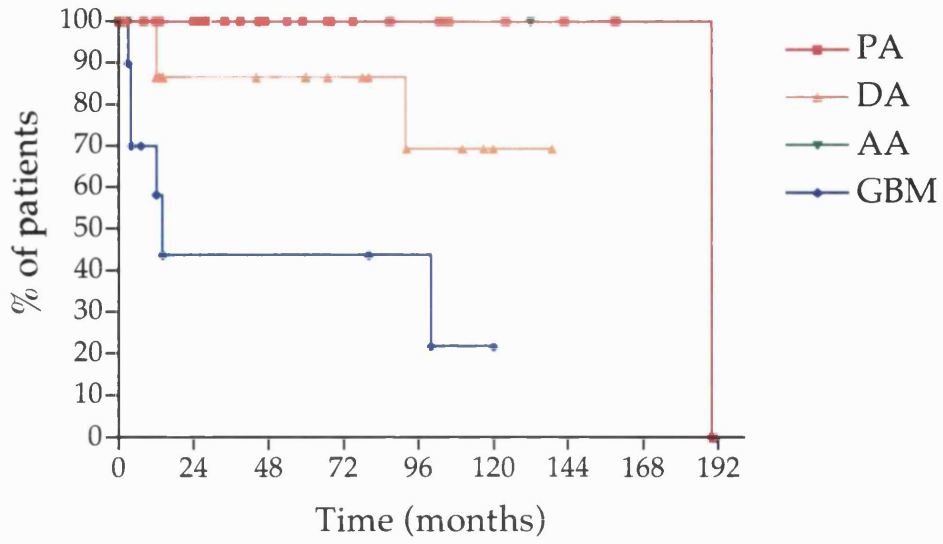


Figure 5.23: Survival in astrocytoma patients according to tumour grade. PA = pilocytic astrocytoma, DA = diffuse astrocytoma, AA = anaplastic astrocytoma, GBM = glioblastoma multiforme. Log-rank test, $p = 0.0003$.

	5 year survival	10 year survival
Pilocytic astrocytoma	100%	100%
Diffuse astrocytoma	84%	68%
Anaplastic astrocytoma	N/A	N/A
Glioblastoma multiforme	42%	21%

Table 5.2: Comparison of 5 and 10 year overall survival in patients with different grades of astrocytoma. There was survival data available for only one anaplastic astrocytoma patient in this study therefore it was not possible to show the five and ten year survival for this group of patients.

The only other clinicopathological feature that affected outcome in these astrocytoma patients was the treatment regimen. The best outcome was seen in those patients who had received no post-operative therapy (see Figure 5.24). The 5 and 10 year survival rates for the different treatment regimens can be seen in Table 5.3. Patients who had received only surgery had a 5 year survival of 100%, compared to 78% in those patients who had received post-operative radiotherapy. The patients who had received post-operative chemotherapy were alive at 12 months post-diagnosis but no further survival

data was available for these patients. The differences in survival between the treatment groups was statistically significant (p = 0.0021). However, the majority of the children who received no post-operative therapy were those with pilocytic astrocytoma, which is known to have a better prognosis than anaplastic astrocytoma and GBM, therefore this data is not independent of tumour grade.

	5 year	10 year
Surgery alone	100%	100%
Surgery & radiotherapy	78%	64%
Surgery & chemotherapy	unknown	unknown

Table 5.3: Five and ten year survival rates for astrocytoma patients depending on treatment received.

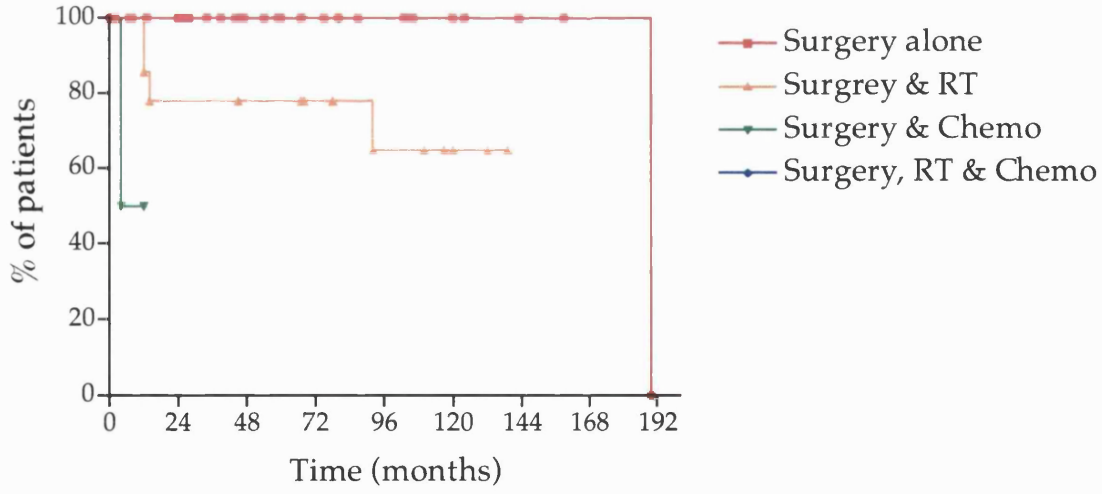


Figure 5.24: Survival in astrocytoma patients according to treatment regimens. Survival is measured in months from the time of initial diagnosis. Log-rank test, p =0.0021.

The presence of CNAs did not influence outcome (log-rank test, p = 0.2420). It was difficult to assess the impact of individual CNAs on survival due to the low number of aberrations and also the difficulty in obtaining survival data

for some of the patients in this study. Four CNAs could be seen to be associated with worse survival, these were gain of 7 (log-rank test, $p = 0.0420$), gain of 5q (log-rank test, $p = 0.0498$), gain of 2q (log-rank test, $p = 0.0017$) and loss of 16p (log-rank test, $p = 0.0092$). The survival curves for these aberrations can be seen in Figures 5.25 – 5.28. Gain of 7q was seen in all grades of astrocytoma, except anaplastic astrocytoma so the effect of gain of 7q on survival occurs independently of tumour grade. The other alterations were seen only in diffuse astrocytoma and GBM, and due to the low number of samples with alterations it is difficult to determine whether the effect of these alterations on survival occur independently of tumour grade.

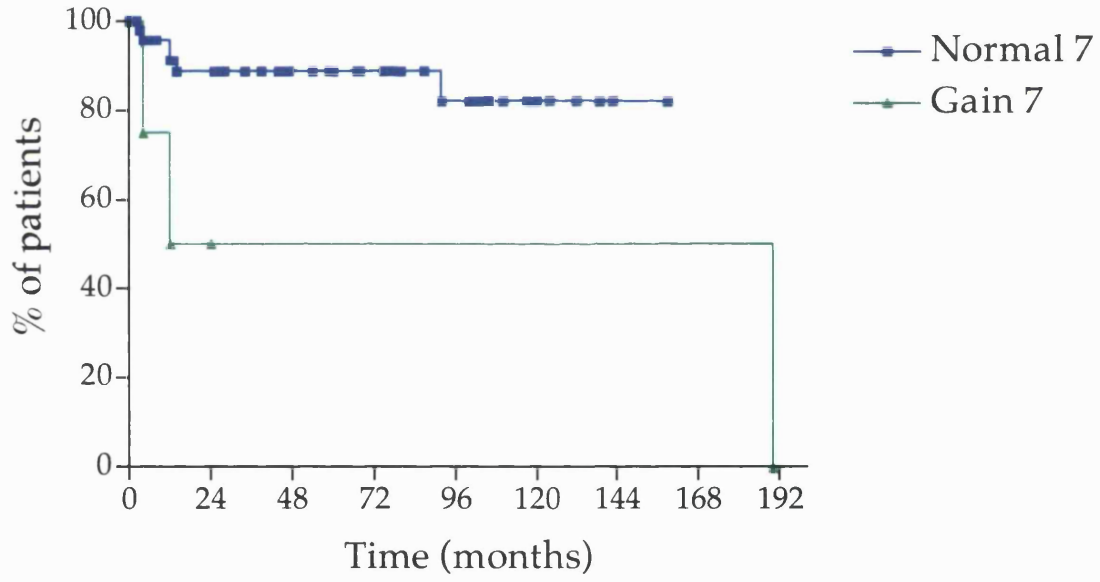


Figure 5.25: The influence of gain of chromosome 7 on survival in astrocytoma patients. Survival is measured in months from the time of initial diagnosis. Log-rank test, $p = 0.0420$.

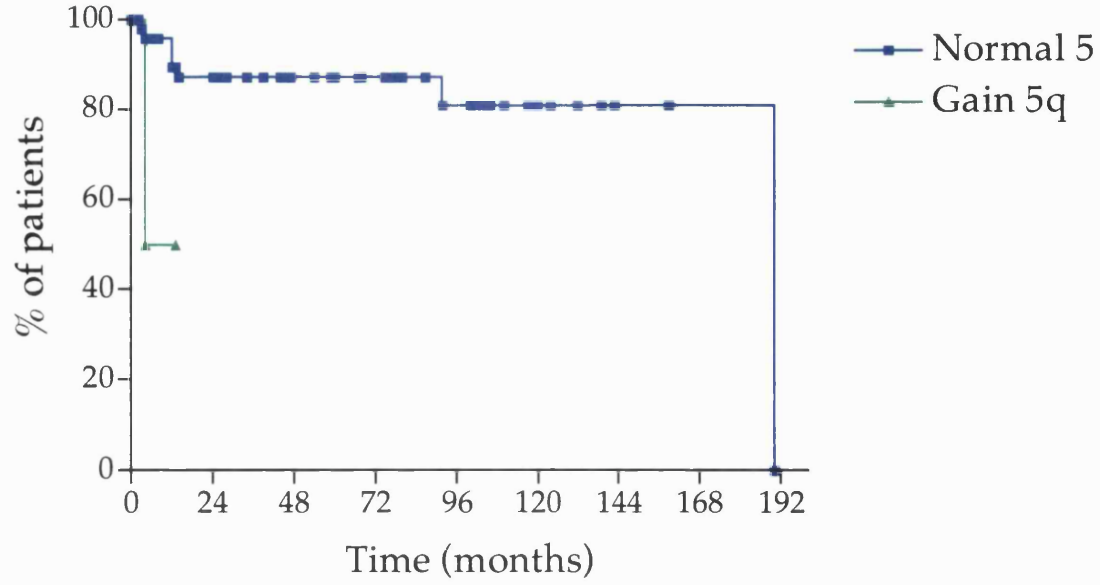


Figure 5.26: The influence of gain of 5q on survival in astrocytoma patients. Survival is measured in months from the time of initial diagnosis. Log-rank test, $p = 0.0498$.

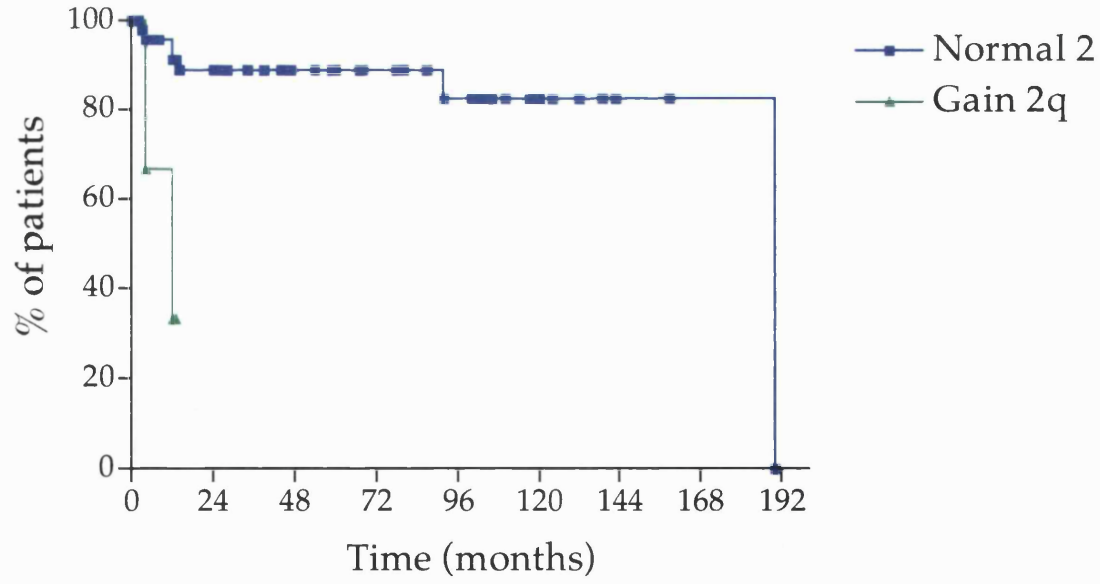


Figure 5.27: The influence of gain of 2q on survival in astrocytoma patients. Survival is measure in months from the time of initial diagnosis. Log-rank test, $p = 0.0017$.

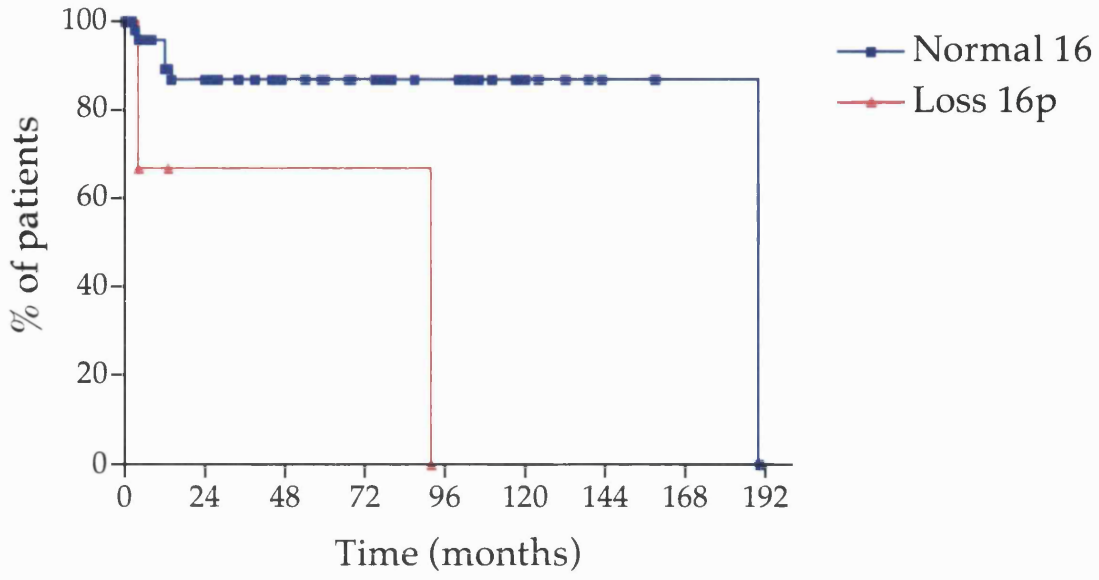


Figure 5.28: The influence of loss of 16p on survival in astrocytoma patients. Survival is measured in months from the time of initial diagnosis. Log-rank test, $p = 0.0092$.

Y-PCR analysis

All the astrocytoma samples in this study were analysed for the presence/absence of Y chromosome material. DNA samples were amplified using two sets of primers for the Y chromosome. Only one sample showed no amplification product with either set of primers, the GBM, IN1786. There was no blood sample available for this patient in order to compare results between normal and tumour, but it would appear that the tumour shows loss of Y.

DISCUSSION

Cytogenetic and molecular genetic studies have shown adult anaplastic astrocytoma and GBM have an increased cytogenetic complexity and acquire specific genetic aberrations, including loss of heterozygosity, amplification and numerical chromosome aberrations, when compared to lower grade astrocytoma (Bigner, Mark et al, 1990). The present study has examined chromosome abnormalities in sixty-four astrocytoma from children and young adults in order to determine novel non-random genetic changes, to establish whether paediatric astrocytoma differs from its adult counterpart and to determine whether any CNAs could be used as prognostic markers. The study was biased towards pilocytic astrocytoma and diffuse astrocytoma due to the rarity of the higher grade tumours in the paediatric population and also due to the previous publication of data pertaining to malignant astrocytoma from the same laboratory (Warr, Ward et al, 2001). The present study found genetic alterations to be infrequent in paediatric astrocytoma; in fact 80% of tumours had no detectable CNAs. The most frequent abnormalities (gain of 4q and 7) were only seen in five cases. There were eleven regions of high copy number amplification seen in five of these astrocytoma, a greater frequency than was observed in the ependymoma in this study.

The regions of alteration seen in these astrocytoma will be discussed in relation to previous findings. These findings will be then be discussed in the light of the alterations seen in adult astrocytoma. The CNAs will then be discussed as potential prognostic markers and markers of malignancy. High copy number amplifications will be discussed separately to low-level gains. A summary of previously reported cytogenetic alterations in paediatric astrocytoma can be seen in Figure 5.29

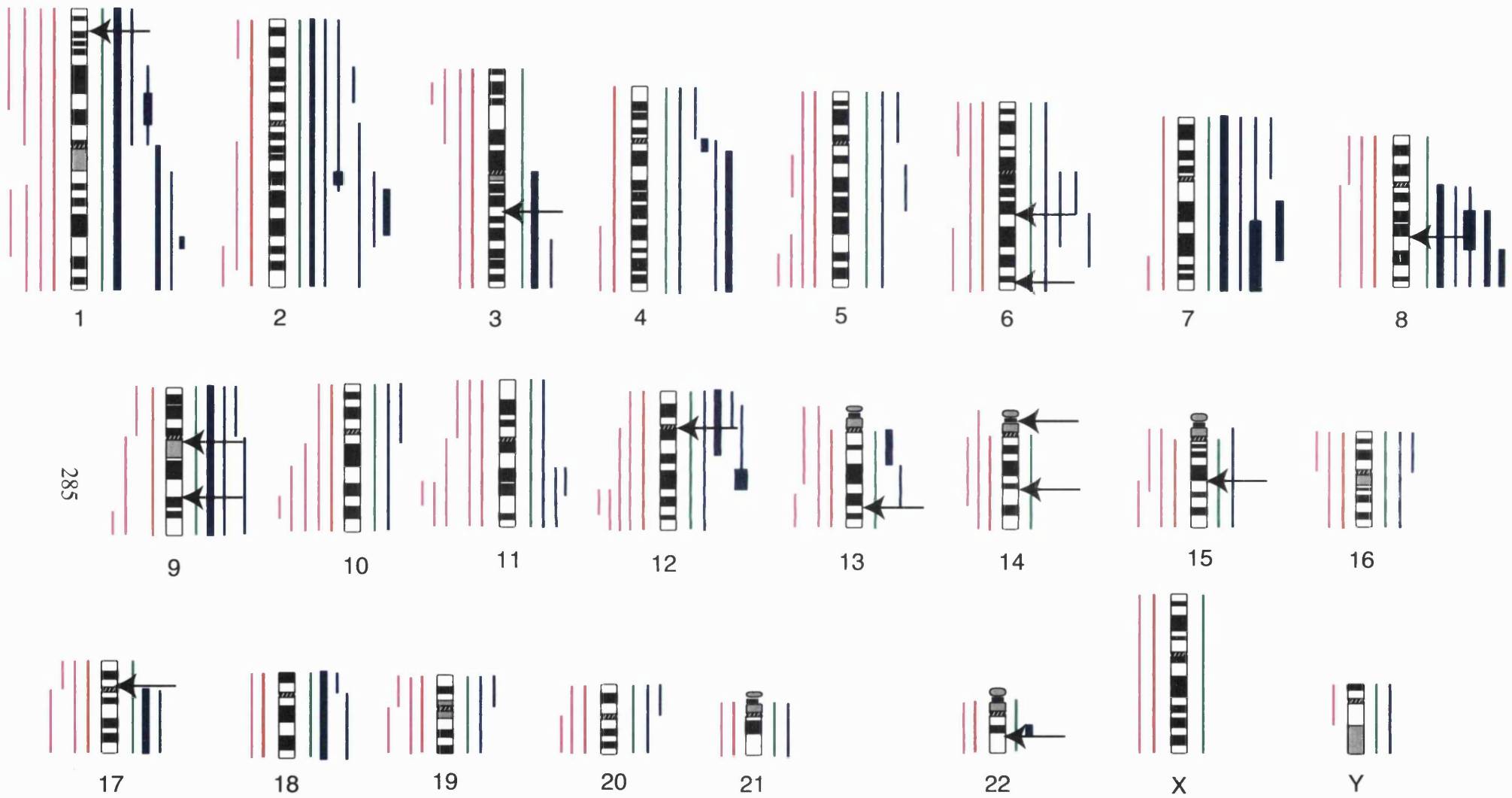


Figure 5.29 : Summary of previously reported abnormalities in paediatric astrocytoma. Red lines indicate regions of deletion as determined cytogenetics; green lines show regions of gain as determined by cytogenetics. Pink lines indicate regions of loss determined by CGH; blue li indicate regions of gain determined by CGH; bold blue lines indicate regionsof high copy number amplification identified by CGH. Arrows indic translocation breakpoints (Griffin, 1988; Karnes, 1992; Sawyer, 1992; Vagner-Capodano, 1992; Neumann, 1993; Fujii, 1994; Schrock, 1994; Agamano 1995; White, 1995; Sainati, 1996; Schrock, 1996; Bhattacharjee, 1997; Bigner, 1997; Wernicke, 1997; Maruno, 2000; Kucerova, 2000; Sanoudou, 20 Shlomit, 2000; Rickert, 2001; Roberts, 2001; Warr, 2001; Orr, 2002; Hirose 2003).

Chromosome 1 abnormalities.

Gain of chromosome 1p was seen only in the diffuse astrocytoma in this study. Two tumours showed gain of 1p, including a region of high copy number amplification at 1p32-34 seen in IN699. Loss of 1p was also an infrequent event, present in only two tumours. Gains of chromosome 1 have been reported in high grade astrocytoma and brain stem astrocytoma (Vagner-Capodano, Gentet et al, 1992; Karnes, Tran et al, 1992; Agamanolis and Malone, 1995; Bigner, McLendon et al, 1997). Reported structural abnormalities of 1p in astrocytoma include add(1)(p11) in a pilocytic astrocytoma and inv(1)(p11p36) in a GBM (Fujii, Hongo et al, 1994; Roberts, Chumas et al, 2001). CGH studies have also shown gains of chromosome 1 in paediatric high grade astrocytoma (Schröck, Thiel et al, 1994; Warr et al, 2001; Rickert et al, 2001).

Within this region of gain on 1p are a number of potential candidate genes. The oncogene *JUN* is located at 1p31-32 and the protein encoded by this gene acts directly to activate gene transcription in response to cell stimulation (Marx, 1988). The product of the *FOS* oncogene co-operates with the *JUN* product in fostering gene transcription. The *TFS1* gene has been localised to 1p22.1-pter and the *RAB3B* gene, a member of the *RAS* oncogene family, localised to 1p31-32 (Stoler & Bouck, 1985; Rousseau-Merck, Zahraoui et al, 1991). Alterations of this region appear to be associated with higher grade tumours suggesting that the gene(s) in this region are likely to be involved with progression rather than tumour initiation.

Monosomy 1 has been reported in recurrent diffuse astrocytoma and malignant astrocytoma (Bigner et al, 1997; Bhattacharjee et al, 1997; Wernicke et al, 1997). CGH analyses have shown loss of 1p in two cases of pilocytic astrocytoma, three anaplastic astrocytoma and three GBM (Shlomit et al, 2000; Maruno et al, 2000; Rickert et al, 2001; Warr et al, 2001). Structural abnormalities involving loss of 1p in astrocytoma include del(1)(p22) in a low-grade astrocytoma, del(1)(p32) in a pilocytic astrocytoma, del(1)(p11) in a recurrent anaplastic astrocytoma, del (1)(p21) in a GBM, t(1;14)(p36;q24) in a diffuse astrocytoma and del (1)(p32) in a

GBM (Griffin et al, 1988; Sainati et al, 1996; Bigner et al, 1997; Wernicke et al, 1997; Orr et al, 2002).

Potential target genes in this region of loss on 1p have been discussed in detail in the ependymoma chapter, but include the *p73* gene at 1p36, *MTS1* (malignant transformation suppression 1) at 1pter-p22.1, *ID3* (inhibitor of DNA binding 3) at 1p36, *NBS* at 1p36, *TNFR2*, *DAN*, *CDC2L1* and *FRAP1* (FKBP12-rapomycin associated protein) that map to 1p36, *MOM1* at 1p35, *HKR3* (human Kruppel-related 3 gene), and *RIZ* (retinoblastoma binding protein) (Knuutila et al, 1999; Miozzo et al, 2000).

Gain of chromosome 2

Four tumours in this study had gain of chromosome 2. Three tumours had alterations of 2q and one tumour had gain of 2p. IN2240, a GBM, had a region of high copy number amplification at 2q24-32. The minimum overlapping region of gain was 2q21-32.

Gain of chromosome 2 has been reported in five anaplastic astrocytoma and five paediatric GBM (Karnes et al, 1992; Fujii et al, 1994; Agamanolis and Malone, 1995; Bigner et al, 1997; Bhattacharjee et al, 1997; Roberts et al, 2001). CGH analyses have also reported gain of the same region in pilocytic astrocytoma and malignant astrocytoma (Shlomit et al, 2000; Warr et al, 2001). Only one structural alteration of chromosome 2 in paediatric astrocytoma has been reported, der(2)t(1;2)(q22;q37) in a brain stem GBM (Neumann et al, 1993).

In the present study, gain of 2q occurred in only the diffuse astrocytoma and GBM. In previous reports, gain of 2q is seen most often in malignant astrocytomas and there have been no reports of diffuse astrocytoma with gains of chromosome 2. There are a number of genes that map within the region of gain at 2q21-32 that may have a role in tumourigenesis. These include *I-TRAF*, *FRZB-1*, and *GRB14* (Rothe et al, 1996; Leyns et al, 1997; Shen et al, 2002). More details of these genes can be found in the ependymoma section of this thesis.

Gain of 4q

Gain of 4q was seen in five tumours in the present study. In four cases it occurred in conjunction with gain of 6q (IN1524, IN2774, IN1566 & IN2240) and

was seen as a sole abnormality in the other case (IN2788). Gain of 4q was seen in all grades of astrocytoma suggesting it is an early event in astrocytoma formation. The minimum overlapping region was 4q13-26.

Trisomy 4 has been reported at low frequency in all grades of paediatric astrocytoma (Karnes et al, 1992; Neumann et al, 1993; Agamanolis and Malone, 1995; White et al, 1995; Schröck et al, 1996; Bhattacharjee et al, 1997; Bigner et al, 1997). CGH analyses have shown high copy number amplification of 4q in anaplastic astrocytoma and GBM (Schröck et al, 1994; Rickert et al, 2001). Low level gains of 4q have been reported in seven GBM and two pilocytic astrocytoma (Schröck et al, 1994; Shlomit et al, 2000; Warr et al, 2001; Rickert et al, 2001). There are no reports of structural alterations involving 4q in paediatric astrocytoma.

In most instances, gain of 4q is reported with either gain of 5q or 6q, or in some instances, both of these alterations. This suggests that an interaction between genes located on these chromosomes may be responsible for the formation of these tumours.

The human *FGF-5* gene maps to 4q21 and is a growth factor with similar properties to those of acidic and basic FGFs (Zhan et al, 1988). FGF-5 is expressed in adult brain where it serves as a continually released trophic factor for neurons and glia (Haub et al, 1990). Increased expression of this gene may lead to increased cell proliferation in the brain. Another gene that maps to 4q21 is *ABCG2*, a member of the ABC superfamily of membrane transporters. *ABCG2* may serve a protective function by preventing the entry of toxins into the cell, as well as playing a role in regulating stem cell differentiation (Ejendal and Hrycyna, 2002). When *ABCG2* is overexpressed it has the ability to confer resistance to anthracyclines, mitoxantrone, bisantrene, and the camptothecins topotecan and SN-38. Positive immunostaining for ABCG2 has been reported in a wide range of human malignancies including glioma (Diestra et al, 2002). The role of this gene in paediatric astrocytoma has not been reported but it is possible it may be involved in the resistance of these tumours to some chemotherapeutic agents. The *TSPAN5* gene has been localised to 4q23 and is a member of the tetraspanin family that are associated with adhesion receptors of the integrin family (Berditchevski, 2001). It has been proposed that tetraspanins can influence

cell migration by one of three mechanisms; firstly by modulating integrin signalling, secondly by the compartmentalisation of integrins on the cell surface and thirdly by directing intracellular trafficking and recycling integrins (Berditchevski, 2001).

Gain of chromosome 5

Three tumours in the present study had gain of chromosome 5. IN1524 and IN2240 had gain of 5q and IN3032 had gain of 5p. The minimum region of gain on 5q is 5q14-23. Trisomy 5 has been reported in pilocytic astrocytoma and anaplastic astrocytoma (Karnes et al, 1992; Neumann et al, 1993; Agamanolis and Malone, 1995; Bigner et al, 1997; Bhattacharjee et al, 1997; Sanoudou et al, 2000; Kucerova et al, 2000; Roberts et al, 2001; Orr et al, 2002).

Gain of 5q has been reported in other CGH analyses of paediatric astrocytoma. Two cases of pilocytic astrocytoma have shown gain of 5q21, a case of GBM with gain 5q11-21 and five further GBM with gain 5q14-21 (Schröck et al, 1994; Shlomit et al, 2000; Rickert et al, 2001; Warr et al, 2001). An anaplastic astrocytoma has been reported with both gain of 5p and 5q11-23. Gain 5q14-23 has been shown in three further cases of anaplastic astrocytoma (Rickert et al, 2001). Structural abnormalities of chromosome 5 include der(5)(5;?)(p14;?) in diffuse astrocytoma (Griffin et al, 1992). There are no reports of gain of 5q as a sole abnormality, and this alteration is usually seen in tumours with multiple chromosome abnormalities.

PAM has been mapped to the 5q14-21 region and has been associated with prostate adenocarcinoma and hyperplasia of the lung (Antonacci et al, 1995; Martinez et al, 1996).

Chromosome 6 alterations

Four tumours had gain of 6q, with a minimum overlapping region of 6q12-16. All of the tumours with gain of 6q also had gain of 4q. Gain of 6q was seen in all grades of astrocytoma except the pilocytic astrocytoma. IN2587, a pleomorphic xanthoastrocytoma had loss of 6q.

Trisomy 6 has been observed at low frequency in all grades of paediatric astrocytoma (Karnes et al, 1992; Neumann et al, 1993; Agamanolis and Malone,

1995; White et al, 1995; Schröck et al, 1996; Bigner et al, 1997; Bhattacharjee et al, 1997; Sanoudou et al, 2000; Kucerova et al, 2000; Roberts et al, 2001). Gain of 6q12-16 has been reported in pilocytic astrocytoma, anaplastic astrocytoma and GBM (Schröck et al, 1994; Shlomit et al, 2000; Warr et al, 2001; Rickert et al, 2001). Structural abnormalities of chromosome 6 in astrocytoma include t(6;9)(q16;q22) in anaplastic astrocytoma, t(6;17)(q21;p11.2) in a GBM and t(6;8)(q27;q21.3) in a low grade astrocytoma (Neumann et al, 1993; Agamanolis and Malone, 1995).

Monosomy 6 has been reported in malignant astrocytoma and in a recurrent diffuse astrocytoma (Agamanolis and Malone, 1995; Bigner et al, 1997; Bhattacharjee et al, 1997; Wernicke et al, 1997; Roberts et al, 2001). Loss of 6q24-ter has been shown in three anaplastic astrocytoma and five GBM (Rickert et al, 2001). *ROS1* and *TTK* both map to the region of gain on 6q seen in these astrocytoma. These genes are described in the ependymoma section.

Gain of chromosome 7

Five tumours in the present study had gain of chromosome 7, including three tumours with high copy number amplification. Gain of 7q occurred more frequently than gain of 7p. One tumour (IN699) had gain of the whole chromosome and IN3032 had amplification of 7p and gain of 7q. Gain of 7q was seen as a sole abnormality in two pilocytic astrocytoma (IN324 & IN2017). The minimum overlapping regions were 7p12-21 and 7q21-36.

Gain of chromosome 7 has been reported at low frequency in all grades of paediatric astrocytoma (Griffin et al, 1988; Vagner-Capodano et al, 1992; Karnes et al, 1992; Schröck et al, 1994; Agamanolis and Malone, 1995; White et al, 1995; Sainati et al, 1996; Schröck et al, 1996; Bhattacharjee et al, 1997; Bigner et al, 1997; Sanoudou et al, 2000; Shlomit et al, 2000; Kucerova et al, 2000; Warr et al, 2001; Rickert et al, 2001; Roberts et al, 2001; Orr et al, 2002). Structural abnormalities involving chromosome 7 include i(7q)(q10) in recurrent anaplastic astrocytoma and t(7;22)(q21;q13.2) in a diffuse astrocytoma(Griffin et al, 1992; Wernicke et al, 1997).

The gene encoding the CDK6 protein maps to 7q21-22. CDK6 has catalytic protein activity and can form complexes with members of the cyclin D family.

CDK6 can be inhibited by both p16 and p15. Overexpression of *CDK6* may mimic mutation of p16/p15 inhibitors and override their function (Cavenee et al, 1993). Another gene that maps to 7q21 is *HGF*, which encodes the hepatocyte growth factor and has been associated with tumour cell differentiation in oesophageal squamous cell carcinoma (Yen et al, 2001).

Chromosome 9 alterations

Three tumours had alterations of chromosome 9. This included a diffuse astrocytoma (IN699) with a region of high copy number amplification at 9q32-34 and gain of the remainder of the q arm, a diffuse astrocytoma with gain of 9p (IN1524) and a pleomorphic xanthoastrocytoma with loss of 9p (IN2587).

Conventional cytogenetic analyses have reported gain of chromosome 9 in all grades of paediatric astrocytoma, albeit at low frequency (Karnes et al, 1992; Vagner-Capodano et al, 1992; Neumann et al, 1993; Bigner et al, 1997; Bhattacharjee et al, 1997; Roberts et al, 2001). CGH analyses have shown gain of chromosome 9 in pilocytic astrocytoma, metastatic pilocytic astrocytoma, diffuse astrocytoma and GBM (Schröck et al, 1996; Sanoudou et al, 2000; Shlomit et al, 2000; Warr et al, 2001). Reported structural alterations of chromosome 9 include inv(9)(p11q12) in a low grade optic nerve astrocytoma, add(9)(p11), t(9;14)(p21;q32.3), t(9;14)(p11;p11) and der(9)t(5;9)(q11;q11) in GBM, i(9q), and der(9)t(9;?)(p21;?), der(9)t(9;17)(q34;q21) in anaplastic astrocytoma(Karnes et al, 1992; Neumann et al, 1993; Agamanolis and Malone, 1995; Bigner et al, 1997; Bhattacharjee et al, 1997). The fact that gain of chromosome 9 is seen in all grades of astrocytoma suggests it is an early event in tumourigenesis. This was also true of the ependymoma in the present study.

There have been infrequent reports of monosomy 9 in paediatric astrocytoma (Fujii et al, 1994; Agamanolis and Malone, 1995; Bigner et al, 1997; Bhattacharjee et al, 1997; Kucerova et al, 2000). CGH analyses have shown loss of 9q in anaplastic astrocytoma and GBM and loss of 9p23-pter in GBM (Schröck et al, 1994; Rickert et al, 2001; Warr et al, 2001). del(9)(p21) was observed in one GBM (Neumann et al, 1993). Deletions of 9p encompassing the *CDKN2A/B* loci occur frequently in adult astrocytoma. The infrequency of this alteration in paediatric

astrocytoma suggests these tumours arise via a different pathway from the adult tumours.

Gain of chromosome 12

Three tumours had gains of chromosome 12. In two cases the gain was confined to 12q, and in one case gain was confined to the short arm and included a region of high copy number amplification at 12p11.2. The minimum region of overlap on 12q was 12q22. None of the tumours showed loss of chromosome 12.

Overrepresentation of chromosome 12 has been reported in four cases of pilocytic astrocytoma, a low grade spinal astrocytoma, five cases of anaplastic astrocytoma and one GBM (Vagner-Capodano et al, 1992; Karnes et al, 1992; Neumann et al, 1993; Agamanolis and Malone, 1995; White et al, 1995; Bigner et al, 1997). Previous CGH analyses have shown six malignant astrocytoma with gains of chromosome 12. In two cases this also included regions of high copy number amplification at 12q13-pter and 12q15-21 (Rickert et al, 2001; Warr et al, 2001). The only structural alteration observed is add(12)(q15) in pilocytic astrocytoma (Roberts et al, 2001). The findings of the present study combined with that of previous data suggests gain of 12q is an infrequent event in paediatric astrocytoma, and that when it does occur it is an early event. Amplification and overexpression of multiple genes from 12q13-13 have been reported in a subset of adult astrocytoma (Reifenberger et al, 1993; Reifenberger et al, 1994). This gene cluster includes *MDM2, GLI, SAS, CDK4* and *A2MR*. Other genes with oncogenic potential have also been mapped to the 12q13-14 regions, including *WNT1, ERBB3* and *CDK2*. In one study, 50% of adult GBM with amplification of 12q13-14 also have deletions of 12q proximal and distal to the amplification site (Reifenberger et al, 1995). In the present study gain of 12q was not accompanied with 12q loss, again suggesting paediatric astrocytoma are a different entity to adult astrocytoma.

Amplification of 12p has also been observed in osteosarcoma and involved the genes *CCND2, ETV6* and *KRAS2* (Gisselsson et al, 2002). *CCND2* maps to 12p13 and is known to interact with cyclin-dependent kinases, such as CDK4, to promote RB1 phosphorylation (Kamb, 1995). This in turn leads to the release of the transcription factor E2F and transition from G_1 to S phase. Therefore, co-

amplification of *CCND2* and *CDK4* may promote cellular proliferation in a synergistic fashion (Gisselsson et al, 2002).

Chromosome 13 alterations

Three tumours had alterations of chromosome 13. In two cases (IN1524 and IN2774) this involved gain of 13q, with a minimum overlapping region of 13q21-24. IN1524 also had a region of high copy number amplification at 13q21-31. IN699 showed loss of chromosome 13 that spanned the whole chromosome.

Cytogenetic analyses have reported gain of 13 all grades of paediatric astrocytoma at a low frequency (Karnes et al, 1992; Vagner-Capodano et al, 1992; Wernicke et al, 1997; Bigner et al, 1997; Bhattacharjee et al, 1997; Roberts et al, 2001). Previous CGH analyses have shown gain of 13q in two cases of pilocytic astrocytoma and five cases of malignant astrocytoma (Shlomit et al, 2000; Roberts et al, 2001; Warr et al, 2001). The only structural abnormality involving chromosome 13 is add(13)(q34) observed in a recurrent high grade astrocytoma (Bigner et al, 1997). A number of genes have been identified in the region of gain at 13q21-24 that may be important in cancer including, *PIBF1, LMO7, KIAA1008, KLF12* and *KLF5*. Further details on these genes can be found in the ependymoma section.

Monosomy 13 has been reported infrequently in all grades of paediatric astrocytoma (Agamanolis and Malone, 1995; Bigner et al, 1997; Bhattacharjee et al, 1997; Kucerova et al, 2000). Loss of 13q13-qter has been observed in a pilocytic astrocytoma, and loss of the whole chromosome in one anaplastic astrocytoma and two GBM (Maruno et al, 2000; Rickert et al, 2001). Structural alterations include del(13)(q22) in a paediatric GBM (Agamanolis and Malone, 1995). The *RB1* gene maps to chromosome 13q14 and is altered in about a third of adult high grade astrocytoma (James et al, 1988; Henson et al, 1994). Mutations in *RB1* can have the same consequences as amplification of *CDK4/CDK6* or mutation of *CDKN2A/B*. In adult astrocytoma these events occur almost exclusively of each (Ueki et al, 1996).

Loss of 16p

Three tumours had loss of 16p and in one case (IN2240) this was accompanied by loss of 16q. No minimum region of loss could be estimated as loss spanned the entire short arm of the chromosome. Cytogenetic analyses of paediatric astrocytoma have reported monosomy 16 in a recurrent diffuse astrocytoma, one anaplastic astrocytoma and three GBM (Griffin et al, 1988; Agamanolis and Malone, 1995; Bhattacharjee et al, 1997; Bigner et al, 1997; Wernicke et al, 1997). Previous

CGH analyses have observed both loss of 16 and loss of 16p in around 20% of paediatric malignant astrocytoma (Warr et al, 2001; Rickert et al, 2001).

Loss of 16 was confined to diffuse astrocytoma and GBM in the present study and the same finding has been reported in other analyses of paediatric astrocytoma. This would suggest that candidate genes on 16p are associated with higher grade tumours. The region of deletion on 16p includes the tuberous sclerosis 2 (*TSC2)* gene locus at 16p13.3, which has been discussed earlier in the ependymoma section.

Chromosome 19 alterations

Four diffuse astrocytoma had alterations of chromosome 19. IN276 had gain of 19p and IN699 had high copy number amplification of 19q. IN380 and IN1524 showed monosomy 19.

Trisomy 19 has been reported at low frequency in pilocytic astrocytoma and malignant astrocytoma (Karnes et al, 1992; Schröck et al, 1994; Agamanolis and Malone, 1995; Schröck et al, 1996; Bigner et al, 1997; Bhattacharjee et al, 1997; Roberts et al, 2001). Structural alterations of chromosome 19 include add(19)(q13) in pilocytic astrocytoma and GBM, and add(19)(p13.3) in GBM (Bigner et al, 1997; Kucerova et al, 2000; Roberts et al, 2001).

Monosomy 19 has been reported in all grades of paediatric astrocytoma at low frequency (Agamanolis and Malone, 1995; Wernicke et al, 1997; Bigner et al, 1997). Previous CGH analyses have observed monosomy 19 in one case of pilocytic astrocytoma and four cases of malignant astrocytoma (Shlomit et al, 2000; Warr et al, 2001). One GBM has also been reported with loss of 19p (Warr et al, 2001).

There is evidence for the presence of a tumour suppressor gene on 19q involved in the progression of adult astrocytoma. LOH at 19q has been reported in around 40% of high-grade astrocytoma, and the minimum region of deletion has been mapped to 19q13.3 (Von Deimling et al, 1992; Smith et al, 2000). There are a number of genes on 19q that may have a role in tumourigenesis including, *PAX, XRCC1, ERCC1* and *ERCC2, p190-A,* and *PLA2G4C.*

Other incidental alterations

A number of alterations were only seen in one tumour including monosomy 4, loss of 6q, loss of 10q, monosomy 15, gain/loss of chromosome 18, gain/loss 20q and monosomy 22.

Alterations of the sex chromosomes

Two tumours in this study had alterations involving the X chromosome. IN2240, a GBM showed gain of X, and IN380, a diffuse astrocytoma, showed loss of the short arm of X. Gain of X has been observed in all grades of astrocytoma (Griffin et al, 1988; Karnes et al, 1992; Agamanolis and Malone, 1995; Schröck et al, 1996; Bigner et al, 1997; Roberts et al, 2001; Orr et al, 2002). Structural abnormalities of chromosome X have only been noted in paediatric GBM and include add(X)(q11) and der(X)t(X;9)(q28)(q12) (Agamanolis and Malone, 1995; Roberts et al, 2001). Loss of X has been reported in a case of recurrent diffuse astrocytoma and one GBM (Wernicke et al, 1997).

Y chromosome specific PCR identified one GBM (IN1786) with loss of Y. Loss of Y has been observed previously in paediatric low-grade astrocytoma (Griffin et al, 1992). The are no reported structural abnormalities of Y in paediatric astrocytoma.

Regions of high copy number amplification

Gene amplification is a frequent event in various types of human tumours, the most well documented of which are amplification of *n-myc* in neuroblastoma and *Erb/Neu* in breast cancer. Gene amplification is a mechanism for up-regulation of

the expression of critical genes such as oncogenes or drug resistance genes. It is known that some amplicons consist of multiple amplified genes, including 7p12 and 20q11-13. CGH is a useful technique for detecting regions of amplification and can assign genes in double minutes to an exact chromosomal location. The height of the peak on the CGH profile does not quantitatively reflect the level of amplification as the fluorescent signal spreads over a region of the chromosome that is actually larger than the length of the amplicon (Kallioniemi et al, 1992). The total amount of amplified DNA (amplicon size times level of amplification) has to be at least 2Mb in order for it to be detected using CGH (Kallioniemi et al, 1994).

Five astrocytoma in this study had regions of high copy number amplification that were located at eleven chromosomal regions. Only two tumours (IN2017 and IN2240) shared a common region of amplification at 7q31. The most amplicons were seen in diffuse astrocytoma, where three tumours showed multiple regions of amplification. IN699 had six amplicons at 1p32-34, 1q21, 9q32-34, 17p, 19q and 20. IN1524 had a single amplicon at 13q21-31 and IN3032 had two amplicons at 7p11-14 and 12p11.2. One pilocytic astrocytoma, IN2017 had a single amplicon at 7q22-31. One GBM, IN2240, had two regions of amplification at 2q24-32 and 7q31-33. There were no amplicons in the anaplastic astrocytoma.

Chromosome 1

Both amplicons on chromosome 1 were seen in the diffuse astrocytoma IN699. The amplicon at 1q21 was also part of the amplicon seen in three ependymoma in this study and this has been discussed previously. Amplification on 1p has been reported in a number of other malignancies at low frequency including, pancreatic carcinoma, leiomyosarcoma, medulloblastoma, and adult GBM multiforme (Solinas-Toldo et al, 1996; Mohapatra et al, 1998; Derré et al, 2001; Michiels et al, 2002).

Chromosome 2

IN2240, a GBM had a region of amplification at 2q24-32. High copy number amplification within this region has been reported in hepatoblastoma, oesophageal carcinoma, rhabdomyosarcoma as well as in three cases of paediatric malignant astrocytoma (Du Plessis et al, 1999; Hu et al, 2000; Rickert et al, 2001; Bridge et al, 2002). This region contains several genes that have potential oncogenic features. These include *ITRAF* (tumour necrosis factor receptor-associated factor) which functions as a general regulator of TRAF-mediated signalling events (Rothe et al, 1996), and *FRZB*, a secreted antagonist of WNT signalling (Leyns et al, 1997; Wang et al, 1997).

Chromosome 7

Amplification was seen on both arms of chromosome 7 in astrocytoma. IN3032, a diffuse astrocytoma, had a region of amplification at 7p11.1 IN2017, a pilocytic astrocytoma, and IN2240, a GBM, shared a common region of amplification at 7q31.

Amplification on 7p has been reported previously in adult grade GBM at high frequencies (Nishizaki et al, 1998), as well as in three paediatric malignant astrocytoma (Rickert et al, 2001). Other malignancies with 7p amplicons include Barrett's adenocarcinoma (Walch et al, 2000). The *EGFR* gene maps to 7p12, precluding it from being the target of the amplicon seen in IN3032. In a recent study of adult GBM, thirteen tumours has amplicons on 7p that excluded the *EGFR* gene (Liu et al, 2000). Twelve of these tumours showed amplification of loci distal to *EGFR* and one tumour had exclusive amplification of proximal loci. From this data it would appear the target for the 7p amplicon in some GBM lies in the region between the ESTs *SGC32574* and *WI-18503*.

Amplification of 7q31 has been reported previously in six paediatric malignant astrocytoma (Warr et al, 2001; Rickert et al, 2001). Adult GBM, choriocarcinoma, adenocarcinoma, germ cell tumours and mixed oligoastrocytoma have been reported with amplicons at 7q21-31 (Weber et al, 1996; Rao et al, 1998b; Kros et al, 1999; Ahmed et al, 2000; Walch et al, 2000; Luk et al, 2001). Located in this region is the *PTPRZ1* gene, which has been shown to be highly up-regulated in

breast carcinoma (Forozan et al, 2000). *CAPZA2* has been mapped to 7q31 and is known to regulate the growth of actin filaments by binding at their barbed end. Also located in this region is *MET*, which encodes a transmembrane growth factor receptor tyrosine kinase whose ligand is the hepatocyte growth factor/scatter factor. Amplification of *MET* is thought to contribute to the formation of glial tumours by the elevated expression of proteins that are involved in mitogenic signalling pathways. One study has shown *MET* to be amplified in 20% of adult GBM (Fischer et al, 1995). However, in a series of 121 adult GBM, no *MET* amplifications were found suggesting *MET* is only amplified at low levels in GBM (Liu et al, 1998). Studies of chromosome 7 abnormalities in hereditary papillary renal cell carcinoma have shown that mechanism of *MET* activation is via mutation and nonrandom duplication of the mutant *MET* allele in trisomy 7 (Zhuang et al, 1998). Three out of eleven GBM have been shown to have duplication of one copy of the *MET* gene, but none of these tumours had *MET* mutations (Moon et al, 2000). Three further GBM with *MET* amplification were screened with eight DNA markers for the region of amplification at 7q31. The domain most commonly amplified in these GBM was restricted to the flanking regions of *MET*. Amplification of exons localised close to *MET* and *CAPZA2* were also seen in GBM (Mueller et al, 1997). There are a number of other, as yet unidentified, genes located within this amplicon at 7q31.

Chromosome 9

IN699, a diffuse astrocytoma had a region of amplification at 9q32-34. Amplification spanning the whole of chromosome 9 has been reported in two cases of paediatric anaplastic astrocytoma (Rickert et al, 2001). This region of amplification spans the *ABL1* gene, *VAV2* (Rho-family guanine-nucleotide exchange factor) gene, and *PBX3* genes (Sanoudou et al, 2000). An EGF-like gene, *EGFL5*, is located at 9q32-33.3 and has been shown to be specifically expressed in the cerebellum (Nakayama et al, 1998). Other neoplasms with amplifications in this region include squamous cell carcinoma of the oesophagus, diffuse B-cell lymphoma and male germ cell tumours (Rao et al, 1998a; Rao et al, 1998b; Shinomiya et al, 1999).

Chromosome 12

A region of amplification at 12p11.2-12 was observed in the diffuse astrocytoma IN3032. Amplification in this region has previously been reported in many other human cancers (see Table 5.4).

Malignancy	No. Cases	Amplicon	Reference
Pancreatic carcinoma	1/27	12p13	Solinas-Toldo et al. (1996)
Gastric cardia adenocarcinoma	1/20	12p11.2	Van Dekken et al. (2001)
Ovarian carcinoma	2/27	12p11-13	Sonoda et al. (1997)
Testicular germ cell tumours	2/15	12p11.1-12.1	Mostert et al. (1996)
Ovarian germ cell tumours	2/23	12p	Bussey et al. (1999)
Seminoma	2/6	12p11.2-12	Rao et al. (1998b)
Oral squamous cell carcinoma	1/19	12p11	Oga et al. (2001)
Oesophageal squamous cell carcinoma	1/29	12p11.2-12	Shinomiya et al. (1999)
Head and neck squamous cell carcinoma	2/13	12p	Speicher et al. (1995)
Fibrosarcoma	1/34	12p12-pter	Schmidt et al. (2002)

Table 5.4: Reported chromosome 12 amplicons in human malignancies.

Amplification at 12p has been reported in adult GBM and in one paediatric GBM (Schröck et al, 1994; Warr et al, 2001). The region of amplification at 12p includes several candidate loci. The *KRAS* proto-oncogene, is overexpressed in 10% of

ovarian carcinoma and has been shown to be associated with malignant progression in endometrial cancer (Sonoda et al, 1997; Koul et al, 2002). The region containing *KRAS* is overrepresented in pancreatic carcinoma and activating mutations in codon 12 are particularly frequent (Heidenblad et al, 2002). Other potential targets in this region include the genes for CD4 and CD8, *TNFR1*, *CCND2* and *FGF6*. *CCND2* has several E boxes, the common binding site of the MYC family proteins, in its promotor region. This gene has been found to be significantly dysregulated in testicular germ cell tumours (Skotheim et al, 2002). The region containing the *CCND2* gene is the most frequently amplified region in osteosarcoma (Gisselsson et al, 2002). This gene is known to be amplified in other malignancies and has also been reported in two GBM and one anaplastic astrocytoma (Büschges et al, 1999). The coamplification of *CCND2* with *CDK4* may promote cellular proliferation in a synergistic manner (Gisselsson et al, 2002). *FGF6* expression has been shown to be increased in prostatic tumours and promote the proliferation of transformed prostatic epithelial cells via paracrine and autocrine mechanisms (Ropiquet et al, 2000). The gene *DEC2* is a putative target of the 12p amplicon in pancreatic cancer (Heidenblad et al, 2002). This gene has recently been cloned and shown to have 97% homology to the basic helix-loop-helix domain of *DEC1* (Fujimoto et al, 2001), which encodes a transcription factor thought to be involved in control of proliferation and differentiation.

Chromosome 13

IN1524, a diffuse astrocytoma, had a region of amplification at 13q21-31. Previous CGH studies have shown one paediatric GBM astrocytoma with an amplicon at 13q11-14 (Warr et al, 2001). Amplification of the 13q21-31 region has been reported in oesophageal carcinoma and follicular lymphoma (Du Plessis et al, 1999; Neat et al, 2001). In lymphoma, amplicons on 13q cluster in the 13q21-33 region and seem to be a result of tandem repeats on a der(13) chromosome seen by G-banding (Neat et al, 2001).

Chromosome 17

IN699, a diffuse astrocytoma, had a region of amplification that spanned the entire short arm of chromosome 17. Amplification of 17q has previously been reported in two cases of paediatric GBM (Rickert et al, 2001) but there have been no reports of amplification of 17p. Amplification of 17p has been reported in other malignancies, details of which can be seen in Table 5.5.

CGH analysis has identified amplification of 17p11-p12 in 20% of glial tumour cell lines (Mohapatra et al, 1995). In comparison amplification of 17p12 has been found in 45% of osteosarcoma. In both tumour types, the presence of the amplification event correlates with aggressive clinical behaviour (Hulsebos et al, 1997).

Malignancy	No. cases	Amplicon	References
Rhabdomyosarcoma	1/45	17p11-12	(Bridge et al, 2002)
Osteosarcoma	2/18 4/10	17p 17p11.2-p12	(Zielenska et al, 2001) (Wolf et al, 1999)
Ovarian germ cell tumours	2/23	17p	(Bussey et al, 1999)
Fibrous histiocytoma	1/3	17p	(Wolf et al, 1999)
Chondrosarcoma	1/3	17p11.2-p12	(Wolf et al, 1999)
Leiomyosarcoma	1/27	17p	(Derré et al, 2001)

Table 5.5: Published reports of amplification of chromosome 17 in human cancers.

Chromosome 19

The entire long arm of chromosome 19 was amplified in tumour IN699. Amplification involving 19q13.1-13.2 has been reported in ovarian carcinoma and gastric cardia adenocarcinoma (Thompson et al, 1996; Sonoda et al, 1997; Perinchery et al, 1999), as well as in GBM (Nishizaki et al, 1998). Amplifications involving the whole of chromosome 19 have been observed in anaplastic oligodendroglioma and anaplastic mixed oligoastrocytoma (Kros et al, 1999). No

paediatric astrocytomas have previously been reported with amplification of chromosome 19. This region of amplification might involve *cyclin E*, which is a candidate gene in the 19q12 amplicon in adenocarcinoma of the gastro-oesophageal junction. Another candidate gene in this region is *AKT2*. *AKT2* encodes a protein serine-threonine kinase that has been implicated in phosphtidylionsitol-3-OH kinase (PI 3-K) signalling. This gene has been found to be amplified and overexpressed in 20% of pancreatic ductal adenocarcinoma (Ruggeri et al, 1998) and 80% of ovarian cancer cell lines have increased *AKT2* copy number (Thompson et al, 1996). Overactivation of *AKT2* by gene amplification or overexpression could intensify signalling through the PI 3-K pathway or may act independently of growth factor mediated signals and contribute to the dysregulation of cell growth (Ruggeri et al, 1998). The *CCNE* gene has been mapped to 19q13.3 and is a regulatory subunit of cyclin-dependent kinase that regulates entry into S phase of the cell cycle (Richter et al, 2000). This gene has been found to be amplified in breast cancer and a small number of urinary bladder cancers (Hoglund et al, 1998; Richter et al, 2000). Other genes in this region that may have a role in tumourigenesis include *FOSB* (19q13.3), *BCL3* (19q13) and *SUPT5H* (19q13.1-13.2).

Chromosome 20

IN699 showed amplification of the whole of chromosome 20. Amplification at 20p11.2-12 has been observed in a GBM that arose from a grade II oligoastrocytoma and in one case of oligoastrocytoma (Giannini et al, 1999; Brunner et al, 2000). There are two genes located in this region, *PCNA* (proliferating cell nuclear antigen) and *PYGB* (human brain glycogen phosphorylase), that may be of some importance in glial tumours. *PCNA* has been shown to be expressed at higher levels in GBM than in pilocytic astrocytoma in two recent studies by oligonucleotide microarray analysis (Rickman et al, 2001).

Gene amplification of 20q has previously been reported in a wide range of malignancies (see Table 5.6), including one case of paediatric anaplastic astrocytoma (Rickert et al, 2001).

Several genes have been identified in the 20q region, including *AIB1* (steroid receptor co-activator), whose altered expression may contribute to the development of steroid dependent cancers, *ZNF217* (Zinc finger transcription factor), which is associated with instability and immortalisation in tumour cells, *STK15*, a centromere-associated serine/threonine protein kinase, *BTAK*, a serine/threonine kinase gene, and nonreceptor tyrosine phosphate gene (*PTPN1*) that has been reported to be overexpressed in 72% of breast carcinomas (Wiener et al, 1994; Anzick et al, 1997; Gray and Collins, 2000; Hu et al, 2000). *STK15* overexpression activates an unknown oncogenic pathway that involves centrosome amplification and results in the mis-segregation of chromosomes. Bladder cancers that have high levels of *STK15* amplification also exhibit pronounced aneuploidy as well as having a more aggressive clinical behaviour (Sen et al, 2002). *ZNF217* has been defined as the core of the 20q amplicon in breast cancer (Tanner et al, 1996). This gene is not amplified alone in ovarian cancer suggesting the involvement of other genes in this region (Tanner et al, 2000). *PTPN1* has been shown to be highly amplified in 29% of ovarian tumours and is overexpressed in both breast and ovarian cancer (Wiener et al, 1994; Tanner et al, 2000). *BTAK* is amplified in 21% of sporadic ovarian cancers and is also the target of the 20q13 amplicon in breast and colon cancers (Tanner et al, 2000). *CYP24*, which encodes for vitamin D24 hydroxylase, is located at 20q13.2 and has been implicated as a potential oncogene in breast carcinoma (Albertson et al, 2000). Another candidate is *MYB12* a 20q13, which encodes a transcription factor and has a role in cell cycle progression. The *hD54* locus is at 20q13.2-13.3 and is a member of the D52-like protein family that are thought to have a role in cell proliferation (Balleine et al, 2000).
The data available for amplification of 20q suggests the coamplification of separate regions at 20q may be the molecular mechanism behind gene amplification in this region.

It is possible that at least some of the amplifications seen in the tumours in this study may involve the coamplification of several genes mapping to the same region. The exact molecular mechanism behind coamplification is unknown but

may involve multiple chromosomal breaks and unbalanced translocations. Coamplification also suggests synergy between genes up regulated by gene amplification; for example the amplified genes may share a common pathway in growth regulation (Gisselsson et al, 2002).

Malignancy	Frequency	Amplicon	Reference
Oesophageal squamous cell carcinoma	3/29	20q13.2-13.3	(Shinomiya et al, 1999)
Gastric carcinoma	3/62	20q11.2-12	(Guan et al, 2000)
Breast carcinoma	15/38 22/41	20q13 20q11	(Forozan et al, 2000) (Tanner et al, 1996)
Ovarian carcinoma	3/27 13/24	20q12-qter 20q	(Sonoda et al, 1997) (Tanner et al, 2000)
Ovarian germ cell tumours	2/23	20	(Bussey et al, 1999)
Male germ cell tumours	1/34	20q11.2-12	(Rao et al, 1998b)
Oligodendroglioma	1/15	20q13	(Kros et al, 1999)
Squamous cell carcinoma of the lung	1/9	20q12-q13.2	(Luk et al, 2001)

Table 5.6: Previous reports of 20q amplification in human cancers

Pleomorphic Xanthoastrocytoma

There was a single case of pleomorphic xanthoastrocytoma (PXA) in this study. PXA is a low-grade glioma that corresponds to WHO grade II. These are rare tumours, accounting for less than 1% of all astrocytic tumours, and hence only a limited number of genetic studies have been performed on PXA (Yin et al, 2002). The majority of PXA have a favourable prognosis with post-operative follow-up periods of up to 18 years being reported (Palma et al, 1985; Ohta et al, 1999). In

some instances, PXA may transform into a malignant phenotype, usually a GBM, with the associated poor prognosis (Kepes et al, 2000).

Molecular genetic analyses of PXA have identified two tumours (one adult and one paediatric) with mutations of the *p53* gene (Paulus et al, 1996). These mutations occurred in codons 220 and 292, outside of the conserved domain of *p53*. Such mutations have not been reported previously in astrocytoma. However, in a study of sixty-two PXA, three tumours had *p53* mutations. (Kaulich et al, 2002). One adult recurrent PXA has also been reported with *EGFR* amplification (Paulus et al, 1996). *EGFR* amplification occurred without LOH at 10q, which differs from the pattern of expression seen in GBM not related to PXA. PXA with *p53* mutations did not have deletion of *CDKN2A* or *CDKN2B* and there was no amplification of *CDK4*, *MDM2* or *EGFR* (Kaulich et al, 2002). Recently CGH has been used to analyse three cases of PXA (Yin et al, 2002). This report showed gain of 7 and loss of 8p in two cases. Gains of 2p, 4p, 12, 15q and 19, as well as loss of 9p, 10p and 13q were each seen in one case. Only one of these tumours was from a paediatric patient, and this tumour had loss of 8p as a sole abnormality. The PXA in the present study showed two abnormalities, loss of 6q and 9p. It is not known whether the region of loss on 9p involves the *CDKN2A/B* genes. Taken together these findings suggest that the genetic aberrations in PXA differ from those typically associated with diffuse astrocytoma.

Comparison of paediatric and adult astrocytoma

Cytogenetic analyses of adult astrocytoma have shown low-grade astrocytomas to have simple numerical alterations, such as trisomy 7 or loss of a sex chromosome, whereas in the higher grade astrocytoma the pattern of abnormalities becomes more complex. In adult GBM consistent, abnormalities have been reported including extra copies of chromosome 7, loss of 9p, monosomy 10, deletion of 22q and double-minute chromosomes (Bigner et al, 1988). Several of the genes associated with these alterations have been identified, including *CDKN2A/B* deletion in tumours with loss of 9p, *PTEN/MMAC1* and *DMBT1* as the targets of 10q deletion and *EGFR* amplification which is typically

present as double minute extra-chromosomal elements (Mollenhauer et al, 1997; Steck et al, 1999).

In comparison to the wealth of studies on the genetic abnormalities in adult astrocytoma, little is known about the genetic pathogenesis of paediatric astrocytoma. Cytogenetic studies of paediatric astrocytoma have been hindered by the low incidence of primary high-grade astrocytoma in the paediatric population. Both cytogenetic and LOH analyses of paediatric astrocytoma have failed to show many of the characteristic alterations seen in adult tumours, and have also been unable to identify consistent numerical and structural alterations in these tumours (Bigner et al, 1997). The same was true in the present study and the findings from this study will be compared to what has been previously reported in adult astrocytoma.

EGFR gene amplification

In the present study only two diffuse astrocytoma had alterations of 7p. This included gain of the entire chromosome in IN699, and high copy number amplification of 7p11-14 in IN3032. The *EGFR* gene maps to 7p12 and has been implicated as an important element mediating neuroglial cell growth and differentiation (Bredel et al, 1999). *EGFR* amplification in paediatric malignant astrocytoma occurs infrequently. In total six diffuse astrocytoma, forty-three anaplastic astrocytoma and seventy-five GBM have been screened for *EGFR* amplification (Raffel et al, 1999; Bredel et al, 1999; Cheng et al, 1999; Sung et al, 2000; Kraus et al, 2002). Only two GBM have been found to have *EGFR* amplification (Bredel et al, 1999). However, elevated immunoreactivity for *EGFR* has been observed in seven out of nineteen anaplastic astrocytoma and twenty-three out of fifty-seven GBM (Cheng et al, 1999; Bredel et al, 1999; Maruno et al, 2000; Sung et al, 2000). This suggests that expression of the EGFR protein is enhanced by mechanisms other than a simple alteration in gene copy number.

Chromosome 9p deletions

One diffuse astrocytoma (IN699) and a pleomorphic xanthoastrocytoma (IN2587) showed loss of 9p, the location of the *CDKN2A/B* genes. Studies investigating the status of these genes in paediatric astrocytoma have found deletions in

approximately 10% of anaplastic astrocytoma and GBM, compared to 50-60% of adult cases (Jen et al, 1994; Raffel et al, 1999; Newcomb et al, 2000; Sung et al, 2000). Two PXA have been shown to have LOH for markers on 9p, but no inactivation of *CDKN2A/B* has been reported (Kaulich et al, 2002). Alterations of *CDKN2A/ARF* have been reported in one anaplastic astrocytoma and one GBM (Raffel et al, 1999). Deletions of $p14^{ARF}$ and $p16^{INK4a}$ have been reported in two anaplastic astrocytoma and one GBM (Sung et al, 2000). As the transition from low-grade to anaplastic astrocytoma in adults is accompanied by allelic loss on 9p, the lack of 9p deletions in paediatric astrocytoma suggests that paediatric diffuse astrocytoma may progress via a different pathway.

Amplification of *MDM2*

The *MDM2* gene is located at 12q14-15 and none of the tumours in this study showed gain within this region of chromosome 12. The MDM2 protein binds to wild type *p53*, inhibiting its ability to activate transcription. Inactivation of the effect of *p53* can be seen through overexpression of the MDM2 protein (Willert et al, 1995). Overexpression of MDM2 has been reported in 50% of adult GBM (Biernat et al, 1997). MDM2 overexpression has been seen in six anaplastic astrocytoma and seventeen GBM (Raffel et al, 1999; Sung et al, 2000). There have been no reports of *MDM2* amplification in paediatric astrocytoma (Sung et al, 2000; Kraus et al, 2002). The low frequency of MDM2 overexpression and the lack of *MDM2* amplification in paediatric astrocytoma suggests this gene does not have a significant role in the pathogenesis of these tumours and that alterations of 12q may be attributed to another target gene(s) in this region. In one study twelve genes in the region of 12q13-15 were found to be amplified in approximately 15% of astrocytoma. These genes include *GLI*, *WNT1*, *MDM2*, *SAS*, *CDK4*, *OS-4*, *GAS16*, *GAS27*, *GAS41*, *GAS56*, *GAS64* and *GAS89* (Fischer et al, 1996). There have been no reports of amplification of these genes in paediatric astrocytoma.

***RB1* gene deletions**

One diffuse astrocytoma (IN699) showed loss of 13q in the region of the *RB1* gene. Wild type pRB is required for the regulation of cell cycle entry as well as

terminal differentiation of different cell type. Mutation of *RB1* is found in a variety of hereditary and sporadic tumours. As many as 75% of paediatric high grade astrocytoma show expression of the retinoblastoma protein but there have been no reports of gene mutations (Sung et al, 2000). Studies of *RB1* deletions in adult astrocytoma have shown that inactivation is restricted to high-grade tumours suggesting this gene is involved in astrocytoma progression rather than initiation (Henson et al, 1994). The lack of *RB1* mutations in paediatric astrocytoma adds further evidence to the argument that paediatric astrocytoma do not progress to a more malignant tumour via the same pathway as adult astrocytoma.

Loss of chromosome 10q

One diffuse astrocytoma (IN380) had loss of 10q. However, this loss was distal to the location of the *PTEN/MMAC1* gene at 10q23. PTEN negatively regulates the PI3K-Akt pathway, thereby affecting the control of the cell cycle and cell survival (Sasaki et al, 2001). It is possible that *PTEN* status may relate to therapeutic sensitivity in malignant glioma. LOH at 10q23 has been reported in two paediatric anaplastic astrocytoma and three GBM (Cheng et al, 1999). Mutations of *PTEN* have been reported in six paediatric GBM and one anaplastic astrocytoma (Raffel et al, 1999; Cheng et al, 1999; Kraus et al, 2002). The reported mutation rate of *PTEN* in paediatric GBM (around 16%) is similar to that seen in adult secondary GBM.

DMBT1 is located at 10q25.3-26.1 and encodes a protein that participates in epithelial differentiation and immune regulation. *DMBT1* has been proposed as a candidate tumour suppressor gene for GBM, medulloblastoma, lung carcinoma and gastrointestinal carcinoma based on homozygous deletions (Mollenhauer et al, 1997). There is concern surrounding *DMBT1* as a tumour suppressor gene based on lack of experimental evidence linking DMBT1 to tumourigenesis and constitutional hemizygous deletions in a subset of normal individuals, seen as germline polymorphisms (Mollenhauer et al, 2000). Deletions of *DMBT1* have been reported in anaplastic oligodendroglioma (Sasaki et al, 2001). LOH at 10q25 has been reported in three paediatric anaplastic astrocytoma and five GBM

(Blaeker et al, 1996; Cheng et al, 1999). Other candidate genes in the 10q25 region include *TNFRSF6*, *MXII* and *h-neu*.

Involvement of *p53*

There was one diffuse astrocytoma (IN3032) with monosomy 17. The *p53* gene maps to 17p13.3 and has a role in several cellular processes including the cell cycle, response to DNA damage, apoptosis, cell differentiation and neovascularisation. Overexpression and mutations of *p53* in paediatric astrocytoma have been reported in around a third of cases (Chozick et al, 1994; Hayes et al, 1999; Raffel et al, 1999; Cheng et al, 1999; Sung et al, 2000; Pollack et al, 2002). Other studies have found no mutations or a low incidence of mutations of the *p53* gene in paediatric astrocytoma (Fults et al, 1992; Litofsky et al, 1994; Walker et al, 2001). It has been suggested that *p53* mutations are associated with increasing tumour malignancy but the similar frequency of mutations in all grades of paediatric astrocytoma would suggest that this is not the case.

Loss of chromosome 22

Approximately 30% of adult astrocytoma have been reported to display loss of 22q, suggesting the presence of a chromosome 22 tumour suppressor gene (Rey et al, 1992; Rey et al, 1993; Ino et al, 1999). LOH at 22q13.3 is associated with the more malignant astrocytoma (anaplastic astrocytoma and GBM), suggesting that loss of this region is associated with astrocytoma progression (Oskam et al, 2000). In the present study only one diffuse astrocytoma (IN1524) had loss of 22q. Monosomy 22 or deletions of 22 have been reported infrequently in paediatric astrocytoma of all grades (Griffin et al, 1988; Karnes et al, 1992; Neumann et al, 1993; Schröck et al, 1994; Blaeker et al, 1996; Shlomit et al, 2000; Rickert et al, 2001; Warr et al, 2001).

Potential prognostic markers

Low-grade paediatric astrocytoma usually has an excellent prognosis but a number of children with these tumours will have tumour recurrence that requires multiple treatment and exposes the child to potential toxicity from

therapy. A number of children will die from their tumour, and in some cases survival at ten years may be less than 50%. A number of studies have shown a survival advantage for patients with pilocytic astrocytoma compared to those with diffuse astrocytoma (Gjerris and Klinken, 1978; Vagner-Capodano et al, 1992; Orr et al, 2002). Paediatric malignant astrocytoma have been shown to have a poor prognosis (Shinoda et al, 1989; Vinchon et al, 2001). In the present study tumour histology was a very strong indicator of prognosis. Patients with GBM had a very poor survival (21% at 10 years), compared to those patients with pilocytic astrocytoma (100% at 10 years).

The effect of age on survival in patients with astrocytoma is a controversial issue. Tumour progression and recurrence has been associated with a young age in some studies (Davis and Joglekar, 1981; Ilgren and Stiller, 1987; Vinchon et al, 2001). Other studies have shown no difference in survival or the risk of progression between infants and older children (Pollack et al, 1995; Smoots et al, 1998; Fisher et al, 2001). In the present study, there was no difference in survival between children aged 3 years and younger and older patients. Neither was there a difference in survival when the patients were divided into three groups based on age: under 3 years, 4-18 years and 19 years and older. This may be due to the relatively small number of samples in the study that came from young adults. It is also difficult to get up to date clinical information and there was no follow up available for nine of the patients in the study, including four of the young adults. With more accurate clinical data it might be possible to see a relationship between patient age and survival.

The extent of surgical resection has been found to be an important prognostic indicator in previous studies of paediatric astrocytoma. Patients who have undergone complete resection are less likely to have tumour recurrence than those who have only had partial removal of tumour (Pencalet et al, 1999). Even children with malignant astrocytoma have been shown to have a better survival if a complete removal of tumour is achieved (Vinchon et al, 2001). In the present study there was no difference in survival between those patients who had received total resection compared to those with incomplete resection.

Studies on the use of post-operative treatment for children with astrocytoma have shown chemotherapy to be effective when used in conjunction with radiotherapy for the treatment of malignant astrocytoma and unresectable low-grade astrocytoma (Sposto et al, 1989; Duffner et al, 1996; López-Aguilar et al, 2000; Tassi et al, 2001). Other studies have reported no increase in patient survival with the use of chemotherapy (Wolff et al, 2000)(Held-Feindt et al, 2002). Radiotherapy has been shown to be useful in increasing survival in patients with low-grade astrocytoma who have had incomplete tumour removal (Dewit et al, 1984; Ilgren and Stiller, 1986; Dirven et al, 1997; Reddy and Packer, 1999). However, in other studies, radiotherapy has been shown not to influence outcome (Marsa et al, 1973; Garcia and Fulling, 1985; Wallner et al, 1988).
In the present study, the best survival was seen in those patients who had received no post-operative therapy (100% at 10 years). Patients who had received post-operative chemotherapy had the worst survival. These findings may be a reflection of the normal progression of disease. Most of the patients who had received no post-operative therapy had pilocytic or diffuse astrocytoma, where the prognosis is usually quite good and no post-operative therapy is required. Children with more aggressive tumours are usually treated with either radiotherapy and/or chemotherapy, and will still have a poor prognosis compared to children with pilocytic astrocytoma. Patients who had only undergone stereotactic biopsy followed by radiotherapy had a better outcome than those patients who had surgical excision followed by radiotherapy. All of the patients who had biopsy were older patients (aged 24-31 years), whereas the patients who had surgical removal of tumour followed by radiotherapy were under 16 years of age. This might suggest astrocytoma in older patients are more radiosensitive than those seen in younger patients. However, for three of the patients with anaplastic astrocytoma no clinical follow-up data was available, therefore this increased survival for patients undergoing stereotactic biopsy might not be a true reflection of the effect of biopsy on survival.

To date there is little information on the prognostic significance of cytogenetic abnormalities in childhood low-grade astrocytoma. In fact, the majority of

reports have shown normal karyotypes to occur in most instances (Griffin et al, 1988; Thiel et al, 1993; Fujii et al, 1994; Agamanolis and Malone, 1995). In a recent study of twenty-nine low-grade astrocytoma, the presence of copy number aberrations conferred a survival advantage compared to those patients with a normal karyotype, though the difference was not statistically significant. (Orr et al, 2002). In the present study, the presence of a normal karyotype conferred a slight survival advantage in the patients with low-grade astrocytoma (see Figure 5.30). In patients with a normal karyotype survival was 98% at 10 years, compared to 58% in those patients with CNAs. Survival data was not available for four patients with normal karyotypes and one patient with multiple CNAs, though even if this data were available it would be unlikely to change the results.

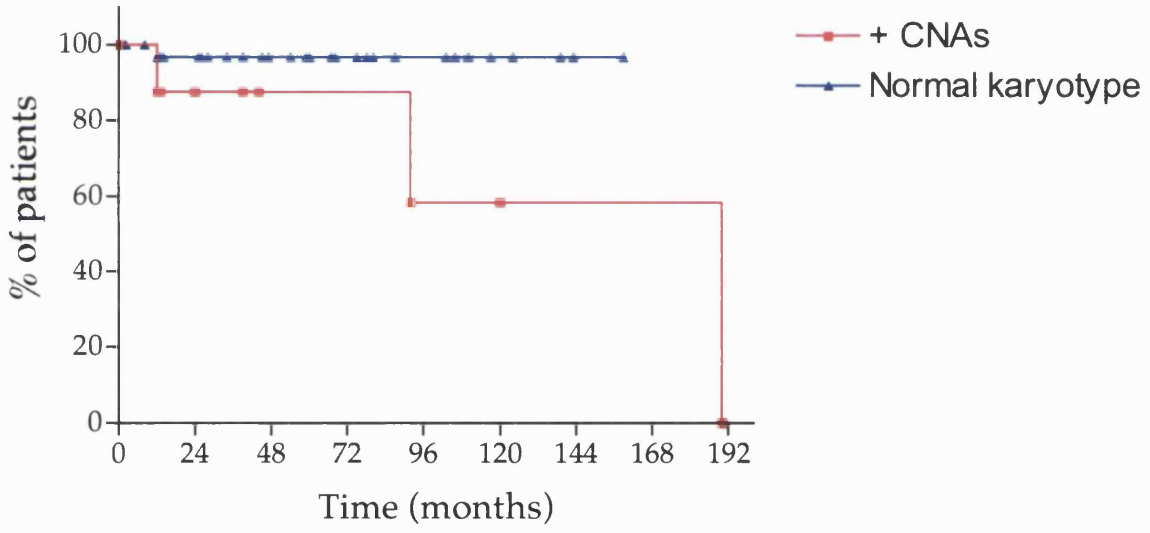


Figure 5.30: The effect of CNAs on survival in patients with low grade (pilocytic and diffuse) astrocytoma. Survival is measured in months from the date of diagnosis. Log-rank test, $p = 0.0706$.

The data for the high-grade astrocytoma in the present study could not be analysed in the same manner, as survival data was not available for a third of the patients in the study.

Four CNAs were seen to be associated with reduced survival in the astrocytoma patients in the present study. These were gain of 2q, 5q, 7 and loss of 16p. However, the validity of these findings is uncertain. Only a small number of tumours had CNAs, and in most cases, the alterations that influenced survival were present in GBM. These findings can not be confirmed as occurring independently of tumour grade. The one exception was gain of chromosome 7, which was seen in all grades of astrocytoma. A greater number of samples would need to be analysed to confirm that these alterations are true prognostic markers. Another reason why the data may be biased is the absence of follow-up data available for ten of the patients in the study, including two patients with multiple CNAs.

In a recent study of adult diffuse astrocytoma, gain of chromosome 7 was found to be significantly associated with shorter survival (Wessels et al, 2002). Gain of chromosome 7 is also associated with shorter survival in adults with malignant astrocytoma (Nishizaki et al, 1998; Kunwar et al, 2001; Burton et al, 2002). However, trisomy 7 has been seen to have no influence on survival in children with pilocytic astrocytoma (White et al, 1995). Trisomy 7 has also be found to correlate with poor survival in patients with prostate carcinoma (Bandyk et al, 1994). Gain of chromosome 7 is associated with the more malignant tumours and with tumours that have the potential to become malignant and it is possible that the patients in the present study with gain of chromosome 7 represent those paediatric tumours that are more aggressive in nature.

In contrast to the finding in the present study that patients with gain of 5q had a poorer survival than those patients with no alteration of 5q, a recent study has reported a prolonged survival for renal cell carcinoma patients with gain 5q (Gunawan et al, 2001).

One chromosome aberration which has been reported as a potential prognostic marker for paediatric astrocytoma is gain of 1q, which has been found to be associated with shorter survival in patients with anaplastic astrocytoma but not GBM (Rickert et al, 2001). Gain of 1q did not influence survival in the patients in

the present study, though this may be due to the low frequency of anaplastic astrocytoma available for analysis.

A small number of studies have looked at the influence of specific gene alterations on survival in children with astrocytoma. A significant association between p53 overexpression and survival has been reported in children with malignant non-brain stem astrocytoma (Pollack et al, 1997; Pollack et al, 2002). In patients with overexpression of p53 (n = 41), PFS at five years was 17% compared to 44% in those patients with low levels of p53 expression (n = 74) (Pollack et al, 2002). EGFR expression and *EGFR* amplification has been investigated in a study of twenty-seven children with malignant astrocytoma (Bredel et al, 1999). This study showed no correlation between EGFR expression and survival. Only two samples showed *EGFR* amplification, so no relationship between survival and amplification could be determined. Bcl-2 expression has also been investigated as a potential prognostic marker in paediatric astrocytoma. Expression of bcl-2 may confer a survival advantage to some cells by preventing cell death or apoptosis from taking place. In a study of twenty-one paediatric astrocytoma survival showed no relationship to the expression of bcl-2 (Newcomb et al, 1997).

Comparison between grade of astrocytoma

In the present study, CNAs occurred infrequently in pilocytic astrocytoma (10%), and were seen at the greatest frequency in anaplastic astrocytoma (40%), but this may be a reflection of the small number of anaplastic tumours in the study. Aberrations in the pilocytic astrocytoma were always in the form of single abnormalities and in 2/3 cases involved gain of 7q. In diffuse astrocytoma, no tumours had sole abnormalities. In diffuse astrocytoma and GBM, tumours multiple aberrations (>6) were accompanied by regions of high copy number amplification. With the exception of IN2017 (pilocytic astrocytoma) and IN1524 (diffuse astrocytoma), at least two regions of amplification were seen in tumours with multiple aberrations. There was no pattern in the alterations seen in the different grades of astrocytoma, and no single CNA could be associated with a particular tumour grade. Chromosome 7 may be an early event in the genesis of

paediatric astrocytoma, as this alteration was seen in all grades of tumour. There appears to be equal heterogeneity both within grades of astrocytoma, and between tumour grades. From the results in this study, it would appear that there is not an individual mechanism of development for each grade of astrocytoma.

In comparison to adult tumours, astrocytoma in children show a different location, a different growth pattern, longer survival times, and a lower tendency for recurrence. The present study combined with the data from previous studies would suggest that paediatric astrocytoma follow genetic pathways that do not fit in with those seen in either adult primary or secondary GBM. It would seem that paediatric astrocytoma is a disease that is genetically distinct to adult astrocytoma.

CHAPTER 6
SUMMARY & CONCLUSIONS

Paediatric glial tumours comprise over half of all childhood brain tumours. Most of these tumours are low-grade tumours that are common in the posterior fossa and diencephalic regions (Packer, 1999). The tumours that comprise this group, astrocytoma and ependymoma, arise from glial cells or the supporting cells of the nervous system. Both ependymoma and astrocytoma shared common regions of alteration, though the ependymoma showed alterations at a much higher frequency than the astrocytoma. The most common region of gain in both tumour types was gain of 4q, seen in twelve ependymoma and five astrocytoma. Gain of 6 was also a frequent finding being seen in ten ependymoma and four astrocytoma. Loss of 16p was the most common region of loss in astrocytoma (two tumours) and was also a frequent finding in ependymoma, being observed in ten tumours. There were other regions of alteration that were common to each group which suggests that there may be similar pathways that lead to the formation of these tumours.

Gains and losses were seen with equal frequency in ependymoma, whereas gains were more common in astrocytoma. This might indicate that overexpression and gene amplification are more common in astrocytoma development than gene deletions or mutations and that both deletion/mutation and gene overexpression are involved in the formation of ependymoma.

In agreement with previously published data, normal karyotypes occurred quite frequently. Seemingly normal karyotypes were seen in nearly half of the ependymoma and 80% of the astrocytoma. Normal karyotypes were seen most frequently (90%) in the pilocytic astrocytoma. The frequency of alterations overall was very low in the astrocytomas, with the most common alteration, gain of 4q, only being seen in five tumours. Despite the low frequency of alterations it was possible to see a pattern in the alterations. Pilocytic astrocytoma had fewer alterations than the glioblastoma, suggesting an increase in genetic instability

with an increase in tumour grade. This trend was not observed in ependymoma, but this may be due to the low frequency of anaplastic ependymoma in this study.

Copy number aberrations occurred more frequently in ependymoma than astrocytoma. Only 20% of astrocytoma had detectable aberrations compared to 49% of ependymoma. Ependymoma had more CNAs per tumour than the astrocytoma. All of the pilocytic astrocytoma with CNAs showed only sole abnormalities, where as sole abnormality occurred infrequently in ependymoma. All of the tumours with high copy number amplification, with exception of IN2017, showed multiple CNAs as well as the region(s) of amplification.

A novel region of amplification was seen in ependymoma at 9p22-pter. This amplicon has not been reported previously in paediatric ependymoma, but has been observed on other malignancies, including squamous cell carcinoma, medulloblastoma and paediatric anaplastic astrocytoma (Nishizaki, Ozaki et al, 1998; Rickert, Strater et al, 2001; Oga, Kong et al, 2001; Eberhart, Kratz et al, 2002). All other amplicons had been reported previously in paediatric astrocytoma and ependymoma.

No single CNA was associated with any clinicopathological feature in the astrocytoma, and the only feature associated with a particular aberration in the ependymoma was tumour recurrence. One of the problems associated with ependymoma is recurrence at the primary tumour site. It would be of great importance therefore to both the neurosurgeon and the neuropathologist if they could predict which patients were more likely to suffer tumour recurrence. In the comparison between primary tumours that had and had not shown recurrence, loss of 16q was found to occur more frequently in tumours with recurrence. This finding has not been reported previously and may be an important prognostic marker. Loss of 16p and gain of 7q were seen more frequently in recurrent ependymoma than primary ependymoma. CNAs were also seen to accumulate between primary and recurrent tumours from the same patient.

There are no definitive markers of survival in use for patients with ependymoma and astrocytoma and one aim of this study was to identify markers, either genetic or clinicopathologic that could be used to predict patient outcome. In ependymoma patients with monosomy 19 showed a better prognosis than those patients with no alterations of chromosome 19. This has not been reported previously in ependymoma. In patients with low grade astrocytoma (pilocytic and diffuse astrocytoma), the presence of CNAs was associated with a poor prognosis, though no individual CNA was found to influence survival in the low grade tumours. In the astrocytoma patients as a whole group, four CNAs were shown to have an influence on survival. Patients with either gain of chromosome gain of 2q, gain of 5q and 7, and loss of 16p had a worse outcome than those patients with no alterations of these chromosomes. Gain of chromosome 7 has been seen to be of prognostic significance in adults with diffuse astrocytoma (Wessels, Twijnstra et al, 2002). There have been no reports of individual chromosome alterations being used as prognostic markers in children with astrocytoma.

In children with ependymoma, treatment with post-operative radiotherapy offered the best survival, whereas in the astrocytoma patients the best survival was seen in patients who had received no post-operative treatment. This finding in the astrocytoma patients may be explained by the fact most of the children who were treated with surgery alone were those patients with pilocytic and diffuse astrocytoma who naturally have a good prognosis. Survival in astrocytoma patients was associated with tumour histology, with those patients with glioblastoma having a worse outcome than those children with pilocytic astrocytoma. Tumour histology could not be used as a marker of outcome in the children with ependymoma. This is probably due to the low numbers of anaplastic ependymoma available for analysis.

The astrocytoma in this study did not show the same characteristic abnormalities that have been reported in adult astrocytoma suggesting they are two different diseases with different genetic backgrounds. Adult astrocytoma show deletions of 9p, 10q and 17, and these alterations occurred infrequently in the paediatric

astrocytoma in this study. Amplification of *EGFR* on 7q and *MDM2* on 12p are another common feature of adult astrocytoma. Gain of 7q did occur quite frequently in the tumours in this study, and amplification of 12p was also observed, but at much lower frequencies than have been reported in adult tumours.

In conclusion this study has found:

- More than half (51%) of ependymoma and 80% of astrocytoma had no detectable copy number aberrations. Sole abnormalities were seen most frequently in pilocytic astrocytoma, and there was an increase in genetic instability with increasing tumour grade in astrocytoma. Gain of 4q and 6q, and loss of 16p were common abnormalities in both ependymoma and astrocytoma, suggesting the same genes may be involved in the development of both tumour types.

- The combination of CNAs seen in ependymoma allowed these tumours to be separated into three groups according to their chromosome abnormalities: those with loss of 1p and associated abnormalities, those with gain of 1q and associated abnormalities, and those tumours with no abnormalities or sole aberrations. This suggests there might be as many as three different genetic pathways that lead to the formation of ependymoma. Due to the low frequency of alterations seen in the astrocytoma, it was not possible to determine if different genetic pathways lead to the formation of different grades of tumour.

- Paediatric astrocytoma do not show the same characteristic abnormalities seen in adult astrocytoma. Deletions of 9p, 10q and 17p were infrequent events and gains of 7q and 12p were seen at lower frequencies than have been reported in adult tumours. This suggests that paediatric astrocytoma and adult astrocytoma are two distinct diseases.

- The presence of monosomy 19 offered a survival advantage to ependymoma patients. Gain of 2q, 5q and 7, and loss of 16p were associated with decreased survival in patients with astrocytoma. Survival was greatest in ependymoma patients who had received some form of post-operative therapy, whereas astrocytoma patients who had received no post-operative therapy had the best outcome. Loss of 16q was associated with the clinical recurrence of tumour in patients with ependymoma.

- Ependymoma do express *MDR1*, but at a lower frequency than has been reported previously, suggesting another mechanism is involved in drug resistance in these tumours.

With regards to the technical aspects investigated in this study, DOP-PCR protocols were seen to differ in their sensitivities for use in CGH. Only one protocol, Huang et al (2000) resulted in CGH profiles with considerable similarity to those produced using conventional CGH methods. All protocols seemed to have difficulty in correctly identifying the patients sex, which may mean other abnormalities may not be detected using this technique. Where possible conventional CGH, using nick translation would be the preferred method for detecting genomic instability.

Of the two methods tested for the extraction of DNA from paraffin embedded tissues, the salting out method described by Jeuken (personal communication) resulted in high molecular weight DNA. The difficulty was in utilising this DNA for CGH analysis. The use of the ULS for labelling the DNA was not successful due to difficulties in obtaining DNA fragments in the correct size range and nick translation also proved difficult due to the range in size of DNA fragments making it difficult to estimate the exact concentration of the DNA. Further investigations are required to optimise the protocol for the use of paraffin embedded section in CGH analysis.

CHAPTER 7
FUTURE WORK

This study has identified a number of tumours that have both losses and gains of the same chromosome arm and also a large number of tumours that may have aberrations that cannot be detected using CGH, such as balanced translocations. Multiplex-fluorescence *in situ* hybridisation (m-FISH) can be used to identify simple and complex translocations, interstitial deletions and insertions, chromosomal aneusomies and double minutes (Speicher, Ballard et al, 1996). The technique is based on combinational labelling strategies, which allows the simultaneous visualisation of several different probes, labelled with different fluorochromes. For example for simultaneous labelling of all 24 human chromosomes five fluorochromes can be combined in the manner outlined in Table 7.1 to give each chromosome a spectral signature (Speicher et al, 1996). This technique will be used to further analyse the tumours from this study that have a large number of CNAs in order to identify breakpoint regions and narrow down regions that harbour genes that are involved in paediatric glial tumours. The diffuse astrocytoma IN699, has recently been analysed using m-FISH and has been found to have a number of translocations and an insertion. The colour profile for this tumour can be seen in Figure 7.1.

A recent publication has shown evidence of an environmental agent (or agents) in the aetiology of paediatric astrocytoma and ependymoma. The data suggests two putative mechanisms that include pre-natal or perinatal exposure to an environmental agent that leads to the formation of pilocytic (grade I) astrocytoma or ependymoma, and a post-natal exposure that contributes to the onset of astrocytoma or ependymoma after a short and constant latent period (McNally et al, 2002)

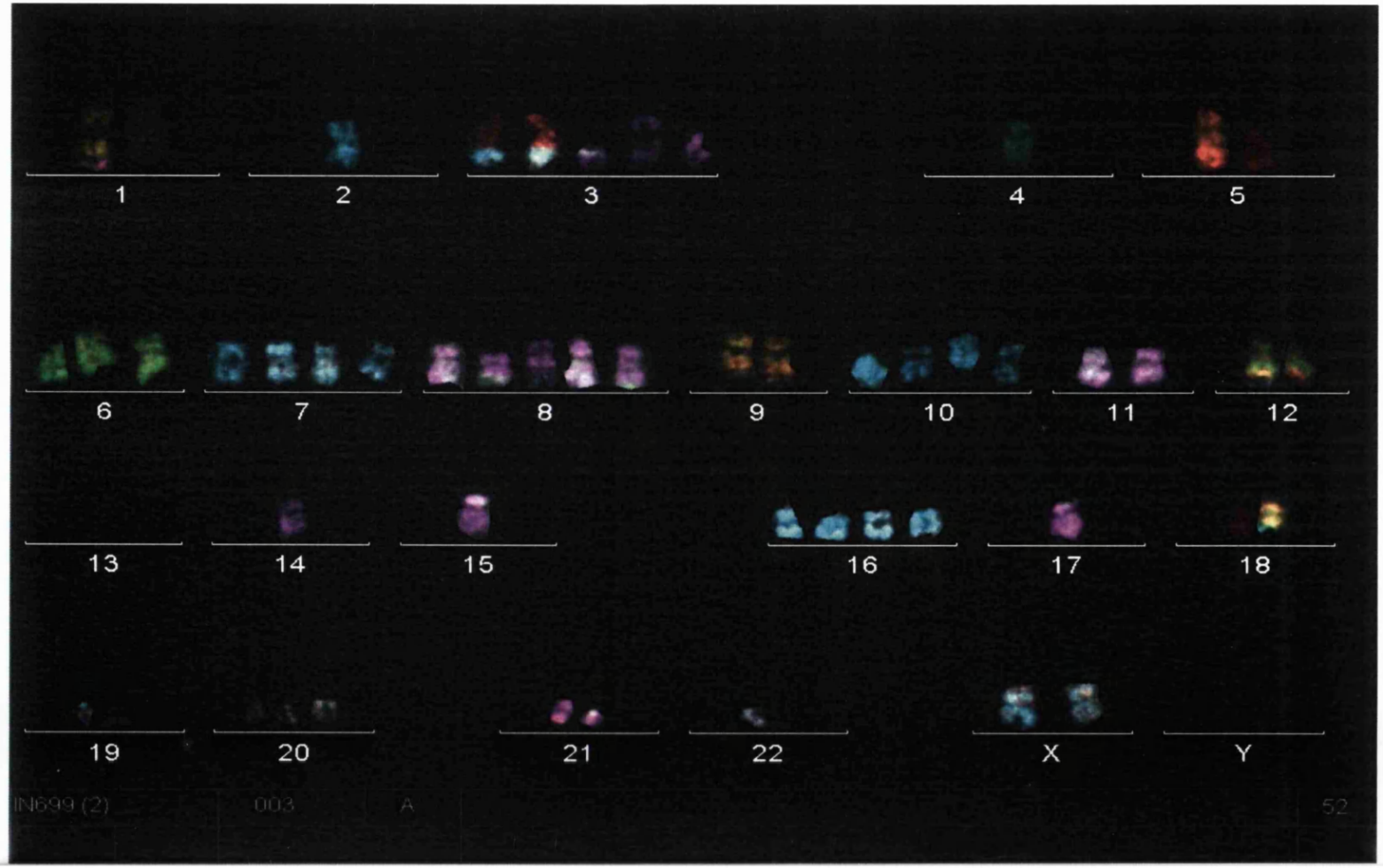
	Fluoroscein	Cy3	Cy3.5	Cy5	Cy7
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					
21					
22					
X					
Y					

Table 7.1: Example of a combinatorial labelling scheme for the simultaneous labelling of the 24 human chromosomes. (Speicher et al, 1996)

.

Human herpes viruses have been implicated in the pathogenesis of several human malignancies although the exact mechanisms of oncogenesis are not fully understood. Recently, human cytomegalovirus (HCMV) nucleic acids and proteins have been found in a high percentage of low and high grade astrocytoma and expression of early and delayed HCMV gene products have also been found (Cobbs et al, 2002).

Figure 7.1: M-FISH composite of diffuse astrocytoma IN699. This tumour shows multiple alterations including extra copies of chromosome 3, 6, 7, 8, 10, 16 and 20. There are a number of translocations; (1q;15), (3q;14), (8q;13), (9q;8), and (12q; 13). There are also two insertions at 8q(4) and 8q(17).



HCMV gene products can transactivate other oncogenic viruses that are associated with gliomas, such as the JC virus (Del Valle et al, 2001). SV40 Tag has been found in ependymoma, choroid plexus tumours, astrocytoma, glioblastoma and in medulloblastoma at varying frequencies (Zhen et al, 1999; Weggen et al, 2000). In these two studies it was interesting to note that all of the SV40 positive tumour samples were from American patients and all the negative samples were from German patients, supporting the suggestion that there are epidemiological differences in SV40 infection. Further research into the role of infectious agents in the aetiology of human brain tumours may help lead to a better understanding of the biology of these tumours and may help identify novel treatments or prevention strategies. The presence of absence of SV40 sequences in the samples used in this study will be investigated in the future using a PCR based analysis in order to determine whether this agent has a role in these particular tumours.

One area of increasing interest is the methylation status of some genes in human cancers. Changes in DNA methylation status are important for regulating gene expression and inducing chromosome instability. CpG islands are regions of the genome with a high density of CpG dinucleotides. These regions are gene-associated and occur throughout the genome. In normal cells, CpG island methylation plays an important role in regulating gene expression in a tissue-specific manner (Shi et al, 2002). In tumours, abnormal hypermethylation of these CpG islands in the promotor regions of some genes is linked to changes in gene silencing, especially in tumour-suppressor genes. Other regions of the genome, such as the pericentromeric heterochromatin, can be subject to hypomethylation, another methylation abnormality that is associated with cancer (see Figure 7.2). In some instances, both changes can be found in the same tumour suggesting that hypermethylation events at specific genes and hypomethylation of pericentromeric heterochromatin can occur concurrently (Tsuda et al, 2002). There has been renewed interest in the application of demethylating drugs for the treatment of cancer. This is because DNA hypermethylation is a common epigenetic event that can be reversed to a demethylated state in neoplasms. In order to exploit this, it is necessary to understand how genes respond to

demethylation. O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that removes promutagenic DNA lesions at the O^6 position of guanine by transferring the alkyl group to one of its own cysteine residues without base excision. MGMT activity varies between tumours and approximately 20-25% of human tumours lack detectable MGMT activity. Tumour cell lines with a methyl repair-deficient (Mer-) phenotype have been shown to have methylation of CpG sequences in the promotor region of the *MGMT* gene. MGMT promotor methylation has been detected in 48% of low grade astrocytomas and 75% of secondary glioblastoma that had progressed from low grade astrocytomas (Nakamura et al, 2001). The resulting loss of *MGMT* expression as a result of promotor methylation appears to be associated with an increased frequency of *TP53* mutations, in particular G: C → A: T transitions.

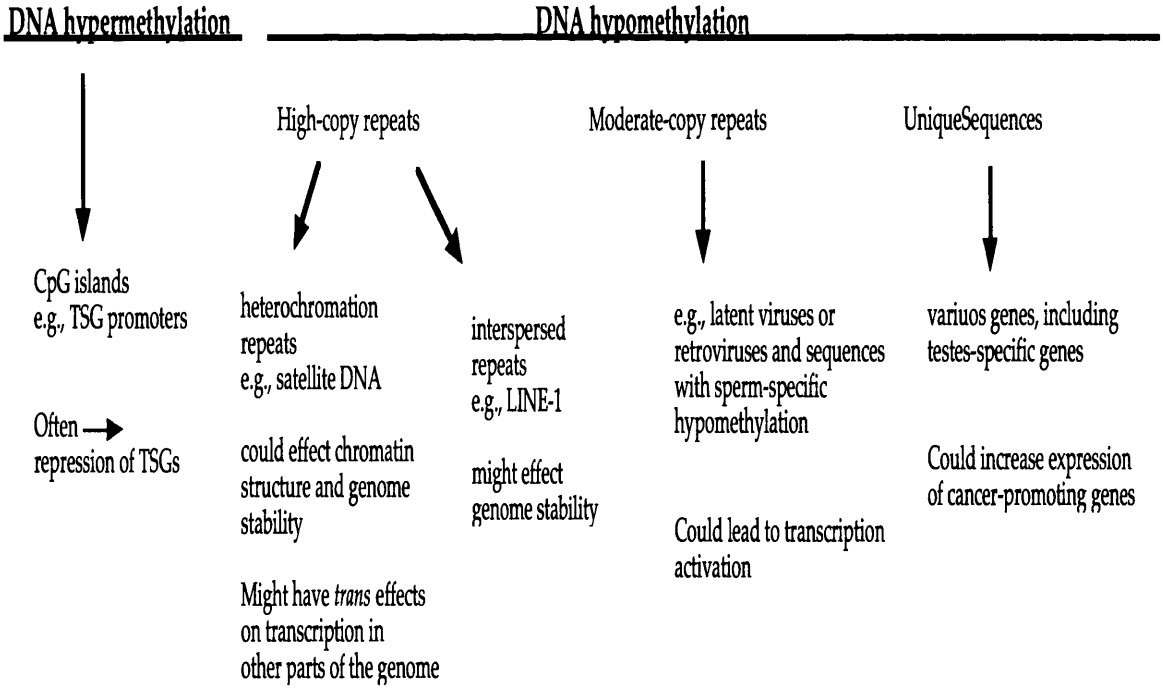


Figure 7.2: A summary of the most frequent types of sequences affected by cancer-specific DNA hypermethylation or hypomethylation. TSG = tumour suppressor gene (Ehrlich, 2002).

There is little information to date about methylation in childhood malignancies. Recently the methylation status of nine genes (*p16^{INK4A}*, *MGMT*, *GSTP1*, *RASSF1A*, *APC*, *DAPK*, *RARβ*, *CDH1* and *CDH13*) was examined in

paediatric solid tumours. The most frequently methylated gene was found to be *RASSF1A*, though frequency of methylation varied with tumour type. The highest frequency of methylation was seen in medulloblastoma (88%), rhabdomyosarcoma (61%), retinoblastoma (59%), neuroblastoma (52%) and Wilms tumour (42%) (Harada et al, 2002). *RASSF1A* is located on chromosome 3p21.3 and has been shown to function as a tumour suppressor gene in lung cancer. Three cases of ependymoma have been analysed for methylation of the *CDKN2A* gene and no promotor methylation was found (Bortolotto et al, 2001).

The methylation status of the *MGMT* gene is currently under investigation in paediatric grade IV astrocytomas. Initial microarray data has shown this gene to be overexpressed in paediatric ependymoma and therefore the gene will not be methylated in this group of tumours (Suarez-Merino, 2002, personal communication). The initial findings of the methylation studies suggest that as many as 20% of paediatric malignant astrocytoma exhibit methylation of *MGMT*.

Another rapidly growing field of research involves the use of high throughput technologies such as arrays. DNA arrays on microchips are being developed for numerous applications, including the ability to screen genes rapidly for mutations/single nucleotide polymorphisms and to study patterns of gene expression, amplification or deletion on a large scale (Daigo et al, 2001). The main limitations of chromosome-based CGH are the limits of its resolution. Replacing metaphase chromosomes as the substrate on to which aberrations are mapped with arrays of well-mapped cloned nucleic acid sequences can eliminate some of the limitations of CGH. Arrays are constructed using a robot to place cloned DNA in high-density arrays on a glass substrate. There may be tens of thousands of clones on an array, each representing a single gene. Microarrays exploit the preferential binding of complementary single-stranded nucleic acid sequences (Brazma and Vilo, 2000). Work has recently begun in our laboratory analysing benign and anaplastic ependymomas using RNA microarray technology in an attempt to identify novel genes involved in the initiation and progression of these tumours. This initial study has identified a cluster of underexpressed genes on chromosome 22 which will be further investigated using quantitative PCR

to determine whether these genes have been lost in paediatric ependymoma, and give further information as to a candidate for the chromosome 22 ependymoma tumour suppressor gene. The results from the present study and from an earlier publication (Warr et al, 2001) identified a number of astrocytoma with regions of high copy number amplification. These tumours are currently under investigation using cDNA microarray technology in order to identify the targets of these regions of amplification.

In order to establish the mechanisms behind the poor response of some paediatric glial tumours to treatment with chemotherapy, it will be necessary to investigate the role of other genes (such as *ABCP*) known to be involved in drug resistance in order to determine the mechanism by which drug resistance arises in this group of tumours.

REFERENCES

Abe T, Mori T, Wakabayashi Y, Nakagawa M, Cole S, Koike K, Kuwano M, Hori S (1998) Expression of multidrug resistance protein gene in patients with glioma after chemotherapy. Journal of Neuro-Oncology 40:11-18

Adra C, Donato J-L, Badovinac R, Syed F, Kheraj R, Cai H, Moran C, Kolker M, Turner H, Weremowicz S, Shirakawa T, Morton C, Schnipper L, Drews R (2000) *SMARCAD1* a novel human helicase family-defining member associated with genetic instability: Cloning, expression and mapping to 4q22-23, a band rich in breakpoints and deletion mutants involved in several human diseases. Genomics 69:162-173

Agamanolis D, Malone JM (1995) Chromosomal abnormalities in 47 pediatric brain tumours. Cancer Genetics & Cytogenetics 81:125-134

Ahmed M, Kim K, Haddad B, Berchuck A, Qumsiyeh M (2000) Comparative genomic hybridisation studies in hydatidiform moles and choriocarcinoma: Amplification of 7q21-31 and loss of 8p12-p21 in choriocarcinoma. Cancer, Genetics & Cytogenetics 116:10-15

Albarosa R, Colombo B, Roz L, Magnani I, Pollo B, Cirenei N, Giani C, Fuhrman Conti A, DiDonato S, Finocchiaro G (1996) Deletion mapping of gliomas suggests the presence of two small regions for candidate tumour suppressor genes in a 17cm interval on chromosome 10q. American Journal of Human Genetics 58:1260-1267

Albertson D, Ylstra B, Segraves R, Collins C, Dairkee S, Kowbel D, Kuo W-L, Gray J, Pinkel D (2000) Quantitative mapping of amplicon structure by array CGH identifies *CYP24* as a candidate oncogene. Nature Genetics 25:144-146

Albright A, Guthkelch A, Packer R (1986) Prognostic factors in pediatric brain-stem gliomas. Journal of Neurosurgery 65:751-755

Alers, JC, Rochat, J, Krijtenburg, P-J, van Dekken, H, Raap, AK, and Rosenberg, C. Universal linkage system: an improved method for labelling archival DNA for comparative genomic hybridisation. Genes Chromosomes & Cancer 25, 301-305. 99.

Allen J, Siffert J, Hukin J (1998) Clinical manifestations of childhood ependymoma: A multitude of syndromes. Pediatric Neurosurgery 28:49-55

Allikmets, R, Schriml, LM, Hutchinson, A, Romano-Spica, V, and Dean, M. A human placenta specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Research 58, 5337-5339. 98.

American Brain Tumour Association (2002) A primer of brain tumours: A patients reference manual. Chapter 1: p12

Antonacci R, Rocchi M, Archidiacono N, Baldini A (1995) Ordered mapping of three alpha satellite DNA subsets on human chromosome 22. Chromosome Research 3:124-127

Anzick S, Kononen J, Walker R, Azorsa D, Tanner M, Guan X-Y, Sauter G, Kallioniemi O, Trent J, Meltzer P (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science

Applegate G, Marymont M (1998) Intracranial ependymomas: A review. Cancer Investigation 16:588-593

Aubele M, Mattis A, Zitzelsberger H, Walch A, Krener M, Hutzler P, Werner M (1999)

Intratumoural heterogeneity in breast carcinoma revealed by laser-microdissection and comparative genomic hybridisation. Cancer, Genetics & Cytogenetics 110:94-102

Balleine R, Fejzo M, Sathasivam P, Basset P, Clarke C, Byrne J (2000) The *hD52* (*TPD52*) gene is a candidate target gene for events resulting in increased 8q21 copy number in human breast carcinoma. Genes Chromosomes & Cancer 29:48-57

Bandyk M, Zhao L, Troncoso P, Pisters L, Palmer J, von Eschenbach A, Chung L, Liang J (1994) Trisomy 7: a potential cytogenetic marker of human prostate cancer progression. Genes Chromosomes & Cancer 9:19-27

Barak Y, Juven T, Haffner R, Oren M (1993) mdm2 expression is induced by wild type p53 activity. EMBO Journal 12:461-468

Barks J, Thompson F, Taetle R, Yang J-M, Stone J, Wymer J, Khavari R, Guan X-Y, Trent J, Pinkel D, Nelson M (1997) Increased chromosome 20 copy number detected by fluorescence in situ hybridisation (FISH) in malignant melanoma. Genes Chromosomes & Cancer 19:278-285

Bayatti N, Engele J (2001) Cyclic AMP modulates the response of central nervous system glia to fibroblast growth factor-2 by redirecting signaling pathways. Journal of Neurochemistry 78:972-980

Beaulieu E, Demeule M, Ghitescu L, Beliveau R (1997) P-glycoprotein is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain. Biochemistry Journal 326:539-544

Becker, I, Becker, K-F, Meyermann, R, and Hollt, V. The multidrug resistance gene MDR1 is expressed in human glial tumours. Acta Neuropathologica 82, 516-519. 91.

Becker (1997) Ependymoblastoma. In: Kleihues P, Cavenee W (eds) Pathology and Genetics: Tumours of the Nervous System. International Agency for Research on Cancer, Lyon, pp 94-95

Bello, MJ, Leone, PE, Nebreda, P, de Campos, JM, Kusak, ME, Vaquero, J, Sarasa, JL, Garcia-Miguel, P, Queizan, A, Hernandez-Moneo, JL, Pestana, A, and Rey, JA. Allelic status of chromosome 1 in neoplasms of the nervous system . Cancer Genetics & Cytogenetics 83, 160-164. 95.

Berditchevski F (2001) Complexes of tetraspanins with integrins: more than meets the eye. Journal of Cell Science 114:4143-4151

Berger M (1996) The impact of technical adjuncts in the surgical management of cerebral hemispheric low-grade gliomas of childhood. Journal of Neuro-Oncology 28:129-155

Berger M, Edwards M, LaMasters D, Davis R, Wilson C (1983) Pediatric brain stem tumours: Radiographic, pathological and clinical correlations. Neurosurgery 12:298-302

Besson A, Yong V (2001) Mitogenic signaling and the relationship to cell cycle regulation in astrocytomas. Journal of Neuro-Oncology 51:245-264

Bhattacharjee, MB, Armstrong, DD, Vogel, H, and Cooley, LD. Cytogenetic analysis of 120 primary pediatric brain tumours and literature review. Cancer Genetics & Cytogenetics 97, 39-53. 97.

Bhattacharjee M, Bruner J (1997) p53 protein in pediatric malignant astrocytomas: A study of 21 patients. Journal of Neuro-Oncology 32:225-233

Bieche I, Lidereau R (1997) A gene dosage effect is responsible for high overexpression of the

MUC1 gene observed in human breast tumours. Cancer, Genetics & Cytogenetics 98:75-80

Biegal, JA. Cytogenetics and molecular genetics of childhood brain tumours. Neuro-Oncology 1, 139-151. 99.

Biernat, W, Kleihues, P, Yonekawa, Y, and Ohgaki, H. Amplification and overexpression of MDM2 in primary (de novo) glioblastomas. Journal of Neuropathology and Experimental Neurology 56, 180-185. 97a.

Biernat W, Tohma Y, Yonekawa Y, Kleihues P, Ohgaki H (1997b) Alterations of cell cycle regulatory genes in primary (de novo) and secondary glioblastoma. Acta Neuropathologica 94:303-309

Bignell G, Barfoot R, Seal S, Collins N, Warren W, Stratton M (1998) Low frequency of somatic mutations in the *LKB1*/Peutz-Jeghers syndrome gene in sporadic breast cancer. Cancer Research 58:1384-1386

Bigner S, Burger P, Wong A, Werner M, Hamilton S, Muhlbaier L, Vogelstein B, Bigner D (1988) Gene amplification in malignant human gliomas: clinical and histopathological aspects. Journal of Neuropathology & Experimental Neurology 47:191-205

Bigner, SH, Mark, J, and Bigner, DD. Cytogenetics of human brain tumours. Cancer Genetics & Cytogenetics 47, 141-154. 90.

Bigner, SH, McLendon, RE, Fuchs, HE, McKeever, PE, and Friedman HS. Chromosomal characteristics of childhood brain tumours. Cancer Genetics & Cytogenetics 97, 125-134. 97.

Bigner, SH and Schrock, E. Molecular cytogenetics of brain tumours. Journal of Neuropathology & Experimental Neurology 56, 1173-1181. 97.

Bijlsma E, Voesten A, Bijleveld E, Troost D, Westerveld A, Merel P, Thomas G, Hulsebos T (1995) Molecular analysis of genetic changes in ependymomas. Genes Chromosomes & Cancer 13:272-277

Billson A, Palmer J, Walker D, Lowe J (1994) Multidrug resistance gene (MDR1) expression in neuro-axial tumours of children and young adults. British Journal of Neurosurgery 8:585-591

Birchmeier C, O'Neill K, Riggs M, Wigler M (1990) Characterisation of ROS1 cDNA from a human glioblastoma cell line. Proceedings of the National Academy of Science USA 87:4799-4803

Birchmeier C, Sharma S, Wigler M (1987) Expression and rearrangement of the *ROS1* gene in human glioblastoma cells. Proceedings of the National Academy of Science USA 84:9270-9274

Blaeker, H, Rasheed, BKA, McLendon, RE, Friedman HS, Batra, SK, Fuchs, HE, and Bigner, SH. Microsatellite analysis of childhood brain tumours. Genes Chromosomes & Cancer 15, 54-63. 96.

Bobola M, Berger M, Ellenbogen R, Roberts T, Geyer J, Silber J (2001) O^6- Methylguanine-DNA methyltransferase in pediatric primary brain tumours: Relation to patient and tumour characteristics. Clinical Cancer Research 7:613-619

Bortolotto S, Chiadò-Piat L, Cavalla P, Bosone I, Mauro A, Schiffer D (2001) CDKN2A/p16 in ependymomas. Journal of Neuro-Oncology 54:9-13

Bouffet, E and Foreman, N. Chemotherapy for intracranial ependymomas. Childs Nervous System 15, 563-570. 99.

Brazma A, Vilo J (2000) Gene expression data analysis. FEBS Letters 480:17-24

Bredel M, Pollack I, Hamilton R, James C (1999) Epidermal growth factor receptor expression and gene amplification in high-grade non-brainstem gliomas of childhood. Clinical Cancer Research 5:1786-1792

Bredel M, Slavc I, Birner P, Czech T, Haberler C, Strobel T, Wolfsberger S, Budka H, Hainfellner J (2002) DNA topoisomerase IIα in optic pathway gliomas of childhood. European Journal of Cancer 38:393-400

Bridge J, Liu J, Qualman S, Suijkerbuijk R, Wenger G, Zhang J, Wan X, Baker K, Sorensen P, Barr F (2002) Genomic gains and losses are similar in genetic and histologic subsets of rhabdomyosarcoma, whereas amplification predominates in embryonal with anaplasia and alveolar subtypes. Genes Chromosomes & Cancer 33:310-321

Brunner C, Jung V, Henn W, Zang K, Urbschat S (2000) Comparative genomic hybridisation reveals recurrent enhancements on chromosome 20 and in one case combined amplification sites on 15q24q26 and 20p11p12 in glioblastomas. Cancer, Genetics & Cytogenetics 121:124-127

Burger P (1996) Pathology of brain stem astrocytomas. Pediatric Neurosurgery 24:35-40

Burger, Paulus W, Kleihues P (1993) Pilocytic Astrocytoma. In: Kleihues P (ed) Pathology and genetics of nervous system tumours. pp 29-33

Burger, Scheithauer B, Paulus W, Szymas J, Giannini C, Kleihues P (2001) Pilocytic astrocytoma. In: Kleihues P, Cavenee W (eds) Pathology and Genetics: Tumours of the Nervous System. IRAC, Lyons,

Burton E, Lamborn K, Feuerstein B, Prados M, Scott J, Forsyth P, Passe S, Jenkins R, Aldape K (2002) Genetic aberrations defined by comparative genomic hybridisation distinguish long-term from typical survivors of glioblastoma. Cancer Research 62:6205-6210

Busca A, Miniero R, Besenzon L, Cordero di Montezemolo L, Cenni M, Fagioli F, Sandri A, Vassallo E, Ricardi U, Madon E (1997) Etoposide-containing regimens with autologous bone marrow transplantation in children with malignant brain tumours. Childs Nervous System 13:572-577

Büschges R, Weber R, Actor B, Lichter P, Collins V, Reifenberger G (1999) Amplification and expression of Cyclin D genes (*CCND1, CCND2* and *CCND3*) in human malignant gliomas. Brain Pathology 9:435-443

Bussey K, Lawce H, Olson S, Arthur D, Kalousek D, Krailo M, Giller R, Heifetz S, Womer R, Magenis R (1999) Chromosome abnormalities of eighty-one pediatric germ cell tumours: Sex-, age-, site-, and histopathology-related differences - A Childrens Cancer Group Study. Genes Chromosomes & Cancer 25:134-146

Cairncross J, Ueki K, Zlatescu M, Lisle D, Finkelstein D, Hammond R, Silver J, Stark P, Macdonald D, Ino Y, Ramsay D, Louis D (1998) Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. Journal of the National Cancer Institute 90:1473-1479

Caldarelli-Stefano R, Boldorini R, Monga G, Merawiglia E, Zorini E&FP (2000) JC virus in human glial-derived tumours. Human Pathology 31:394-395

Calender A, Giraud S, Porchet N, Gaudray P, Cadiot G, Mignon M (1998) Clinicogenetic study of MEN1: recent physiopathological data and clinical applications. Study group of multiple endocrine neoplasia (GENEM). Ann Endocrinol (Paris) 59:444-451

Carter M, Nicholson J, Ross F, Crolla J, Allibone R, Balaji V, Perry R, Walker D, Gilbertson R,

Ellison D (2002) Genetic abnormalities detected in ependymomas by comparative genomic hybridisation. British Journal of Cancer 86:929-939

Castello M, Schiavetti A, Varrasso G, Clerico A, Cappelli C (1998) Chemotherapy in low-grade astrocytoma management. Childs Nervous System 14:6-9

Cavenee, Bigner D, Newcomb E, Paulus W, Kleihues P (1993) Diffuse Astrocytoma. In: Kleihues P (ed) Pathology and Genetics of Nervous System tumours.

Cavenee, Furnari F, Nagane M, Huang H-J, Newcomb E, Bigner D, Weller M, Beren M, Plate. KH, Israel M, Noble M, Kleihues P (2000) Diffusely infiltrating astrocytomas. In: Kleihues P, Cavenee W (eds) Pathology and Genetics: Tumours of the Nervous System. IRAC, Lyons,

Chakrabarty A, Mitchell P, Bridges L, Franks A (1999) Malignant transformation in pleomorphic xanthoastrocytoma - a report of two cases. British Journal of Neurosurgery 13:516-519

Chamberlain M (1997) recurrent cerebellar gliomas: Salvage therapy with oral etoposide. Journal of Child Neurol 12:200-204

Chen Y-J, Ko J-Y, Chen P-J, Shu C-H, Hsu M-T, Tsai S-F, Lin C-H (1999) Chromosomal aberrations in nasopharyngeal carcinoma analysed by comparative genomic hybridisation. Genes Chromosomes & Cancer 25:

Cheng Y, Ng H, Zhang S, Ding M, Chung-Sean Pang J, Zheng J, Poon WS (1999) Genetic alterations in pediatric high grade astrocytomas. Human Pathology 30:1284-1290

Cheung V&NS (1996) Whole genome amplification using a degenerate oligonucleotide primer allows hundreds of genotypes to be performed on less than one nanogram of genomic DNA. Proceedings of the National Academy of Science USA 93:14676-14679

Chiu J, Woo S, Ater J, Connelly J, Bruner J, Maor M, van Eys J, Oswald M, Shallenberger R (1992) Intracranial ependymoma in children: analysis of prognostic factors. Journal of Neuro-Oncology 13:283-292

Chou P, Sanz C, Tomita T, Reyes-Mugica M (1996) Ependymomas in children express the multidrug resistance gene: Immunohistochemical and molecular biologic study. Pediatric Pathology & Laboratory Medicine 16:551-561

Chozick B, Weicker M, Pezzullo J, Jackson C, Finkelstein S, Ambler M, Epstein M, Finch P (1994) Pattern of mutant p53 expression in human astrocytomas suggests the existence of alternative pathways of tumourigenesis. Cancer 73:406-415

Chung R, Whaley J, Kley N (1991) TP53 mutation and 17p deletion in human astrocytomas. Genes Chromosomes & Cancer 3:323-331

Clark G, Henry J, McKeever P (1985) Cerebellar pilocytic astrocytoma. Cancer 56:1128-1133

Cobbs C, Harkins L, Samanta M, Gillespie G, Bharara S, King P, Nabors L, Cobbs C, Britt W (2002) Human cytomegalovirus infection and expression in human malignant glioma. Cancer Research 62:3347-3350

Cobbs, McDonald J, Edwards M (1996) Ependymomas. In: Youmans J (ed) Neurological Surgery: A comprehensive reference guide to the diagnosis and management of neurosurgical problems, 4th edn. WB Saunder Co, Philadelphia, pp 2552-2569

Cogen P, McDonald J (1996) Tumour suppressor gene and medulloblastoma. Journal of Neuro-Oncology 29:103-112

Collins C, Rommens J, Kowbel D, Godfrey T, Tanner M, Hwang S, Polikoff D, Nonet G, Cochran J, Myambo J, Jay K, Froula J, Cloutier T, Kuo W, Yaswen P, Dairkee S, Giovanola J, Hutchinson G, Isola J, Kallioniemi O, Palazzolo M, Martin C, Ericsson C, Pinkel D, Gray J (1998) Positional cloning of *ZNF217* and *NABC1*: genes amplified at 20q13.2 and overexpressed in breast carcinoma. Proceedings of the National Academy of Science USA 95:8703-8708

Cordon-Cardo C, O'Brien J, Casals D, Rittman-Grauer L, Biedler J, Melamed M, Bertino J (1989) Multidrug resistance gene (P-Glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. Proceedings of the National Academy of Science USA 86:695-698

Costello J, Berger M, Huang H-J, Cavenee W (1996) Silencing of p16/CDKN2 expression in human gliomas by methylation and chromatin condensation. Cancer Research 56:2405-2410

Costello J, Plass C, Arap W, Chapman V, Held W, Berger M, Huang H-JS, Cavenee W (1997) Cyclin-dependent kinase 6 (CDK6) amplification in human gliomas identified using two-dimensional separation of genomic DNA. Cancer Research 57:1250-1254

Couldwell W, Hinton D, Surnock A (1996) Treatment of recurrent malignant gliomas with chronic oral high-dose tamoxifen. Clinical Cancer Research 2:619-623

Daigo Y, Chin S-F, Gorringe K, Bobrow L, Ponder B, Pharoah P, Caldas C (2001) Degenerate oligonucleotide primed-polymerase chain reaction-based array comparative genomic hybridisation for extensive amplicon profiling of breast cancers: A new approach for the molecular analysis of paraffin-embedded cancer tissue . American Journal of Pathology 158:1623-1631

Dal Cin P, Van Den Berghe H, Buonamici L, Losi L, Roncaroli F, Calbucci F (1999) Cytogenetic investigation in subependymoma. Cancer, Genetics & Cytogenetics 108:84

Dam A, Fock J, Hayes V, Molenaar W, van den Berg E (2000) Recurrent astrocytoma in a child: A report of cytogenetics and TP53 gene mutation screening. Neuro-Oncology 2:184-189

Davis C, Joglekar V (1981) Cerebellar astrocytomas in children and young adults. Journal of Neurol Neurosurg Psychiatry 44:820-828

Debiec-Rychter M, Lasota J, Alwasiak J, Liberski (1995) Recurrent anaplastic ependymoma with an abnormal karyotype and c-*myc* proto-oncogene overexpression. Acta Neuropathologica 89:270-274

Del Valle L, Gordon J, Assimakopoulou M, Enam S, Geddes J, Varakis J, Katsetos C, Croul S, Khalili K (2001) Detection of JC virus DNA sequences and expression of the viral regulatory protein T-antigen in tumours of the central nervous system. Cancer Research 61:4287-4293

Derré J, Lagacé R, Nicolas A, Mairal A, Chibon F, Coindre J-M, Terrier P, Sastre X, Aurias A (2001) Leiomyosarcomas and most malignant fibrous histiocytomas share very similar comparative genomic hybridisation imbalances: An analysis of a series of 27 leiomyosarcomas. Laboratory Investigation 81:211-215

Dewit L, Van der Schueren E, Ang K (1984) Low grade astrocytomas in children treated by surgery and radiation therapy. Acta Radiol Oncol 23:1-8

Diestra J, Scheffer G, Català I, Maliepaard M, Schellens J, Scheper R, Germà-Lluch J, Izquierdo M (2002) Frequent expression of the multi-drug resistance-associated protein BCRP/MXR/ABCP/ABCG2 in human tumours detached by the BXP-21 monoclonal antibody in paraffin-embedded material. Journal of Pathology 198:213-219

Dirks P, Jay V, Becker L, Drake J, Humphreys R, Hoffman H, Rutka J (1994) Development of

anaplastic changes in low-grade astrocytomas of childhood. Neurosurgery 34:68-78

Dirven C, Mooij J, Molenaar W (1997) Cerebellar pilocytic astrocytoma: A treatment protocol based upon analysis of 73 cases and a review of the literature. Childs Nervous System 13:17-23

Dohrmann G, Farwell J, Flannery J (1976) Ependymomas and ependymoblastomas in children. Journal of Neurosurgery 45:273-283

Dong J, Kukula A, Toyoshima M, Nakajima M (2000) Genomic organization and chromosome localization of the newly identified human heparanase gene. Gene 253:171-178

Dong S, Pang J-S, Hu J, Zhou L-F, Ng H-K (2002) Transcriptional inactivation of TP73 expression in oligodendroglial tumours. International Journal of Cancer 98:370-375

Douglass E, Green A, Hayes F, Etcubanas E, Horowitz M, Wilimas J (1985) Chromosome 1 abnormalities: A common feature of paediatric solid tumours. Journal of the National Cancer Institute 75:51-53

Drach L, Kammermeier M, Neirich U, Jacobi G, Kornhuber B, Lorenz R, Schlote W (1996) Accumulation of nuclear p53 protein and prognosis of astrocytomas in childhood and adolescence. Clinical Neuropathology 15:67-73

Dropcho E, Soong S (1996) The prognostic impact of prior low grade histology in patients with anaplastic gliomas: a case-control study. Neurology 47:684-690

Du Plessis L, Dietzsch E, Van Gele M, Van Roy N, van Helden P, Parker M, Mugwanya D, De Groot M, Marx M, Kotze M, Speleman F (1999) Mapping of novel regions of DNA gain and loss by comparative genomic hybridisation in esophageal carcinoma in the black and coloured populations of South Africa. Cancer Research 59:1877-1883

Duffner P, Cohen M, Myers M, Heise H (1986) Survival of children with brain tumours: SEER program 1973-1980. Neurology 36:597-601

Duffner P, Horowitz M, Krischer J, Friedman H, Burger P, Cohen M, Sanford R, Mulhern R, James H, Freeman C, Seidel F, Kun L (1993) Postoperative chemotherapy and delayed radiation in children less than three years of age with malignant brain tumours. The New England Journal of Medicine 328:1725-1731

Duffner P, Krischer J, Burger P, Cohen M, Backstrom J, Horowitz M, Sanford R, Friedman H, Kun L, The Pediatric Oncology Group (1996) Treatment of infants with malignant gliomas: The Pediatric Oncology Group Experience. Journal of Neuro-Oncology 28:245-256

Dumanski J (1996) The human chromosome 22 located genes and malignancies of the central nervous system. Neuropathology and Applied Neurobiology 22:425-433

Dyer S, Prebble E, Davison V, Davies P, Ramani P, Ellison D, Grundy R (2002) Genomic imbalances in pediatric intracranial ependymomas define clinically relevant groups. American Journal of Pathology 161:2133-2141

Eberhart C, Kratz J, Schuster A, Goldthwaite P, Cohen K, Perlman E, Burger P (2002) Comparative genomic hybridisation detects an increased number of chromosomal alterations in large cell/anaplastic medulloblastomas. Brain Pathology 12:36-44

Ebert C, Von Haken M, Meyer-Puttlitz B, Wiestler O, Reifenberger G, Pietsch T, Von Deimling A (1999) Molecular genetic analysis of ependymal tumours. American Journal of Pathology 155:627

Edwards M, Wara W, Ciricillo S, Barkovich A (1994) Focal brain-stem astrocytomas causing

symptoms of involvement of the facial nerve nucleus: long-term survival in six paediatric cases. Journal of Neurosurgery 80:20-25

Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21:5400-5413

Eifel P, Cassady J, Belli J (1987) Radiation therapy of tumours of the brainstem and midbrain in children: Experience of the Joint Centre for Therapy and Children's Hospital Medical Centre. International Journal Radiation Oncology Biol Phys 13:847-852

Ejendal K, Hrycyna C (2002) Multidrug resistance and cancer: the role of the human ABC transporter ABCG2. Curr Protein Pept Sci 3:503-511

El-Naggar A, Dinh M, Tucker S, Swanson D, Steck K, Vielh P (1999) Numerical chromosomal changes in DNA hypodiploid solid tumours: Restricted loss and gain of certain chromosomes. Cytometry 37:107-112

Esteller M, Garcia-Foncillas J, Andion E, Goodman S, Hidalgo O, Vanaclocha V, Baylin S, Herman J (2000) Inactivation of the DNA-repair gene *MGMT* and the clinical response of gliomas to alkylating agents. The New England Journal of Medicine 343:1350-1354

Evans A, Anderson J, Lefkowitz-Boudreaux I, Finlay JL (1996) Adjuvant chemotherapy of childhood posterior fossa ependymoma: Cranio-spinal irradiation with or without adjuvant CCNU, Vincristine and Prednisone: A Childrens cancer group study. Medical & Pediatric Oncology 27:8-14

Fink K, Rushing E, Schold SJ, Nisen P (1996) Infrequency of p53 gene mutations in ependymomas. Journal of Neuro-Oncology 27:111-115

Finlay J, Goins S, Uteg R, Giese WL (1987) Progress in the management of childhood brain tumours. Hematology/Oncology Clinics of North America 1:753-776

Finlay J, Wisoff J (1999) The impact of extent of resection in the management of malignant gliomas of childhood. Childs Nervous System 15:786-788

Fischer U, Meltzer P, Meese E (1996) Twelve amplified and expressed genes localised in a single domain in gliomas. Human Genetics 98:625-628

Fischer U, Mueller H, Sattler H, Feiden K, Zang K, Meese E (1995) Amplification of the MET gene in glioma. Genes Chromosomes & Cancer 12:63-65

Fisher B, Leighton C, Vujovic O, MacDonald D, Stitt L (2001) Results of a policy of surveillance alone after surgical management of pediatric low-grade gliomas. International Journal Radiation Oncology Biol Phys 51:704-710

Fisher P, Breiter S, Carson B, Wharam M, Williams J, Weingart J, Foer D, Goldthwaite P, Tihan T, Burger P (2000) A clinicopathologic reappraisal of brain stem tumour classification. Cancer 89:1569-1576

Forozan F, Karhu R, Kononen J, Kallioniemi A, Kallioniemi O (1997) Genome screening by comparative genomic hybridisation. Trends in Genetics 13:405-409

Forozan F, Mahlamaki E, Monni O, Chen Y, Veldman R, Jiang Y, Gooden GC, Ethier S, Kallioniemi A, Kallioniemi O (2000) Comparative genomic hybridisation analysis of 38 breast cancer cell lines: A basis for interpreting complementary DNA microarray data. Cancer Research 60:4519-4525

Forus A, Berner J-M, Meza-Zepeda L, Saeter G, Mischke D, Fodstad Ø, Myklebost O (1998)

Molecular characterization of a novel amplicon at 1q21-q22 frequently observed in human sarcomas. British Journal of Cancer 78:495-503

Fouladi M, Baruchel S, Chan H, Grant R, Malkin D, Weitzman S, Greenberg M (1998) Use of adjuvant ICE chemotherapy in the treatment of anaplastic ependymomas. Childs Nervous System 14:590-595

Frankel R, Bayona W, Koslow M, Newcomb E (1992) p53 mutations in human malignant gliomas: Comparison of loss of heterozygosity with mutation frequency. Cancer Research 52:1427-1433

Freeman C, Krischer J, Sanford R (1993) Final results of a study of escalating doses of hyperfractionated radiotherapy in brain stem tumours in children: a Pediatric Oncology Group study. International Journal Radiation Oncology Biol Phys 27:197-206

Friedman H, Krischer J, Burger P, Oakes W, Hockenberger B, Weiner M, Falletta J, Norris D, Ragab A, Mahoney D (1992) Treatment of children with progressive or recurrent brain tumours with carboplatin or iproplatin: A Pediatric Oncology Group randomized phase II study. Journal of Clinical Oncology 10:249-256

Friend S, Dryja T, Weinberg R (1988) Oncogenes and tumour-suppressing genes. The New England Journal of Medicine 318:618-622

Fueyo J, Gomez-Manzano C, Liu T-J, Yung W (2001) Delivery of cell cycle genes to block astrocytoma growth. Journal of Neuro-Oncology 51:277-287

Fujii Y, Hongo T, Hayashi Y (1994) Chromosome analysis of brain tumours in childhood. Genes Chromosomes & Cancer 11:205-215

Fujimoto K, Shen M, Noshiro M, Matsubara K, Shingu S, Honda K, Yoshida E, Suardita K, Matsuda YKY (2001) Molecular cloning and characterisation of DEC2, a new member of basic helix-loop-helix proteins. Biochemical and Biophysical Research Communications 280:164-171

Fujimoto M, Sheridan PJ, Sharp Z, Weaker F, Kagan-Hallet K, Story J (1989) Proto-oncogene analysis in brain tumours. Journal of Neurosurgery 70:910-915

Fulci G, Labuhn M, Maier D, Lachat Y, Hausmann O, Hegi M, Janzer R, Merlo A, Van Meir E (2000) *p53* gene mutation and *ink-4a-arf* deletion appear to be two mutually exclusive events in human glioblastoma. Oncogene 19:3816-3822

Fults D, Brockmeyer D, Tullous M, Pedone C, Cawthorn R (1992) p53 mutation and loss of heterozygosity on chromosomes 17 and 10 during human astrocytoma progression. Cancer Research 52:674-679

Garcia D, Fulling K (1985) Juvenile pilocytic astrocytoma of the cerebrum in adults. A distinctive neoplasm with favorable prognosis. Journal of Neurosurgery 63:382-386

Gebhart E, Liehr T (2000) Patterns of genomic imbalance in human solid tumours (review). International Journal of Oncology 16:383-399

Geddes J, Vowles G, Ashmore S, Cockburn H, Darling J (1994) Detection of multidrug resistance gene product (P-glycoprotein) expression in ependymomas. Neuropathology and Applied Neurobiology 20:118-121

Georges E, Sharom F, Ling V (1990) Multidrug resistance and chemosensitization: Therapeutic implications for cancer chemotherapy. Adv. Pharmacol 21:185-220

Giannini C, Scheithauer B, Burger P, Brat D, Wollan P, Lach B, O'Neill B (1999) Pleomorphic

Xanthoastrocytoma: What do we really know about it? Cancer 85:2033-2045

Gilles F, Leviton A, Tavare C, Adelman L, Rorke L, Sobel E, Hedley-Whyte E, Davis R (2000) Definitive classes of childhood supratentorial neuroglial tumours. Pediatric and Developmental Pathology 3:126-139

Ginsberg D, Mechta F, Yaniv M, Oren M (1991) Wild-type p53 can down regulate the activity of various promoters. Proceedings of the National Academy of Science USA 88:9979-9983

Giraud S, Choplin H, Teh B, Lespinasse J, Jouvet A, Labat-Moleur F, Lenoir G, Hamon B, Hamon P, Calender A (1997) A large multiple endocrine neoplasia type 1 family with clinical expression suggestive of anticipation. Journal of Clin Endocrinol Metab 82:3487-3492

Gisselsson D, Pålsson E, Höglund M, Domanski H, Mertens F, Pandis N, Sciot R, Dal Cin P, Bridge J, Mandahl N (2002) Differentially amplified chromosome 12 sequences in low- and high-grade osteosarcoma. Genes Chromosomes & Cancer 33:133-140

Gjerris F, Klinken L (1978) Long-term prognosis in children with benign cerebellar astrocytoma. Journal of Neurosurgery 49:179-184

Goldwein J, Leahy J, Packer R, Sutton L, Curran W, Rorke L, Schut L, Littman P, D'Angio G (1990) Intracranial ependymomas in children. International Journal Radiation Oncology Biol Phys 19:1497-1502

Gonzales (1995) Classification and pathogenesis of brain tumours. In: Kaye A, Laws E (eds) Brain Tumours. Churchill Livingstone, New York, pp 31-46

Gray J, Collins C (2000) Genome changes and gene expression in human solid tumours. Carcinogenesis 21:443-452

Griffin C, Hawkins A, Packer R, Rorke L, Emanuel B (1988) Chromosome abnormalities in pediatric brain tumours. Cancer Research 48:175-180

Griffin C, Long P, Carson B, Brem H (1992) Chromosome abnormalities in low grade central nervous system tumours. Cancer, Genetics & Cytogenetics 60:67-73

Grill J, Avet-Loiseau H, Lellouch-Tubiana A, Sévenet N, Terrier-Lacombe M-J, Vénuat A-M, Doz F, Sainte-Rose C, Kalifa C, Vassal G (2002) Comparative genomic hybridisation detects specific cytogenetic abnormalities in pediatric ependymomas and choroid plexus papillomas. Cancer, Genetics & Cytogenetics 136:121-125

Grill J, Le Deley M-C, Gambarelli D, Raquin M-A, Couanet D, Pierre-Khan A, Habrand J-L, Doz F, Gentet J-C, Edan C, Chastagner P, Kalifa C (2001) Postoperative chemotherapy without irradiation for ependymoma in children under 5 years of age: A multicentre trial of the French Society of Pediatric Oncology. Journal of Clinical Oncology 19:1288-1296

Guan X-Y, Fu S-B, Xia J-C, Fang Y, Sham J, Du B-D, Zhou H, Lu S, Wang B-Q, Lin Y-Z, Liang Q, Li X-M, Du B, Ning X-M, Du J-R, Li P, Trent J (2000) Recurrent chromosome changes in 62 primary gastric carcinomas detected by comparative genomic hybridisation. Cancer, Genetics & Cytogenetics 123:27-34

Gunawan B, Huber W, Holtrup M, von Heydebreck A, Efferth T, Poustka A, Ringert R-H, Jakse G, Füzesi L (2001) Prognostic impacts of cytogenetic findings in clear cell renal cell carcinoma: Gain of 5q-31-qter predicts a distinct clinical phenotype with favourable prognosis. Cancer Research 61:7731-7738

Gutmann D, Zhang Y, Hasbani M, Goldberg M, Plank T, Henske E (2000) Expression of the

tuberous sclerosis complex gene products, hamartin and tuberin, in central nervous system tissues. Acta Neuropathologica 99:223-230

Hahn H, Christiansen J, Wicking C, Zaphiropoulos P, Chidambaram A, Gerrard B, Vorechovsky I, Bale A, Toftgard R, Dean M, Wainwright B (1996) A mammalian *patched* homolog is expressed in target tissues of *sonic hedgehog* and maps to a region associated with developmental abnormalities. The Journal of Biological Chemistry 271:

Hall W, Merrill M, Walbridge S, Youle R (1990) Epidermal growth factor receptors on ependymomas and other brain tumours. Journal of Neurosurgery 72:641-646

Harada K, Nishizaki T, Ozaki S, Kubota H, Harada K, Okamura T, Ito H, Sasaki K (1999) Cytogenetic alterations in pituitary adenomas detected by comparative genomic hybridisation. Cancer, Genetics & Cytogenetics 112:38-41

Harada K, Toyooka S, Maitra A, Maruyama R, Toyooka K, Timmons C, Tomlinson G, Mastangelo D, Hay R, Minna J, Gazdar A (2002) Aberrant promotor methylation and silencing of the *RASSF1A* gene in paediatric tumours and cell lines. Oncogene 21:4345-4349

Hartmann C, Johnk L, Sasaki H, Jenkins R, Louis D (2002) Novel *PLA2G4C* polymorphism as a molecular diagnostic assay for 19q loss in human gliomas. Brain Pathology 12:178-182

Haub O, Drucker B, Goldfarb M (1990) Expression of the murine fibroblast growth factor 5 gene in the adult central nervous system. Proceedings of the National Academy of Science USA 87:8022-8026

Hayashi Y, Ueki K, Waha A, Wiestler O, Louis D, von Deimling A (1997) Association of *EGFR* gene amplification and *CDKN2 (p16/MTS1)* gene deletion in glioblastoma multiforme. Brain Pathology 7:871-875

Hayes V, Dirven C, Dam A, Verlind E, Molenaar W, Mooij J, Hofstra R, Buys C (1999) High frequency of TP53 mutations in juvenile pilocytic astrocytomas indicates role of TP53 in the development of these tumours. Brain Pathology 9:463-467

Hayostek C, Shaw E, Scheithauer B, O'Fallon J, Weiland T, Schomberg P, Kelly PHT (1993) Astrocytomas of the cerebellum. A comparative clinicopathologic study of pilocytic and diffuse astrocytomas. Cancer 72:856-869

Healey E, Barnes P, Kupsky W, Scott R, Sallan S, Black P, Tarbell N (1991) The prognostic significance of postoperative residual tumour in ependymoma. Neurosurgery 28:666-672

Hecht B, Turc-Carel C, Chatel M, Paquis P, Gioanni J, Attias R, Gaudray P, Hecht F (1995) Cytogenetics of malignant gliomas: The sex chromosomes with reference to X isodisomy and the role of numerical X/Y changes. Cancer, Genetics & Cytogenetics 84:9-14

Hegmann E, Bauer H, Kerbel R (1992) Expression and functional activity of P-glycoprotein in cultured cerebral capillary endothelial cells. Cancer Research 52:6969-6975

Heidenblad M, Jonson T, Mahlamäki E, Gorunova L, Karhu R, Johansson B, Höglund M (2002) Detailed genomic mapping and expression analyses of 12p amplifications in pancreatic carcinomas reveal a 3.5Mb target region for amplification. Genes Chromosomes & Cancer 34:211-223

Held-Feindt J, Mentlein R (2002) CD70/CD27 ligand, a member of the TNF family, is expressed in human brain tumours. International Journal of Cancer 98:352-356

Helseth E, Due-Tonnessen B, Lote K, Skullerud K, Storm-Mathisen I, Wesenberg F, Lundar T

(2001) Ependymoma in children and young adults (0-19 years): Report of 25 consecutive cases. Childs Nervous System 17:24-30

Hemminki A, Markie D, Tomlinson I, Avizienyte E, Roth S, Loukola A, Bignell G, Warren W, Aminoff M, Höglund P, Järvinen H, Kristo P, Pelin K, Ridanpää M, Salovaara S, Toro T, Bodmer W, Olschwang S, Olsen A, Stratton M, de la Chapelle A, Aaltonen L (1998) A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. Nature 391:184-187

Henson J, Cordon-Cardo C, Posner J (1992) P-glycoprotein expression in brain tumours. Journal of Neuro-Oncology 14:37-43

Henson JSB, Correa K, von Deimling A, Fassbender F, Xu H, Benedict W, Yandell D, Louis D (1994) The retinoblastoma gene is involved in malignant progression of astrocytomas. Annals of Neurology 36:714-721

Herpers M, Freling G, Beuls E (1994) Pleomorphic xanthoastrocytoma in the spinal cord. Case report. Journal of Neurosurgery 80:564-569

Hirose, Y, Aldape, KD, Bollen, A, James, CD, Brat, DJ, Lamborn, KR, Berger, MS, and Feuerstein, BG. Chromosomal abnormalities subdivides ependymal tumours into clinically relevant groups. American Journal of Pathology 158(3), 1137-1143. 2001.

Hirose Y, Aldape K, Chang S, Lamborn K, Berger M, Feuerstein B (2003) Grade II astrocytomas are subgrouped by chromosome aberrations. Cancer, Genetics & Cytogenetics 142:1-7

Hoang-Xuan K, He J, Huguet S, Mokhtari K, Marie Y, Kujas M, Leuraud P, Capelle L, Delattre J, Poirier J, Broët P, Sanson M (2001) Molecular heterogeneity of oligodendrogliomas suggests alternative pathways in tumor progression. Neurology 57:1278-1281

Hoglund M, Gorunova L, Andren-Sandberg A, Dawiskiba S, Mitelman F, Johansson B (1998) Cytogenetic and fluorescence in situ hybridisation analyses of chromosome 19 aberrations in pancreatic carcinomas: Frequent loss of 19p13.3 and gain of 19q13.1-13.2. Genes Chromosomes & Cancer 21:8-16

Horio M, Gottesman M, Pastan I (1988) ATP-dependent transport of vinblastine in vesicles from human multi-drug resistant cells. Proceedings of the National Academy of Science USA 85:3580-3584

Hu J, Wills M, Baker B, Perlman E (2000) Comparative genomic hybridisation analysis of hepatoblastomas. Genes Chromosomes & Cancer 27:196-201

Huang B, Starostik P, Kühl J, Tonn J, Roggendorf W (2002) Loss of heterozygosity on chromosome 22 in human ependymomas. Acta Neuropathologica 103:415-420

Huang H, Colella S, Kurrer M, Yonekawa Y, Kleihues P, Ohgaki H (2000a) Gene expression profiling of low-grade diffuse astrocytomas by cDNA arrays. Cancer Research 60:6868-6874

Huang Q, Schantz S, Rao P, Mo J, McCormick S, Chaganti R (2000b) Improving degenerate oligonucleotide primed PCR-comparative genomic hybridisation for analysis of DNA copy number changes in tumours. Genes Chromosomes & Cancer 28:395-403

Hukin J, Epstein F, Lefton D, Allen J (1998) Treatment of intracranial ependymoma by surgery alone. Pediatric Neurosurgery 29:40-45

Hulsebos T, Oskam N, Bijleveld E, Westerveld A, Hermsen M, van den Ouweland A, Hamel B, Tijssen C (1999) Evidence for an ependymoma tumour suppressor gene in chromosome region 22pter-22q11.2. British Journal of Cancer 81:1150-1154

Hunter T (1991) Cooperation between oncogenes. Cell 64:249

Hurtt M, Moossy J, Donovan-Peluso M, Locker J (1992) Amplification of epidermal growth factor receptor gene in gliomas: Histopathology and prognosis. Journal of Neuropathology and Experimental Neurology 51:84-90

Ichimura K, Schmidt E, Miyakawa A, Goike H, Collins V (1998) Distinct patterns of deletion on 10p and 10q suggest involvement of multiple tumour suppressor genes in the development of astrocytic gliomas of different malignancy grades. Genes Chromosomes & Cancer 22:9-15

Ichimura K, Schmidt E, Yamaguchi N, James C, Collins V (1994) A common region of homozygous deletion in malignant human gliomas lies between IFNa/w gene cluster and the D9S171 locus. Cancer Research 54:3127-3130

Ilgren E, Stiller C (1986) Cerebellar astrocytomas. Therapeutic management. Acta Neurochir 81:11-26

Ilgren E, Stiller C (1987) Cerebellar astrocytomas: Clinical characteristics and prognostic indices. Journal of Neuro-Oncology 4:293-308

Ino Y, Silver J, Blazejewski L, Nishikawa R, Matsutani M, Von Deimling A, Louis D (1999) Common regions of deletion on chromosome 22q12.3-q13.1 and 22q13.2 in human astrocytomas appear related to malignancy grade. Journal of Neuropathology & Experimental Neurology 58:881-885

International Human Genome Sequencing Consortium (2001) Initial sequencing and analysis of the human genome. Nature 409:860-921

Irwin M, Kaelin W (2001) p53 family update: p73 and p63 develop their own identities. 12:337-349

Isaka S, Takei Y, Tokino T, Koyama K, Miyoshi Y, Suzuki M, Takahashi E, Azuma C, Murata Y, Nakamura Y (2000) Isolation and characterization of a novel TP53-Inducible gene, *TP53TG5*, which suppresses growth and shows cell cycle dependent transition of expression. Genes Chromosomes & Cancer 27:345-352

Jallo G, Zagzag D, Epstein F (1996) Intramedullary subependymoma of the spinal cord. Neurosurgery 38:251-257

James C, Carlbom E, Dumanski J, Hansen M, Nordenskjold M, Collins V, Cavenee WK (1988) Clonal genomic alterations in glioma malignancy stages. Cancer Research 48:5546-5551

James C, He J, Carlbom E, Mikkelsen T, Ridderheim P, Cavenee W, Collins V (1990) Loss of genetic information in CNS tumours common to children and young adults. Genes Chromosomes & Cancer 2:94-102

Jen J, Harper W, Bigner S, Bigner D, Papadopoulos N, Markowitz S, Willson J, Kinzler K, Vogelstein B (1994) Deletion of *p16* and *p15* genes in brain tumours. Cancer Research 54:6353-6358

Jenkin D, Danjoux C, Greenberg M (1998) Subsequent quality of life for children irradiated for a brain tumour before age four years. Medical & Pediatric Oncology 31:506-511

Jenkins R, Kimmel D, Moertel C, Schulz C, Scheithauer B, Kelly P, Dewald G (1989) A cytogenetic study of 53 human gliomas. Cancer, Genetics & Cytogenetics 39:253-279

Jeuken J, Sprenger S, Gilhuis J, Teepen H, Grotenhuis A, Wesseling P (2002) Correlation between localization, age and chromosomal imbalances in ependymal tumours as detected by CGH.

Journal of Pathology 197:238-244

Jeuken J, Sprenger S, Wesseling P, Macville M, Von Deimling A, Teepen H, van Overbeeke J, Boerman R (1999) Identification of subgroups of high-grade oligodendroglial tumours by comparative genomic hybridisation. Journal of Neuropathology & Experimental Neurology 58:606-612

Johnson E, Iyer L, Rich S, Orr H, Gilnagel A, Kurth J, Zabranski J, Marchuk D, Weisenbach J, Clericuzio C, Davies L, Hart B, Gusella J, Kosofsay B, Louis D, Morrison L, Green E, Webber J (1995) Refined localisation of the cerebral cavenous malformation gene (CCM1) to a 4-cM interval of chromosome 7q contained in a well defined YAC contig . Genome Research 5:368-380

Jones M, Hamana N, Nezu J, Shimane M (2000) A novel family of bromodomain genes. Genomics 63:40-45

Kadota R, Allen J, Hartman G, Spruce W (1989) Brain tumours in children. The Journal of Pediatrics 114:511-519

Kalapurakal J, Kepka A, Bista T, Goldman S, Tomita T, Marymont M (2000) Fractionated stereotactic radiotherapy for pediatric brain tumours: the Chicago childrens experience. Childs Nervous System 16:296-303

Kalifa C, Valteau D, Pizer B, Vassal G, Grill J, Hartmann O (1999) High-dose chemotherapy in childhood brain tumours. Childs Nervous System 15:498-505

Kallioniemi A, Kallioniemi O, Piper J, Isola J, Waldman F, Gray J, Pinkel D (1994) Optimizing comparative genomic hybridisation for analysis of DNA sequence copy number changes in solid tumours. Genes Chromosomes & Cancer 10:231-243

Kallioniemi A, Kallioniemi O, Sudar D, Rutovitz D, Gray J, Waldman F, Pinkel D (1992) Comparative genomic hybridisation for molecular cytogenetic analysis of solid tumours. Science 258:818-821

Kamb A (1995) Cell-cycle regulators and cancers. Trends in Genetics 11:136-140

Karnes, Raffel C (1993) Pediatric Brain Tumours. In: Levine A, Schmidek H (eds) Molecular Genetics of Nervous System Tumours. Wiley-Liss, New York, pp 217-221

Karnes P, Tran T, Cui M, Raffel C, Gilles F, Barranger J, Ying K (1992) Cytogenetic analysis of 39 paediatric central nervous system tumours. Cancer, Genetics & Cytogenetics 59:12-19

Kastan M, Onyekere O, Sidransky D, Vogelstein B, Craig R (1991) Participation of p53 protein in the cellular response to DNA damage. Cancer Research 51:6304-11

Kastan M, Zhan Q, El-Deiry W (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 71:587-597

Kato H, Uchimura I, Morohoshi M, Fujisawa K, Kobayashi Y, Numano F, Goseki N, Endo M, Tamura A, Nagashima C (1996) Multiple endocrine neoplasia type 1 associated with spinal ependymoma. Intern Med 35:285-289

Kaulich K, Blaschke B, Numann A, von Deimling A, Wiestler O, Weber R, Reifenberger G (2002) Genetic alterations commonly found in diffusely infiltrating cerebral gliomas are rare or absent in pleomorphic xanthoastrocytoma. Journal of Neuropathology & Experimental Neurology 61:1092-1099

Kawano Y, Sasaki M, Nakahira K, Yoshimine T, Shimizu K, Wada H, Ikenaka K (2001) Structural

characterization and chromosomal localization of the *MAGE-E1* gene. Gene 277:129-137

Kepes, Louis D, Giannini C, Paulus W (2000) Pleomorphic Xanthoastrocytoma. In: Kleihues P, Cavenee W (eds) Pathology and Genetics: Tumours of the Nervous System. IRAC, Lyon,

Kepes, Louis D, Paulus W (1993) Pleomorphic xanthoastrocytoma. In: Kleihues P (ed) Pathology and genetics of nervous system tumours. pp 34-36

Kirches E, Oda Y, Von Bassanyi P, Diete S, Schneider T, Warich-Kirches M, Dietzmann K (1997) Mdr1 mRNA expression differs between grade III astrocytomas and glioblastomas. Clinical Neuropathology 16:

Kirkwood (1998) Further Methods for Contingency Tables. In: Kirkwood B (ed) Essentials of Medical Statistics. Blackwell Scientific, London, UK, pp 94-105

Kirla R, Salminen E, Huhtala S, Nuutinen J, Talve L, Haapasalo H, Kalimo H (2000) Prognostic value of the expression of tumour suppressor genes p53, p21, p16 and pRB, and Ki-67 labelling in high grade astrocytomas treated with radiotherapy. Journal of Neuro-Oncology 46:71-80

Kittler R, Stoneking M, Kayser M (2002) A whole genome amplification method to generate long fragments from low quantities of genomic DNA. Analytical Biochemistry 300:237-244

Kleihues, Burger P, Collins V, Newcomb E, Ohgaki H, Cavenee W (2000) Glioblastoma. In: Kleihues P, Cavenee W (eds) Pathology and Genetics: Tumours of the Nervous System. IRAC, Lyons,

Kleihues, Burger P, Plate K, Ohgaki H, Cavenee W (1993) Glioblastoma. In: Kleihues P (ed) Pathology and genetics of nervous system tumours. pp 16-29

Kleihues, Davies R, Ohgaki H, Burger P, Westphal M, Cavenee W (2001a) Diffuse Astrocytoma. In: Kleihues P, Cavenee W (eds) Pathology and Genetics: Tumours of the Nervous System. IRAC, Lyons,

Kleihues, Davis R, Coons S, Burger P (2001b) Anaplastic astrocytoma. In: Kleihues P, Cavenee W (eds) Pathology and Genetics: Tumours of the Nervous System. IRAC, Lyons,

Kleihues P, Ohgaki H (1999) Primary and secondary glioblastomas: From concept to clinical diagnosis. Neuro-Oncology 1:

Klein G (1987) The approaching era of the tumour suppressor genes. Science 238:1539

Knudsen A (1971) Mutation and cancer: Statistical study of retinoblastoma. Proceedings of the National Academy of Science USA 68:820-823

Knutsen T (1998) Cytogenetic and molecular characterization of random chromosomal rearrangements activating the drug resistance gene MDR1/P-Glycoprotein in drug selected cell lines and patients with drug refractory ALL. Genes Chromosomes & Cancer 23:44-54

Knutsen T, Rao V, Ried T, Mickley L, Schneider E, Miyake K, Ghadimi B, Padilla-Nash H, Pack S, Greenberger L, Cowan K, Dean M, Fojo T, Bates S (2000) Amplification of 4q21-q22 and the MXR gene in independently derived mitoxantrone resistant cell lines. Genes Chromosomes & Cancer 27:110-116

Knuutila S, Aalto Y, Autio K, Bjorkqvist A-M, El-Rifai W, Hemmer S, Huhta T, Kettunen E, Kiuru-Kuhlefelt S, Larramendy M, Lushnikova T, Monni O, Pere H, Tapper J, Tarkkanen M, Varis A, Wasenius V-M, Wolf M, Zhu Y (1999) DNA copy number losses in human neoplasms. American Journal of Pathology 155:683-694

Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, Trapman J, Cleutjens K, Noordzij A, Visakorpi T, Kallioniemi O (1997) Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Research 57:314-319

Korshunov A, Golanov A (2001) Pleomorphic xanthoastrocytomas: immunohistochemistry, grading and clinico-pathologic correlations. An analysis of 34 cases from a single Institute. Journal of Neuro-Oncology 52:63-72

Korshunov A, Sycheva R, Timirgaz V, Golanov A (1999) Prognostic value of immunoexpression of the chemoresistance related proteins in ependymomas: an analysis of 76 cases. Journal of Neuro-Oncology 45:219-227

Koschny R, Koschny T, Froster U, Krupp W, Zuber M (2002) Comparative genomic hybridisation in glioma: a meta-analysis of 509 cases. Cancer, Genetics & Cytogenetics 135:147-159

Koul D, Willén R, Bendahl P-O, Nilbert M, Borg A (2002) Distinct sets of gene alterations in endometrial carcinoma implicate alternate modes of tumourigenesis. Cancer 94:2369-2379

Kovalic J, Flaris N, Grigsby P, Pirkowski M, Simpson J, Roth K (1993) Intracranial ependymoma long term outcome, patterns of failure. Journal of Neuro-Oncology 15:125-131

Kramer D, Parmiter A, Rorke L, Sutton L, Biegal J (1998) Molecular cytogenetic studies of paediatric ependymomas. Journal of Neuro-Oncology 37:25-33

Kraus J, Bolln C, Wolf H, Neumann J, Kindermann D, Fimmers R, Forster F, Baumann A, Schlegel U (1994) *TP53* alterations and clinical outcome in low grade astrocytomas. Genes Chromosomes & Cancer 10:

Kraus J, de Millas W, Sorensen N, Herbold C, Schichor C, Tonn J, Wiestler O, von Deimling A, Pietsch T (2001) Indications for a tumour suppressor gene at 22q11 involved in the pathogenesis of ependymal tumours and distinct from *hsNF5/INI1*. Acta Neuropathologica 102:69-74

Kraus J, Felsberg J, Tonn J, Reifenberger G, Pietsch T (2002) Molecular genetic analysis of the *TP53, PTEN, CDKN2A, EGFR, CDK4* and *MDM2* tumour-associated genes in supratentorial primitive neuroectodermal tumours and glioblastomas of childhood. Neuropathology and Applied Neurobiology 28:325-333

Kraus J, Koopman J, Kaskel P, Maintz D, Bradner S, Schramm J, Louis D, Wiestler O, Von Deimling A (1995) Shared allelic losses on chromosome 1p and 19q suggest a common origin of oligodendroglioma and oligoastrocytoma. Journal of Neuropathology & Experimental Neurology 54:91-95

Kretschmar C, Tarbell N, Barnes P (1993) Pre-irradiation chemotherapy and hyperfractionated radiation therapy 66 Gy for children with brain stem tumours. A phase II study of the Pediatric Oncology Group. Protocol 8833. Cancer 72:1404-1413

Kros J, Van Run P, Alers J, Beverloo H, Van den Bent M, Avezaat C, van Dekken H (1999) Genetic aberrations in oligodendroglial tumours: An analysis using comparative genomic hybridisation (CGH). Journal of Pathology 188:282-288

Kucerova H, Stejskalova E, Vicha A, Tichy M, Chanova M, Sumerauer D, Koutecky J, Eckschlager T (2000) Gene aberrations in childhood brain tumours. Folia Biologica (Praha) 46:187-190

Kun L, Kovnar E, Sanford R (1988) Ependymomas in children. Pediatric Neuroscience 14:57-63

Kunwar S, Mohapatra G, Bollen A, Lamborn K, Prados M, Feuerstein B (2001) Genetic subgroups

of anaplastic astrocytomas correlate with patient age and survival. Cancer Research 61:7683-7688

Kuukasjarvi T, Tanner M, Pennanen S, Karhu R, Visakorpi T, Isola J (1997) Optimizing DOP-PCR for universal amplification of small DNA samples in comparative genomic hybridisation. Genes Chromosomes & Cancer 18:94-101

Lamszus K, Lachenmayer L, Heinemann U, Kluwe L, Finckh U, Hoppner W, Stavrou D, Fillbrandt R, Westphal M (2001) Molecular genetic alterations on chromosomes 11 and 22 in ependymoma. International Journal of Cancer 91:803-808

Land H, Parada L, Weinberg R (1983) Cellular oncogenes and multistep carcinogenesis. Science 322:

Lane D (1993) A death in the life of p53. Nature 362:786-787

Lang F, Miller D, Pisharody S, Koslow M, Newcomb E (1994) High frequency of p53 protein accumulation without p53 gene mutation in human juvenile pilocytic, low grade and anaplastic astrocytomas. Oncogene 9:949-954

Lehnert M (1994) Multidrug resistance in human cancer. Journal of Neuro-Oncology 22:239-243

Leon S, Zhu J, McL.Black P (1994) Genetic aberrations in human brain tumours. Neurosurgery 34:708-722

Levine A, Momand J (1990) Tumour suppressor genes: The p53 and retinoblastoma sensitivity genes and gene products. Biochem. Biophys. Acta. 1032:119

Leyns L, Bouwmeester T, Kim S-H, Piccolo S, De Robertis E (1997) Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. Cell 88:747-756

Liang B (1996) Effects of hypoxia on drug resistance phenotype and genotype in human glioma cell lines. Journal of Neuro-Oncology 29:149-155

Lim J, Kuroki T, Ozaki K, Kohsaki H, yamori T, Tsuruo T, Nakamori S, Imaoka S, Endo M, Nakamura Y (1997) Isolation of murine and human homologues of the fission-yeast dis3+ gene encoding a mitotic-control protein and its overexpression in cancer cells with progressive phenotype. Cancer Research 57:921-925

Litofsky N, Hinton D, Raffel C (1994) The lack of a role for p53 in astrocytomas in pediatric patients. Neurosurgery 34:967-972

Littman P, Jarret P, Bilanuk L (1982) Pediatric brainstem gliomas. Cancer 45:2787-2792

Liu H, Boogs J, Kidd J (1976) Ependymomas of childhood: Histological survey and clinicopathological correlation. Childs Brain 2:92-110

Liu L, Ichimura K, Pettersson E, Collins V (1998) Chromosome 7 rearrangements in glioblastomas; Loci adjacent to EGFR are independently amplified. Journal of Neuropathology & Experimental Neurology 57:1138-1145

Liu L, Ichimura K, Pettersson E, Goike H, Collins V (2000) The complexity of the 7p12 amplicon in human astrocytic gliomas: Detailed mapping of 246 tumours. Journal of Neuropathology & Experimental Neurology 59:1087-1093

Lopes, VandenBerg S, Scheithauer B (1993) The World Health Organisation Classification of Nervous System Tumours in Experimental Neuro-Oncology. In: Levine A, Schmidek H (eds) Molecular Genetics of Nervous System Tumours. Wiley-Liss, New York, pp 1-36

Lopes, VandenBerg S, Scheithauer B (1995) Histopathology, immunochemistry and ultrastructure of brain tumours. In: Kaye A, Laws E (eds) Brain Tumours. Churchill Livingstone, New York, pp 125-162

López-Aguilar E, Sepúlveda-Vildósola A, Rivera-Márquez H, Cerecedo-Díaz F, Hernández-Contreras I, Ramón-García G, Diegopérez-Ramírez J, Santacruz-Castillo E (2000) Preirradiation Ifosfamide, Carboplatin and Etoposide for the treatment of anaplastic astrocytomas and glioblastoma multiforme: A phase II Study. Archives of Medical Research 31:186-190

Louis D (1994) The p53 gene and protein in human brain tumours. Journal of Neuropathology & Experimental Neurology 53:11-21

Louis D (1997) A molecular genetic model of astrocytoma histopathology. Brain Pathology 7:755-764

Lowe S, Ruley H (1993) Stabilization of the p53 tumour suppressor is induced by adenovirus 5 EIA and accompanies apoptosis. Genes Development 7:535-545

Lowe S, Schmit E, Smith S, Osborne B, Jacks T (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362:847-849

Lu Q, Park J, Noll E, Chan J, Alberta J, Yuk D, Alzamora M, Louis D, Stiles C, Rowitch D, Black P (2001) Oligodendrocyte lineage genes (*OLIG*) as molecular markers for human glial brain tumours. Proceedings of the National Academy of Science USA 98:10851-10856

Luk C, Tsao M-S, Bayani J, Shepherd F, Squire J (2001) Molecular cytogenetic analysis of non-small cell lung carcinoma by spectral karyotyping and comparative genomic hybridisation. Cancer, Genetics & Cytogenetics 125:87-99

Lyons M, Kelly P (1991) Posterior fossa ependymomas: Report of 30 cases and review of the literature. Neurosurgery 28:659-665

Madden J, Fenton L, Weil M, Winston K, Partington M, Foreman N (2001) Experience with tamoxifen/etoposide in the treatment of a child with myxopapillary ependymoma. Medical & Pediatric Oncology 37:67-69

Maier H, Jones C, Jasani B, Ofner D, Zelger B, Schmid K, Budka H (1997) Metallothionein overexpression in human brain tumours. Acta Neuropathologica 94:599-604

Mannoji H, Becker L (1988) Ependymal and choroid plexus tumours. Cytokeratin and GFAP expression. Cancer 61:1377-1385

Mao X, Hamoudi R (2000) Molecular and cytogenetic analysis of glioblastoma multiforme. Cancer, Genetics & Cytogenetics 122:87-92

Marsa G, Probert J, Rubinstein L (1973) Radiation therapy in the treatment of childhood astrocytic gliomas. Cancer 32:646-655

Martinez A, Treston A, Saldise L, Montuenga L, Linnoila R (1996) Expression of peptidyl-glycine alpha-amidating mono-oxygenase (PAM) enzymes in morphological abnormalities adjacent to pulmonary tumours. American Journal of Pathology 149:707-716

Maruno M, Ninomiya H, Muhammad A, Hirata M, Kato A, Yoshimine T (2000) Whole genome analysis of human astrocytic tumours by comparative genomic hybridisation. Brain Tumor Pathology 17:21-27

Marx J (1988) "Jun" is bustin' out all over (Research News). Science 242:1377-1378

Mashiyama S, Murakami Y, Yoshimoto T, Sekiya T, Hayashi K (1991) Detection of p53 mutations in human brain tumours by single-strand conformation polymorphism analysis of polymerase chain reaction products. Oncogene 6:1313-1318

Mastronardi L, Puzzilli F, Couldwell W, Farah J, Lunardi P (1998) Tamoxifen and carboplatin combinational treatment of high-grade glioma. Results of a clinical trial on newly diagnosed patients. Journal of Neuro-Oncology 38:59-68

Matsumoto R, Tada M, Nozaki M, Zhang C, Sawamura Y, Abe H (1998) Short alternative splice transcripts of the mdm2 oncogene correlate to malignancy in human astrocytic neoplasms. Cancer Research 58:609-613

Matsuo M, Yonemitsu N, Zaitsu M, Ishii K, Hamasaki Y, Fukuyama K, Tabuchi K, Miyazaki S (2001) Expression of prostaglandin H synthase-2 in human brain tumours. Acta Neuropathologica 102:181-187

Mautner V, Tatagiba M, Guthoff R, Samii M, Pulst S-M (1993) Neurofibromatosis 2 in the paediatric age group. Neurosurgery 33:92-96

Mazewski C, Soukup S, Ballard E, Gotwals B, Lampkin B (1999) Karyotype studies in 18 ependymomas with literature review of 107 cases. Cancer, Genetics & Cytogenetics 113:1-8

McCormick P, Torres R, Post K, Stein B (1990) Intramedullary ependymoma of the spinal cord. Journal of Neurosurgery 72:523-532

McCowage G, Tien R, McLendon R, Felsberg G, Fuchs H, Graham M, Kurtzberg J, Moghrabi A, Ferrell L, Kerby T, Duncan-Brown M, Stewart E, Robertson P, Colvin O, Golembe B, Bigner D, Friedman H (1996) Successful treatment of childhood pilocytic astrocytomas metastatic to the leptomeninges with high-dose cyclophosphamide. Medical & Pediatric Oncology 27:32-39

McGirr S, K, Scheithauer B, Elly P (1987) Stereotactic resection of juvenile pilocytic astrocytomas of the thalamus and basal ganglia. Neurosurgery 20:447-452

McLaughlin M, Marcus R, Buatti J, McCollough W, Mickle J, Kedar A, Maria B, Million R (1998) Ependymoma: Results, prognostic factors and treatment recommendations. International Journal Radiation Oncology Biol Phys 40:845-850

McLendon R, Fung K, Bentley R, Rasheed BKA, Trojanowski J, Bigner S, Bigner D, Friedman H (1996) Production and characterization of two ependymoma xenografts. Journal of Neuropathology & Experimental Neurology 55:540-548

McNally R, Cairns D, Eden O, Alexander F, Taylor G, Kelsey A, Birch J (2002) An infectious aetiology for childhood brain tumours? Evidence from space-time clustering and seasonality analyses. British Journal of Cancer 86:1070-1077

Mercer W, Shields M, Amin M, Sauve G, Appella E, Romano J, Ullrich S (1990) Negative growth regulation in a glioblastoma tumour cell line that conditionally expresses human wild-type p53. Proceedings of the National Academy of Science USA 87:6166-6170

Mertens F, Johansson B, Hoglund M, Mitelman F (1997) Chromosomal imbalance maps of malignant solid tumours: A cytogenetic survey of 3185 neoplasms. Cancer Research 57:2765-2780

Metzger A, Sheffield V, Duyk G, Daneshvar L, Edwards M, Cogen P (1991) Identification of a germ line mutation in the p53 gene in a patient with an intracranial ependymoma. Proceedings of the National Academy of Science USA 88:7825-7829

Michiels E, Weiss M, Hoovers J, Baak J, Voûte P, Baas F, Hermsen M (2002) Genetic alterations in

childhood medulloblastoma analysed by comparative genomic hybridisation. Journal of Pediatric Haematology/Oncology 24:205-210

Mills G, Schmandt R, McGill M, Amendola A, Hill M, Jacobs K, May C, Rodricks A, Campbell S, Hogg D (1992) Expression of TTK, a novel human protein kinase, is associated with cell proliferation. The Journal of Biological Chemistry 267:16000-6

Minehan K, Shaw E, Scheithauer B, Davis D, Onofiro B (1995) Spinal cord astrocytoma: Pathological and treatment considerations. Journal of Neurosurgery 83:590-595

Miozzo M, Dalpra L, Riva P, Volonte M, Macciardi F, Pericotti S, Tibiletti M, Cereti M, Rohde K, Larizza L, Fuhrman Conti A (2000) A tumour suppressor locus in familial and sporadic chordoma maps to 1p36. International Journal of Cancer 87:68-72

Missiaglia E, Moore P, Williamson J, Lemoine N, Falconi M, Zamboni G, Scarpa A (2002) Sex chromosome anomalies in pancreatic endocrine tumours. International Journal of Cancer 98:532-538

Miyake K, Mickley L, Litman T, Zhan Z, Robey R, Cristensen B, Brangi M, Greenberger L, Dean M, Fojo T, Bates S (1999) Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: Demonstration of homology to ABC transport genes. Cancer Research 59:8-13

Mohapatra G, Bollen A, Kim D, Lamborn K, Moore D, Prados M, Feuerstein B (1998) Genetic analysis of glioblastoma multiforme provides evidence for subgroups within the grade. Genes Chromosomes & Cancer 21:195-206

Mohapatra G, Kim D, Feuerstein B (1995) Detection of multiple gains and losses of genetic material in 10 glioma cell lines by comparative genomic hybridisation. Genes Chromosomes & Cancer 13:86-93

Mohri M, Nitta H, Yamashita J (2000) Expression of multidrug resistance-associated protein (MRP) in human gliomas. Journal of Neuro-Oncology 49:105-115

Mollenhauer J, Herbetz S, Holmskov U, Tolnay M, Krebs I, Merlo A, Schroder H, Maier D, Breitling F, Wiemann S, Grone H, Poustka A (2000) DMBT1 encodes a protein involved in the immune defense and in epithelial differentiation and is highly unstable in cancer. Cancer Research 60:1704-1710

Mollenhauer J, Wiemann S, Scheurlen W, Korn B, Hayashi Y, Wilgenbus K, von Deimling A, Poustka A (1997) DMBT1, a new member of the SRCR superfamily on chromosome 10q25.3-26.1 is deleted in malignant brain tumours. Nature Genetics 17:32-39

Moon Y-W, Weil R, Pack S, Park W-S, Pak E, Pham T, Karkera J, Kim H-K, Vortmeyer A, Fuller B, Zhuang Z (2000) Missense mutation of the *MET* gene detected in human glioma. Modern Pathology 13:973-977

Mora J, Cheung N-K, Chen L, Qin J, Gerald W (2001) Loss of heterozygosity at 19q13.3 is associated with locally aggressive neuroblastoma. Clinical Cancer Research 7:

Mostert M, van de Pol. M, Weghuis D, Suijkerbuijk R, van Kessel A, van Echten J, Oosterhuis J, Looijenga L (1996) Comparative genomic hybridisation of germ cell tumours of the adult testis: Confirmation of karyotype findings and identification of a 12p-amplicon. Cancer, Genetics & Cytogenetics 89:146-152

Mueller H, Michel A, Heckel D, Fischer U, Tonnes M, Tsui L, Scherer S, Zang K, Meese E (1997) Identification of an amplified gene cluster in glioma including two novel amplified genes isolated

by exon trapping. Human Genetics 101:190-197

Nabors M, Griffin C, Zehnbauer B, Hruban R, Phillips P, Grossman S, Brem H, Colvin O (1991) Multidrug resistance gene (MDR1) expression in human brain tumours. Journal of Neurosurgery 75:941-946

Nagane M, Asai A, Shibui S, Oyama H, Nomura K, Kuchino Y (1999) Expression pattern of chemoresistance-related genes in human malignant brain tumours: a working knowledge for proper selection of anticancer drugs. Japanese Journal of Clinical Oncology 29:527-534

Nagane M, Coufal F, Lin H, Bogler O, Cavenee W, Huang H (1996) A common mutant epidermal growth factor receptor confers enhanced tumourigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. Cancer Research 56:5079-5086

Nakamura M, Watanabe T, Klangby U, Asker C, Wilman K, Yonekawa Y, Kleihues P, Ohgaki H (2001a) *p14^{ARF}* deletion and methylation in genetic pathways to glioblastoma. Brain Pathology 11:159-168

Nakamura M, Watanabe T, Yonekawa Y, Kleihues P, Ohgaki H (2001b) Promotor methylation of the DNA repair gene *MGMT* in astrocytomas is frequently associated with G:C ∅ A:T mutations of the *TP53* tumour suppressor gene. Carcinogenesis 22:1715-1719

Nakayama M, Nakajima D, Nagase T, Nomura N, Seki N, Ohara O (1998) Identification of high-molecular weight proteins with multiple EGF-like motifs by motif-trap screening. Genomics 51:27-34

Nazar G, Hoffman H, Becker L, Jenkin D, Humphreys R, Hendrick EB (1990) Infratentorial ependymomas in childhood: Prognostic factors and treatment. Journal of Neurosurgery 72:408-417

Neat M, Foot N, Jenner M, Goff L, Ashcroft K, Burford D, Dunham A, Norton A, Lister T, Fitzgibbon J (2001) Localisation of a novel region of recurrent amplification in follicular lymphoma to an ~6.8Mb region of 13q32-33. Genes Chromosomes & Cancer 32:236-243

Neumann E, Kalousek D, Norman M, Steinbok P, Cochrane D, Goddard K (1993) Cytogenetic analysis of 109 pediatric central nervous system tumours. Cancer, Genetics & Cytogenetics 71:40-49

Newcomb E, Alonso M, Sung T, Miller D (2000) Incidence of p14^{ARF} gene deletion in high grade adult and pediatric astrocytomas. Human Pathology 31:115-119

Newcomb E, Bhalla S, Parrish C, Hayes R, Cohen H, Miller D (1997) bcl-2 protein expression in astrocytomas in relation to patient survival and p53 gene status. Acta Neuropathologica 94:639-

Nijssen P, Deprez R, Tijssen C, Hagemeijer A, Arnoldus E, Teepen J, Holl R, Niermeyer M (1994) Familial anaplastic ependymoma: Evidence of loss of chromosome 22 in tumour cells. Journal of Neurology, Neurosurgery & Psychiatry 57:1245-1248

Nishikawa R, Furnari F, Lin H, Arap W, Berger M, Cavenee W, Su H (1995) Loss of P16INK4 expression is frequent in high grade gliomas. Cancer Research 55:1941-1945

Nishizaki T, Ozaki S, Harada K, Ito H, Arai H, Beppu T, Sasaki K (1998) Investigation of genetic alterations associated with grade of astrocytic tumour by comparative genomic hybridisation. Genes Chromosomes & Cancer 21:340-346

Nitiss J, Beck W (1996) Antitopoisomerase drug action and resistance. European Journal of Cancer 32A:958-966

Noonan K, Beck C, Holzmayer T, Chin J, Wunder J, Andrulis I, Gazdar A, Willman C, Griffith B, Von Hoff D, Roninson I (1990) Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumours by polymerase chain reaction. Proceedings of the National Academy of Science USA 87:7160-7164

Nozaki M, Tada M, Matsumoto R, Sawamura Y, Abe H, Iggo R (1998) Rare occurrence of inactivating p53 gene mutations in primary non-astrocytic tumours of the central nervous system: reappraisal by yeast functional assay. Acta Neuropathologica 95:291-296

Nupponen N, Isola J, Visakorpi T (2000) Mapping the amplification of *EIF3S3* in breast and prostate cancer. Genes Chromosomes & Cancer 28:203-210

O'Driscoll L, Daly C, Saleh M, Clynes M (1993) The use of reverse transcriptase-polymerase chain reaction (RT-PCR) to investigate specific gene expression in multidrug resistant cells. Cytotechnology 12:289-314

Oda Y, Rose I, Radig K, Wagemann W, Mittler U, Roessner A (1997) Expression of MDR1/P-glycoprotein and multidrug resistance associated protein in childhood solid tumours. Virchows Archiv 430:99-105

Oga A, Kong G, Tae K, Lee Y, Sasaki K (2001) Comparative genomic hybridisation analysis reveals 3q gain resulting in genetic alteration in 3q in advanced oral squamous cell carcinoma. Cancer, Genetics & Cytogenetics 127:24-29

Ohgaki H, Eibl R, Schwab M, Reichel M, Mariani L, Gehring M, Petersen I, Holl T, Wiestler O, Kleihues P (1993) Mutations of the p53 tumour suppressor gene in neoplasms of the human nervous system. Molecular Carcinogenesis 8:74-80

Ohgaki H, Eibl R, Wiestler O, Yasargil M, Newcomb E, Kleihues P (1991) p53 mutations in non-astrocytic human brain tumours. Cancer Research 51:6202-6205

Ohgaki H, Watanabe K, Peraud A, Biernat W, von Deimling A, Yasargil G, Yonekawa Y, Kleihues P (1999) A case history of glioma progression. Acta Neuropathologica

Ohta S, Ryu H, Miura K (1999) Eighteen-year survival of a patient with malignant pleomorphic xanthoastrocytoma associated with von Recklinghausen neurofibromatosis. British Journal of Neurosurgery 13:420-422

Olopade O, Jenkins R, Ransom D, Malik K, Pomykala H, Nobori T, Cowan J, Rowley J, Diaz. MO (1992) Molecular analysis of deletions of the short arm of chromosome 9 in human gliomas. Cancer Research 52:2523-2529

Orellana C, Hernandez-Marti M, Martinez F, Castel V, Millan J, Alvarez-Garijo J, Prieto F, Badia L (1998) Pediatric brain tumours: Loss of heterozygosity at 17p and TP53 gene mutations. Cancer, Genetics & Cytogenetics 102:93-99

Orr L, Fleitz J, McGavran L, Wyatt-Ashmead J, Handler M, Foreman N (2002) Cytogenetics in pediatric low-grade astrocytomas. Medical & Pediatric Oncology 38:173-177

Oskam N, Bijleveld E, Hulsebos T (2000) A region of common deletion in 22q13.3 in human glioma associated with astrocytoma progression. International Journal of Cancer 85:336-339

Packer R (1994) Diagnosis and treatment of pediatric brain tumours. Current Opinion in Neurology 7:484-491

Packer R (1999) Brain tumours in children. Archives of Neurology 56:421-425

Packer R, Boyett J, Zimmermann R (1993) Hyperfractionated radiation therapy (72 Gy) for children with brain stem gliomas. A Childrens Cancer Group Phase I/ /II trial. Cancer 72:1414-1421

Packer R, Boyett J, Zimmermann R (1994) Outcome of children with brain stem gliomas after treatment with 7800 cGy of hyperfractionated radiotherapy. A Childrens Cancer Group Phase I/II trial. Cancer 74:1827-1834

Palma L, Celli P, Mariottini A, Zalaffi A, Schettini G (2000) The importance of surgery in supratentorial ependymomas: Long term survival in a series of 23 cases. Childs Nervous System 16:170-175

Palma L, Maleci A, Di Lorenzo N, Lauro G (1985) Pleomorphic xanthoastrocytoma with 18 year survival. Case report. Journal of Neurosurgery 63:808-810

Park J, Chaffee S, Noll W, Rhodes C (1996) Constitutional de novo t(1;22)(p22;q11.2) and ependymoma. Cancer, Genetics & Cytogenetics 86:150-152

Parry L, Maynard J, Patel A, Hodges A, von Deimling A, Sampson J, Cheadle J (2000) Molecular analysis of the *TSC1* and *TSC2* tumour suppressor genes in sporadic glial and glioneuronal tumours. Human Genetics 107:350-356

Paulino A (2001) The local field in infrantentorial ependymoma: Does the entire posterior fossa need to be treated? International Journal Radiation Oncology Biol Phys 49:757-761

Paulino A, Wen B-C (2000) The significance of radiotherapy treatment duration in intracranial ependymoma. International Journal Radiation Oncology Biol Phys 47:585-589

Paulino A, Wen B-C, Buatti J, Hussey D, Zhen W, Mayr N, Menezes A (2002) Intracranial ependymomas. An analysis of prognostic factors and patterns of failure. American Journal of Clinical Oncology 25:117-122

Paulus W, Lisle D, Tonn J, Wolf H, Roggendorf W, Reeves S, Louis D (1996) Molecular genetic alterations in pleomorphic xanthoastrocytoma. Acta Neuropathologica 91:293-297

Pencalet P, Maixner W, Sainte-Rose C, Lellouch-Tubiana A, Cinalli G, Zerah M, Pierre-Kahn A, Hoppe-Hirsch E, Bourgeois M, Renier D (1999) Benign cerebellar astrocytomas in children. Journal of Neurosurgery 90:265-273

Perinchery G, Bukurov N, Nakajima K, Chang J, Li L-C, Dahiya R (1999) High frequency of deletion on chromosome 9p21 may harbour several tumour suppressor genes in human prostate cancer. International Journal of Cancer 83:610-614

Phelan C, Liu L, Ruttledge M, Muntzning K, Ridderheim P, Collins V (1995) Chromosome 17 abnormalities and lack of TP53 mutations in pediatric central nervous system tumours. Human Genetics 96:684-690

Pierre-Khan A, Hirsch J, Roux F, Renier D, Sainte-Rose C (1983) Intracranial ependymomas in childhood. Survival and functional results of 47 cases. Childs Brain 10:145-156

Pollack I (1994) Brain tumours in children. New England Journal of Medicine 331:1500-1507

Pollack I (1999) The role of surgery in pediatric gliomas. Journal of Neuro-Oncology 42:271-288

Pollack I, Claassen D, Al-Shboul Q, Janosky J, Deutsch M (1995) Low grade gliomas of the cerebral hemispheres in children: An analysis of 71 cases. Journal of Neurosurgery 82:536-547

Pollack I, Finkelstein S, Burnham J, Holmes E, Hamilton R, Yates A, Finlay J, Sposto R (2001) Age and *TP53* mutation frequency in childhood malignant gliomas: Results in a Multi-institutional cohort. Cancer Research 68:7404-7407

Pollack I, Finkelstein S, Woods J, Burnham J, Holmes E, Hamilton R, Yates A, Boyett J, Finlay J, Sposto R, The Childrens Cancer Group (2002) Expression of p53 and prognosis in children with malignant gliomas. The New England Journal of Medicine 346:420-427

Pollack I, Gerszten P, Martinez A, Lo K-H, Shultz B, Albright L, Janosky J, Deutsch M (1995) Intracranial ependymomas of childhood: Long term outcome and prognostic factors. Neurosurgery 37:655-667

Pollack I, Hamilton R, Finkelstein S, Campbell J, Martinez A, Sherwin R, Bozik M, Gollin S (1997) The relationship between TP53 mutations and overexpression of p53 and prognosis in malignant gliomas of childhood. Cancer Research 57:304-309

Pollack I, Hoffman H, Humphreys R, Becker L (1993) The long term outcome after surgical treatment of dorsally exophytic brainstem gliomas. Journal of Neurosurgery 78:859-863

Pollack, PM, Welch, J, and Hayward, NK. Evidence for three tumour suppressor loci on chromosome 9p involved in melanoma development. Cancer Research 61, 1154-1161. 2001.

Punt (1995) Management of brain tumours in childhood. In: Thomas D, Graham D (eds) Malignant Brain tumours, 1st edn. Springer-Verlag, London, pp 171-186

Raco A, Bristot R, Salvati M, Delfini R (1997) Malignant supratentorial astrocytomas of late childhood. Childs Nervous System 13:341-344

Raffel C (1996) Molecular biology of pediatric gliomas. Journal of Neuro-Oncology 28:121-128

Raffel C, Frederick L, O'Fallon J, Atherton-Skaff P, Perry A, Jenkins R, James C (1999) Analysis of oncogene and tumour suppressor gene alterations in pediatric malignant astrocytomas reveals reduced survival for patients with PTEN mutations. Clinical Cancer Research 5:4085-4090

Raffel C, Jenkins R, Frederick L, Hebrink D, Alderete B, Fults D, James C (1997) Sporadic medulloblastomas contain PTCH mutations. Cancer Research 57:842-845

Ragoussis J, Senger G, Trowdale J, Campbell I (1992) Genomic organisation of the human folate receptor genes on chromosome 11q13. Genomics 14:423-430

Ransom D, Ritland S, Kimmel D, Moertel C, Dahl R, Scheithauer B, Kelly P, Jenkins R (1992) Cytogenetic and loss of heterozygosity studies in ependymomas, pilocytic astrocytomas and oligodendrogliomas. Genes Chromosomes & Cancer 5:348-356

Rao P, Houldsworth J, Dyomina K, Parsa N, Cigudosa J, Louie D, Popplewell L, Offit K, Jhanwar S, Chaganti R (1998a) Chromosomal and gene amplification in diffuse large B-cell lymphoma. Blood 92:234-240

Rao P, Houldsworth J, Palanisamy N, Murty V, Reuter V, Motzer R, Bosl G, Chaganti R (1998b) Chromosomal amplification is associated with cisplatin resistance of human male germ cell tumours. Cancer Research 58:4260-4263

Rasheed B, McLendon R, Herndon J, Friedman HS, Friedman A, Bigner D, Bigner S (1994) Alterations of the TP53 gene in human gliomas. Cancer Research 54:1324-1330

Rawlings C, Giangaspero F, Burger P, Bullard D (1988) Ependymoma: a clinicopathologic study. Surgical Neurology 29:271-281

Reardon D, Entrekin R, Sublett J, Ragsdale S, Li H, Boyett J, Kepner J, Look A (1999) Chromosome arm 6q is the most common recurrent autosomal alteration detected in primary paediatric ependymoma. Genes Chromosomes & Cancer 24:230-237

Reddy A, Packer R (1999) Chemotherapy for low-grade gliomas. Childs Nervous System 15:506-513

Rees (1994) Further tests for percentages. In: Rees D (ed) Essential statistics for medical practice: A case study approach, First edn. Chapman & Hill, London, pp 163-166

Reifenberger G, Ichimura K, Reifenberger J, Elkahloun A, Meltzer P, Collins V (1996) Refined mapping of 12q13-q15 amplicons in human malignant gliomas suggests *CDK4/SAS* and *MDM2* as independent amplification targets. Cancer Research 5141-5145:

Reifenberger G, Liu L, Ichimura K, Schmidt E, Collins V (1993) Amplification and overexpression of the MDM2 gene in a subset of human malignant gliomas without p53 mutations. Cancer Research 53:2736-2739

Reifenberger G, Reifenberger J, Ichimura K, Collins V (1995) Amplification at 12q13-14 in human malignant gliomas is frequently accompanied by loss of heterozygosity at loci proximal and distal to the amplification site. Cancer Research 55:731-734

Reifenberger G, Reifenberger J, Ichimura K, Meltzer P, Collins V (1994a) Amplification of multiple genes from chromosomal region12q13-14 in human malignant gliomas: preliminary mapping of the amplicons shows preferential involvement of CDK4, SAS and MDM2. Cancer Research 54:4299-4303

Reifenberger J, Reifenberger G, Liu L, James C, Wechsler W, Collins V (1994b) Molecular genetic analysis of oligodendroglial tumours shows preferential allelic deletions on 19q and 1p. American Journal of Pathology 145:1175-1190

Rey J, Bello J, de Campos J, Vaquero J, Kusak M, Sarasa J, Pestana A (1993) Abnormalities of chromosome 22 in human brain tumours determined by combined cytogenetic and molecular approaches. Cancer, Genetics & Cytogenetics 66:1-10

Rey J, Bello M, Jimenez-Lara A, Vaquero J, Kusak M, De Campos J, Sarasa J, Pestana A (1992) Loss of heterozygosity for distal markers on 22q in human gliomas. International Journal of Cancer 51:703-706

Richter J, Wagner U, Kononen J, Fijan A, Bruderer J, Schmid U, Ackerman D, Maurer R, Alund G, Knönagel H, Rist M, Wilber K, Anabitarte M, Hering F, Hardmeier T, Schönonberger A, Flury R, Jäger P, Fehr J, Schraml P, Moch H, Mihatsch M, Gasser T, Kallioniemi O, Sauter G (2000) High-throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. American Journal of Pathology 157:787-794

Rickert C, Strater R, Kaatsch P, Wassman H, Jurgens H, Dockhorn-Dworniczak B, Paulus W (2001) Pediatric high grade astrocytomas show chromosomal imbalances distinct from adult cases. American Journal of Pathology 158:1525-1532

Rickman D, Bobek M, Misek D, Kuick R, Blaivas M, Kurnit D, Taylor J, Hanash S (2001) Distinctive molecular profiles of high-grade and low-grade gliomas based on oligonucleotide microarray analysis. Cancer Research 61:6885-6981

Roberts P, Chumas P, Picton S, Bridges L, Livingstone J, Sheridan E (2001) A review of the cytogenetics of 58 pediatric brain tumours. Cancer, Genetics & Cytogenetics 131:1-12

Robertson P, Allen J, Abbott I, Miller D, Fidel J, Epstein F (1994) Cervicomedullary tumours in

children: A distinct subset of brainstem gliomas. Neurology 44:1798-1803

Robertson P, Zeltzer P, Boyett J, Rorke L, Allen J, Geyer J, Stanley P, Li H, Albright A, McGuire-Cullen P, Finlay J, Stevens K, Milstein J, Packer R, Wisoff J, The Childrens Cancer Group (1998) Survival and prognostic factors following radiation therapy and chemotherapy for ependymoma in children: A report of the children cancer study group. Journal of Neurosurgery 88:695-703

Rodriguez C, Causse A, Ursule E, Theillet C (2000) At least 5 regions of imbalance on 6q in breast tumours, combining losses and gains. Genes Chromosomes & Cancer 27:76-84

Rodriguez L, Edwards M, Levin V (1990) Management of hypothalamic gliomas in children: An analysis of 33 cases. Neurosurgery 26:242-246

Roepe P (2000) What is the precise role of human MDR1 protein in chemotherapeutic drug resistance. Curr Pharm Design 6:341-360

Rogatto S, Casartelli C, Rainho C, Barbieri-Neto J (1993) Chromosome in the genesis and progression of ependymoma. Cancer, Genetics & Cytogenetics 69:146-152

Rolhion C, Penault-Llorca F, Kemeny J-L, Kwiatkowski F, Lemaire J-J, Chollet P, Finat-Duclos F, Verrelle P (1999) O^6-methylgunanine-DNA methyltransferase gene (*MGMT*) expression in human glioblastomas in relation to patient characteristics and p53 accumulation. International Journal of Cancer 416:416-420

Rollbrocker B, Waha A, Louis D, Wiestler O, von Deimling A (1996) Amplification of the cyclin-dependent kinase 4 (*CDK4*) gene is associated with high cdk4 protein levels in glioblastoma multiforme. Acta Neuropathologica 92:70-74

Ropiquet F, Giri D, Kwabi-Addo B, Mansukhani A, Ittmann M (2000) increased expression of fibroblast growth factor 6 in human prostatic intraepithelial neoplasia and prostate cancer. Cancer Research 60:4245-4250

Rosenstock J, Evans A, Schut L (1976) Response to vincristine of recurrent brain tumours in children. Journal of Neurosurgery 45:135-140

Rostomily R, Hoyt J, Berger M, Kros J, Alvord E, Wilkins P, Rabinovitch P (1997) Pleomorphic Xanthoastrocytoma: DNA flow cytometry and outcome analysis of 12 patients. Cancer 80:2141-2150

Rothe M, Xiong J, Shu H-B, Williamson K, Goddard A, Goeddel D (1996) I-TRAF is a novel TRAF-interacting protein that regulates TRAF-mediated signal transduction. Proceedings of the National Academy of Science USA 93:8241-8246

Rousseau-Merck M-F, Versteege I, Zattara-Cannoni H, Figarella D, Lena G, Aurias A, Vagner-Capodano A (2000) Fluorescence In Situ Hybridisation determination of 22q12-q13 deletion in two intracranial ependymomas. Cancer, Genetics & Cytogenetics 121:223-227

Rousseau-Merck M-F, Zahraoui A, Touchot N, Tavitian A, Berger R (1991) Chromosome assignment of four RAS-related RAB genes. Human Genetics 86:350-354

Rozenblum E, Vahteristo P, Sandberg T, Bergthorsson J, Syrjakoski K, Weaver D, Haraldsson K, Johannsdottir H, Vehmanen P, Nigam S, Golberger N, Robbins C, Pak E, Dutra A, Gillander E, Stephan D, Bailey-Wilson J, Juo S-H, Kainu T, Arason A, Barkardottir R, Nevanlinna H, Borg A, Kallioniemi O (2002) A genomic map of a 6-Mb region at 13q21-q22 implicated in cancer development: identification and characterisation of candidate genes. Human Genetics 110:111-121

Rubio M, Correa K, Ramesh V, MacCollin M, Jacoby L, Von Deimling A, Gusella J, Louis D (1994a) Analysis of the neurofibromatosis 2 gene in human ependymomas and astrocytoma. Cancer Research 54:45-47

Rubio M, Correa K, Ueki K, Mohrenweiser H, Gusella J, Von Deimling A, Louis D (1994b) The putative glioma tumour suppressor gene on chromosome 19q maps between *APOC2* and *HRC*. Cancer Research 54:4760-4763

Ruggeri B, Huang L, Wood M, Cheng J, Testa J (1998) Amplification and overexpression of the *AKT2* oncogene in a subset of human pancreatic ductal adenocarcinomas. Molecular Carcinogenesis 21:81-86

Ruley (1993) Oncogenes. In: Levine A, Schmidek H (eds) Molecular Genetics of Nervous System Tumours. Wiley-Liss, New York, pp 89-100

Sager R (1989) Tumour suppressor genes: The puzzle and the promise. Science1406-1412

Sainati L, Bolcato S, Montaldi A, Celli P, Stella M, Leszl A, Silvestro L, Perilongo G, Cordero di Montezemolo L, Basso G (1996) Cytogenetics of pediatric central nervous system tumours. Cancer, Genetics & Cytogenetics 91:13-27

Salazar O (1983) A better understanding of CNS seeding and a brighter outlook for postoperatively irradiated patients with ependymoma. International Journal Radiation Oncology Biol Phys 9:

Salazar O, Castro-Vita H, VanHoutte P, Rubin P, Aygun C (1983) Improved survival in cases of intracranial ependymoma after radiation therapy. Journal of Neurosurgery 59:652-659

Sanchez-Cespedes M, Cairns P, Jen J, Sidransky D (1998) Degenerate oligonucleotide-primed PCR (DOP-PCR): Evaluation of its reliability for screening of genetic alterations in neoplasia. Biotechniques 25:1036-1038

Sanoudou D, Tingby O, Ferguson-Smith M, Collins V, Coleman N (2000) Analysis of pilocytic astrocytoma by comparative genomic hybridisation. British Journal of Cancer 82:1218-1222

Sasaki H, Zlatescu M, Betensky R, Ino Y, Cairncross J, Louis D (2001a) PTEN is a target of chromosome 10q loss in anaplastic oligodendrogliomas and PTEN alterations are associated with poor prognosis. American Journal of Pathology 159:359-367

Sasaki M, Nakahira K, Kawano Y, Katakura H, Yoshimine T, Shimizu K, Kim S, Ikenaka K (2001b) MAGE-E1, a new member of melanoma-associated antigen gene family and its expression in human glioma. Cancer Research 61:4809-4814

Sato K, Schäuble B, Kleihues P, Ohgaki H (1996) Infrequent alteration of the p15, p16, CDK4 and cyclin D1 genes in non-astrocytic human brain tumours. International Journal of Cancer 66:305-308

Satoh H, Yoshida M, Matsushime H, Shibuya M, Sasaki M (1987) Regional localization of the human c-ros-1 on 6q22 and flt on 13q12. Japanese Journal of Cancer Research 78:772-775

Sawyer J (1994) Chromosome aberrations in 4 ependymomas. Cancer, Genetics & Cytogenetics 74:132-138

Sawyer J, Swanson C, Roloson G, Longee D, Chadduck W (1992) Cytogenetic findings in a case of pediatric glioblastoma. Cancer, Genetics & Cytogenetics 64:75-79

Schäfer B, Heizmann CW (1996) The S100 family of EF hand calcium binding proteins: function

and pathology. Trends in Biological Sciences 21:134-140

Scheil S, Bruderlein S, Eicker M, Herms J, Herold-Mende C, Steiner H, Barth T, Moller P (2001) Low frequency of chromosomal imbalances in anaplastic ependymomas as detected by comparative genomic hybridisation. Brain Pathology 11:133-143

Schiffer D, Chio A, Giordana M, Migheli A, Palma L, Pollo B, Soffietti R, Tribolo A (1991) Histologic prognostic factors in ependymoma. Childs Nervous System 7:177-182

Schiffer D, Giordana M (1998) Prognosis of ependymoma. Childs Nervous System 14:357-361

Schiffer, Wiestler O (1997) Ependymoma. In: Kleihues P, Cavenee W (eds) Pathology and Genetics: Tumours of the Nervous System. International Agency for Research on Cancer, Lyon, France, pp 49-56

Schmidt H, Taubert H, Würl P, Kappler M, Lange H, Bartel F, Bache M, Holzhausen H-J, Hinze R (2002) Gains of 12q are the most frequent genomic imbalances in adult fibrosarcoma and are correlated with a poor outcome. Genes Chromosomes & Cancer 34:69-77

Schneider J, Raffel C, McComb J (1992) Benign cerebellar astrocytomas of childhood. Neurosurgery 30:58-62

Schraml P, Struckmann K, Bednar R, Fu W, Gasser T, Wilber K, Kononen J, Sauter G, Mihatsch M, Moch H (2001) *CDKN2A* mutation analysis, protein expression, and deletion mapping of chromosome 9p in conventional clear-cell renal carcinomas. American Journal of Pathology 158:593-601

Schröck E, Blume C, Meffert M-C, du Manoir S, Bersch W, Kiessling M, Lozanowa T, Thiel G, Witkowski R, Ried T, Cremer T (1996) Recurrent gain of chromosome arm 7q in low grade astrocytic tumours studied by comparative genomic hybridisation. Genes Chromosomes & Cancer 15:199-205

Sen S, Zhou H, Zhang R-D, Yoon D, Vakar-Lopez F, Ito S, Jiang F, Johnston D, Grossman H, Ruifrok A, Katz R, Brinkley W, Czerniak B (2002) Amplification/overexpression of a mitotic kinase gene in human bladder cancer. Journal of the National Cancer Institute 94:1320-1329

Sevenet N, Lellouch-Tubiana A, Schofield D, Hoang-Xuan K, Gessler M, Birnbaum D, Jeanpierre C, Jouvet A, Delattre O (1999) Spectrum of *hSNF5/INI1* somatic mutations in human cancer and genotype-phenotype correlations. Human Molecular Genetics 8:2359-2368

Shapiro W (1975) Chemotherapy of primary malignant brain tumours in children. Cancer 35:965-972

Shayesteh L, Yiling L, Kuo W, Collins C, Yang-Feng T, Pinkel D, Mills G, Gray J (1999) *PIK3CA* is implicated as an oncogene in ovarian cancer. Nature Genetics 21:99-102

Shen T-L, Han D, Guan J-L (2002) Association of Grb7 with phosphoinositides and it's role in the regulation of cell migration. The Journal of Biological Chemistry 277:29069-29077

Shepherd C, Scheithauer B, Gomez M, Altermatt H, Katzmann J (1991) Subependymal giant cell astrocytoma: A clinical, pathological and flow cytometric study. Neurosurgery 28:864-868

Sherley J (1991) Guanine nucleotide biosynthesis is regulated by the cellular p53 concentration. The Journal of Biological Chemistry 266:24815-24828

Shi H, Yan P, Chen C-M, Rahmatpanah F, Lofton-Day C, Caldwell C, Huang T-M (2002) Expressed CpG island sequence Tag microarray for dual screening of DNA hypermethylation

and gene silencing in cancer cells. Cancer Research 62:3214-3220

Shinoda J, Yamada H, Sakai N, Hirata A, Hirayama H (1989) Malignant cerebellar astrocytic tumours in children. Acta Neurochirurgica 98:1-8

Shinomiya T, Mori T, Ariyama Y, Sakabe T, Fukuda Y, Murakami Y, Nakamura Y, Inazawa J (1999) Comparative genomic hybridisation of squamous cell carcinoma of the esophagus: The possible involvement of the *DP1* gene in the 13q34 amplicon. Genes Chromosomes & Cancer 24:337-344

Shlomit R, Ayala A-G, Michal D, Ninett A, Frida S, Boleslaw G, Gad B, Gideon R, Shlomit C (2000) Gains and losses of DNA sequences in childhood brain tumours analysed by comparative genomic hybridisation. Cancer, Genetics & Cytogenetics 121:67-72

Shono T, Tofilon P, Bruner J, Owolabi O, Lang F (2001) Cyclooxygenase-2 expression in human gliomas: Prognostic significance and molecular correlations. Cancer Research 61:4375-4381

Shuman R, Alvord E, Leech R (1975) The biology of childhood ependymomas. Arch Neurol 32:731-739

Singh P, Gutmann D, Fuller C, Newsham I, Perry a (2002) Differential involvement of protein 4.1 family members *DAL-1* and *NF2* in intracranial and intraspinal ependymomas. Modern Pathology 15:526-531

Skotheim R, Monni O, Mousses S, Fosså, Kallioniemi O, Lothe R, Kallioniemi A (2002) New insights into testicular germ cell tumourigenesis from gene expression profiling. Cancer Research 62:2359-2364

Slavc I, MacCollin M, Dunn M, Jones S, Sutton L, Gusella J, Biegal J (1995) Exon scanning for mutations of the NF2 gene in pediatric ependymomas, rhabdoid tumours and meningiomas. International Journal of Cancer 64:243-247

Smith J, Peery A, Borell T, Lee H, O'Fallon J, Hosek S, Kimmel D, Yates A, Burger P, Scheithauer B, Jenkins R (2000) Alterations of chromosome arms 1p and 19q as predictors of survival in oligodendrogliomas, astrocytomas and mixed oligoastrocytomas. Journal of Clinical Oncology 18:636-645

Smoots D, Geyer J, Lieberman D, Berger M (1998) Predicting disease progression in childhood cerebellar astrocytoma. Childs Nervous System 14:636-648

Solinas-Toldo S, Wallrapp C, Muller-Pillasch F, Bentz M, Gress T, Lichter P (1996) Mapping of chromosomal imbalances in pancreatic carcinoma by comparative genomic hybridisation. Cancer Research 56:3803-3807

Sonneland P, Scheithauer B, Onofrio B (1985) Myxopapillary ependymoma. A clinicopathological and immunocytochemical study of 77 cases. Cancer 56:883-893

Sonoda G, Palazzo J, du Manoir S, Godwin A, Feder M, Yakushiji M, Testa J (1997) Comparative genomic hybridisation detects frequent overrepresentation of chromosomal material from 3q26, 8q24 and 20q13 in human ovarian carcinomas. Genes Chromosomes & Cancer 20:320-328

Sonoda Y, Ozawa T, Aldape K, Deen D, Berger M, Pieper R (2001) Akt pathway activation converts anaplastic astrocytoma to glioblastoma multiforme in a human astrocyte model of glioma. Cancer Research 61:6674-6678

Speicher M, Ballard S, Ward D (1996) Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nature Genetics 12:368-375

Speicher M, du Manoir S, Schrock E, Holtgreve-Grez H, Schoell B, Lengauer C, Cremer T, Ried T (1993) Molecular cytogenetic analysis of formalin-fixed, paraffin embedded solid tumours by comparative genomic hybridisation after universal DNA amplification. Human Molecular Genetics 2:1907-1914

Speicher M, Howe C, Crotty P, du Manoir S, Costa J, Ward D (1995) Comparative genomic hybridisation detects novel deletions and amplifications in head and neck squamous cell carcinomas. Cancer Research 55:1010-1013

Sposto R, Ertel I, Jenkin R, Boesel C, Venes J, Ortega J, Evans A, Wara W, Hammond D (1989) The effectiveness of chemotherapy for treatment of high grade astrocytomas in children: Results of a randomized trial. Journal of Neuro-Oncology 7:165-177

Stafford S, Pollack B, Foote R, Gorman D, Nelson D, Schomberg P (2000) Stereotactic radiosurgery for recurrent ependymoma. Cancer 88:870-875

Steck P, Lin H, Langford L, Jasser S, Koul D, Yung W, Pershouse M (1999) Functional and molecular analyses of 10q deletions in human gliomas. Genes Chromosomes & Cancer 24:135-143

Stoecklein N, Erbersdobler A, Schmidt-Kittler O, Diebold J, Schardt J, Izbicki J, Klein C (2002) SCOMP is superior to degenerate oligonucleotide primed-polymerase chain reaction for global amplification of minute amounts of DNA from microdissected archival tissue samples. American Journal of Pathology 161:43-51

Stoler A, Bouck N (1985) Identification of a single chromosome in the normal human genome essential for suppression of hamster cell transformation. Proceedings of the National Academy of Science USA 82:570-574

Strange P, Wohlert L (1982) Primary brain stem tumours. Acta Neurochirurgica 62:219-232

Stratton M, Collins N, Lakhani S, Sloane J (1995) Loss of heterozygosity in ductal carcinoma in situ of the breast. Journal of Pathology 175:195-201

Stratton M, Darling J, Lantos P, Cooper C, Reeves B (1989) Cytogenetic abnormalities in human ependymomas. International Journal of Cancer 44:579-581

Strojan P, Petric-Grabnar G, Zupancic N, Jereb B (1999) Concomitant chemoradiotherapy for incompletely resected supratentorial low-grade astrocytoma in children: Preliminary report. Medical & Pediatric Oncology 32:112-116

Suehiro Y, Sakamoto M, Umayahara K, Iwabuchi H, Sakamoto H, Tanaka N, Takeshima N, Yamauchi K, Hasumi K, Akiya T, Sakunaga H, Muroya T, Numa F, Kao H, Tenjin Y, Sugishita T (2000) Genetic aberrations detected by comparative genomic hybridisation in ovarian clear cell adenocarcinomas. Oncology 59:50-56

Sugawara I, Watanabe M, Masunaga A, Itoyama S, Ueda K (1992) Primer-dependent amplification of *mdr*1 mRNA by polymerase chain reaction. Japanese Journal of Cancer 83:548-553

Sung T, Miller D, Hayes R, Alonso M, Yee H, Newcomb E (2000) Preferential inactivation of the p53 tumour suppressor pathway and lack of EGFR amplification distinguish *de novo* high grade paediatric astrocytomas from *de novo* adult astrocytomas. Brain Pathology 10:249-259

Sutton L, Goldwein J, Perilongo G, Lang B, Schut L, Rorke L, Packer R (1990-1991) Prognostic factors in childhood ependymomas. Pediatric Neurosurgery 16:

Szénásy J, Slowik F (1983) Prognosis of benign cerebellar astrocytomas in children. Childs Brain

10:39-47

Szymanska J, Virolainen M, Tarkkanen M, Wiklund T, Asko-Seljavaara S, Tukiainen E, Elomaa I, Blomqvist C, Knuutila S (1997) Overrepresentation of 1q21-23 and 12q13-21 in lipoma-like liposarcomas but not in benign lipomas: A comparative genomic hybridisation study. Cancer, Genetics & Cytogenetics 99:14-18

Takeda O, Homma C, Maseki N, Sakurai M, Kanda N, Schwab M, Nakamura Y, Kaneko Y (1994) There may be two tumour suppressor genes on chromosome arm 1p closely associated with biologically distinct subtypes of neuroblastoma. Genes Chromosomes & Cancer 10:30-39

Takeichi M (1987) Cadherins: a molecular family essential for selective cell-cell adhesion and animal morphogenesis. Trends in Genetics 3:213-217

Tanaka S, Kamitani H, Amin M, Watanabe T, Oka H, Fujii K, Nagashima T, Hori T (2000) Preliminary individual adjuvant therapy for gliomas based on the results of molecular biological analyses for drug-resistance genes. Journal of Neuro-Oncology 46:157-171

Tanner M, Grenman S, Koul A, Johannsson O, Meltzer P, Pejovic T, Borg Å, Isola J (2000) Frequent amplification of chromosomal region 20q12-13 in ovarian cancer. Clinical Cancer Research 6:1833-1839

Tanner S, Austin J, Leone G, Rush L, Plass C, Heinonen K, Mrózek K, Sill H, Knuutila S, Kolitz J, Archer K, Caligiuri M, Bloomfield C, de la Chapelle A (2001) BAALC, the human member of a novel mammalian neuroectoderm gene lineage, is implicated in hematopoiesis and acute leukemia. Proceedings of the National Academy of Science USA 98:13901-13906

Tanner S, Tirkkonen M, Kallioniemi A, Isola J, Kuukasjarvi T, Collins C, Kowbel D, Guan X-Y, Trent J, Gray J, Meltzer P, Kallioniemi O (1996) Independent amplification and frequent co-amplification of three nonsyntenic regions on the long arm of chromosome 20 in human breast cancer. Cancer Research 56:3441-3445

Tao W, Levine A (1999) p19ARF stabilizes p53 by blocking nucleocytoplasmic shuttling of mdm2. Proceedings of the National Academy of Science USA 96:6937-6941

Taratuto, Rorke L (1997) Desmoplastic cerebral astrocytoma of infancy and desmoplastic infantile ganglioglioma. In: Kleihues P, Cavenee W (eds) Pathology and genetics of nervous system tumours. International Agency for Research on Cancer, Lyon, France, pp 70-72

Tassi E, Al-Attar A, Aigner A, Swift M, McDonnell K, Karavanov A, Wellstein A (2001) Enhancements of fibroblast growth factor (FGF) activity by an FGF-binding protein. The Journal of Biological Chemistry 276:40247-40253

Teng D, Hu R, Lin H, Davis T, Iliev D, Frye C, Swedlund B, Hansen K, Vinson V, Gumpper K, Ellis L, El-Nagger A, Frazier M, Jasser S, Langford L, Lee J, Mills G, Pershouse MA, Pollack R, Tornos C, Troncoso P, Yung W, Fujii G, Berson A, Bookstein R, Bolen J, Tavtigian S, Steck P (1997) *MMAC1/PTEN* mutations in primary tumour specimens and tumour cell lines. Cancer Research 57:5221-5225

Thiebaut F, Tsuruo T, Hamada H, Gottesman M, Pastan I, Willingham M (1989) Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: Evidence for localization in brain capillaries and crossreactivity of one antibody with a muscle protein. Journal of Histochemistry and Cytochemistry 37:159-164

Thiel G, Lozanova T, Vogel S, Kintzel D, Janisch W, Witkowski R (1993) Age related nonrandom chromosomal abnormalities in human low grade astrocytomas. Human Genetics 91:547-550

Thompson F, Nelson M, Trent J, Guan X-Y, Liu Y, Yang J-M, Emerson J, Adair L, Wymer J, Balfour C, Massey K, Weinsein R, Alberts D, Taetle R (1996) Amplification of 19q13.1-q13.2 sequences in ovarian cancer. G-Band, FISH and molecular studies. Cancer, Genetics & Cytogenetics 87:55-62

Thompson L, Brookman K, Jones N, Allen S, Carrano A (1990) Molecular cloning of the human XRCC1 gene, which corrects defective DNA strand break repair and sister chromatid exchange. Molecular Cell Biology 10:6160-6171

Thomson S, Rasmussen S, Zhang J, Wallace M (1999) A new hereditary cylindromatosis family associated with *CYLD1* on chromosome 16. Human Genetics 105:171-173

Tikoo A, Czekay S, Viars C, White S, Heatk J, Arden K, Maruta H (2000) p190-A, a human tumour suppressor gene, maps to the chromosomal region 19q13.3 that is reportedly deleted in some gliomas. Gene 257:23-31

Timmermann B, Kortmann R-D, Kuhl J, Meisner C, Slavc I, Pietsch T, Bamberg M (2000) Combined postoperative irradiation and chemotherapy for anaplastic ependymomas in childhood: Results of the German prospective trials HIT 88/89 and HIT 91. International Journal Radiation Oncology Biol Phys 46:287-295

Tishler D, Weinbergm KI, Sender LS, Nolta J, Raffel C (1992) Multidrug resistance gene expression in pediatric primitive neuroectodermal tumours of the central nervous system. Journal of Neurosurgery 76:507-512

Tominaga T, Kayama T, Kumabe T, Sonoda Y, Yoshimoto T (1995) Anaplastic ependymomas: Clinical features and tumour suppressor gene p53 analysis. Acta Neurochir (Wien) 135:163-170

Tomita T (1998) neurosurgical perspectives in pediatric neuro-oncology. Childs Nervous System 14:94-96

Tong C, Ng H-K, Pang J, Hui A, Ko H, Lee J (1999) Molecular genetic analysis of non-astrocytic tumours. Histopathology 34:331-341

Torres, J, Navarro, S, Rogla, I, Ripoll, F, Lluch, A, Garcia-Conde, J, Llomart-Bosch, A, Cervera, J, and Pulido, R. Heterogeneous lack of expression of the tumour suppressor PTEN protein in human neoplastic tissues. European Journal of Cancer 37, 114-121. 2001.

Toth K, Vaughan MPN, Slocum H, Rustum Y (1996) MDR1 P-Glycoprotein is expressed by endothelial cells of newly formed capillaries in human gliomas but is not expressed in the neovasculature of other primary tumours. American Journal of Pathology 149:853-858

Traversari V, van der Bruggen P, Luescher I, Lurquin C, Chomez P, Van Mel A, De Plaen E, Amar-Costesec A, Boon T (1992) A nonapeptide encoded by human gene MAGE-1 is recognised on HLA-A1 by cytolytic T lymphocytes directed against tumour antigene MZ2-E. Journal of Experimental Medicine 176:1453-1457

Tsuda H, Takarabe T, Kanai Y, Fukutomi T, Hirohashi S (2002) Correlation of DNA hypomethylation at pericentromeric heterochromatin regions of chromosomes 16 and 1 with histologic features and chromosomal abnormalities of human breast carcinomas. American Journal of Pathology 161:859-866

Tsujii M, Kawano S, Tsujii S, Sawaoka H, Hori M, DuBois R (1998) Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell 93:705-716

Ueki K, Ono Y, Henson J, Efird J, Von Deimling A, Louis D (1996) *CDKN2/p16* or *RB* alterations occur in the majority of glioblastomas and are inversely correlated. Cancer Research 56:150-153

Ueki K, Ramaswamy S, Billings S, Mohrenweiser H, Louis D (1997) ANOVA, a putative astrocytic RNA-binding protein gene that maps to chromosome 19q13.3. Neurogenetics 1:31-36

Uhrbom L, Dai C, Celestino J, Rosenblum M, Fuller G, Holland E (2002) *Ink4a-Arf* loss co-operates with KRas activation in astrocytes and neural progenitors to generate glioblastomas of various morphologies depending on activated Akt. Cancer Research 62:5551-5558

Underwood (1992) Carcinogenesis. In: Underwood J (ed) General and systematic pathology. Churchill Livingstone, Edinburgh, pp 201-222

Undjian S, Marinov M, Georgiev K (1989) Long-term follow-up after surgical treatment of cerebellar astrocytomas in 100 children. Childs Nervous System 5:99

Urioste M, Martínez-Ramírez A, Cigudosa J, Colmenero I, Madero L, Robledo M, Martínez-Delgado B, Benítez J (2002) Complex cytogenetic abnormalities including telomeric associations and *MEN1* mutation in a pediatric ependymoma. Cancer, Genetics & Cytogenetics 138:107-110

Vagner-Capodano A, Gentet J, Gambarelli D, Pellissier J, Gouzien M, Lena G, Genitori L, Choux M, Raybaud C (1992) Cytogenetic studies in 45 pediatric brain tumours. Pediatric Haematology and Oncology 9:223-235

Vagner-Capodano A, Zattara-Cannoni H, Gambarelli D, Figarella-Branger D, Lena G, Dufour H, Grisoli F, Choux M (1999) Cytogenetic study of 33 ependymomas. Cancer, Genetics & Cytogenetics 115:96-99

van Dekken H, Alers J, Riegman P, Rosenberg C, Tilanus H, Vissers K (2001) Molecular cytogenetic evaluation of gastric cardia adenocarcinoma and precursor lesions. American Journal of Pathology 158:1961-1967

Van Gele M, Van Roy N, Jauch A, Laureys G, Benoit Y, Schelfhout V, De Potter C, Brock P, Uyttebroeck A, Sciot R, Schuuring E, Versteeg R, Speleman F (1997) Sensitive and reliable detection of genomic imbalances in human neuroblastomas using comparative genomic hybridisation analysis. European Journal of Cancer 33:1979-1982

van Tilborg A, de Vries A, Zwarthoff E (2001) The chromosome 9q genes *TGFBR1*, *TSC1* and *ZNF189* are rarely mutated in bladder cancer. Journal of Pathology 194:76-80

Verhagen P, Zhu X, Rohr L, Cannon-Albright L, Tavtigian S, Skolnick M, Brothman A (2000) Microdissection, DOP-PCR, and comparative genomic hybridisation of paraffin-embedded familial prostate cancers. Cancer, Genetics & Cytogenetics 122:43-48

Verstegen M, Bosch D, Troost D (1997) Treatment of ependymoma. Clinical and non-clinical factors influencing prognosis: a review. British Journal of Neurosurgery 11:542-553

Vinchon M, Ruchoux M-M, Soto-Ares G, Nicolas R, Dhellemmes P (2001) Cerebellar gliomas in infants: specificity, pathology and outcome. Childs Nervous System 17:31-36

Visakorpi T, Kallioniemi A, Syvanen A-C, Hyytinen E, Karhu R, Tammela T, Isola J, Kallioniemi O (1995) Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridisation. Cancer Research 55:342-347

von Bossanyi P, Diete S, Dietzmann K, Warich-Kirches M, Kirches E (1997) Immunohistochemical expression of P-glycoprotein and glutathione S-transferases in cerebral glioma and responses to chemotherapy. Acta Neuropathologica 94:605-611

Von Deimling A, Bender B, Jahnke R, Waha A, Kraus J, Albrecht S, Wellenreuther R, Fassbender F, Nagel J, Menon A, Louis D, Lenartz D, Schramm J, Wiestler O (1994) Loci associated with

malignant progression in astrocytomas: A candidate on chromosome 19q. Cancer Research 54:1397-1401

Von Deimling A, Fimmers R, Schmidt M, Bender B, Fassbender F, Nagel J, Jahnke R, Kaskel P, Duerr E, Koopman J, Maintz D, Steinbeck S, Wick W, Platten M, Mueller D, Przkora R, Waha A, Blumcke B, Wellenreuther R, Meyer-Puttlitz B, Schmidt O, Mollenhauer J, Poustka A, Stangl A, Lenartz D, Von Ammon K, Henson J, Schramm J, Louis D, Wiestler O (2000) Comprehensive allelotype and genetic analysis of 466 human nervous system tumours. Journal of Neuropathology & Experimental Neurology 59:544-558

Von Deimling A, Louis D, Von Ammon K, Petersen I, Hoell T, Chung R, Martuza R, Schoenfeld D, Yasargil G, Wiestler O, Seizinger B (1992a) Association of epidermal growth factor receptor gene amplification with loss of chromosome 10 in human glioblastoma multiforme. Journal of Neurosurgery 77:295-301

Von Deimling A, Louis D, Von Ammon K, Petersen I, Wiestler O, Seizinger B (1992b) Evidence for a tumour suppressor gene on chromosome 19q associated with human astrocytomas, oligodendrogliomas and mixed gliomas. Cancer Research 52:4277-4279

Von Haken M, White E, Daneshvar-Shyesther L, Sih S, Choi E, Kalra R, Cogen P (1996) Molecular cytogenetic analysis of chromosome arm 17p and chromosome arm 22q in sporadic pediatric ependymomas. Genes Chromosomes & Cancer 17:37-44

Waber P, Chen J, Nisen P (1993) Infrequency of MDM2 gene amplification in paediatric solid tumours and lack of association with p53 mutations in adult squamous cell carcinomas. Cancer Research 53:6028-6030

Walch A, Zitzelsberger H, Bruch J, Keller G, Angermeier D, Aubele M, Mueller J, Stein H, Braselmann H, Siewert J, Höfler H, Werner M (2000) Chromosomal imbalances in Barrett's adenocarcinoma and the metaplasia-dysplasia-carcinoma sequence. American Journal of Pathology 156:555-566

Walker C, Joyce K, Thompson-Hehir J, Davies M, Gibbs F, Halliwell N, Lloyd B, Machell Y, Roebuck M, Salisburym J, Sibson D, Du Plessis D, Broome J, Rossi M (2001) Characterisation of molecular alterations in microdissected archival gliomas. Acta Neuropathologica 101:321-333

Wallner K, Gonzales M, Edwards M, Wara W, Sheline G (1988) Treatment results of juvenile pilocytic astrocytoma. Journal of Neurosurgery 69:171-176

Walter A, Gajjar A, Reardon D, Thompson S, Langston J, Jones-Wallace D, Kun L, Heideman R (2000) Tamoxifen and carboplatin for children with low-grade gliomas: A pilot study at St. Jude Childrens Research Hospital. J Ped Hemat Oncol 22:247-251

Wang S, Krinks M, Lin K, Luyten F, Moos M (1997) Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. Cell 88:757-766

Wang X, Michael D, de Murcia G, Oren M (2002) p53 activation by nitric oxide involves down-regulation of Mdm2. The Journal of Biological Chemistry 277:15697-15702

Wang X-Y, Smith D, Liu W, James C (1998) *GBAS*, a novel gene encoding a protein with tyrosine phosphorylation sites and a transmembrane domain, is co-amplified with EGFR. Genomics 49:448-451

Warnick R, Raisanen J, Adornato B, Prados M, Davis RLD, Gutin P (1993) Intracranial myxopapillary ependymoma: A case report. Journal of Neuro-Oncology 15:251-256

Warr, T, Ward, S, Burrows, J, Harding, B, Wilkins, P, Harkness, W, Hayward, R, Darling, J, and

Thomas, D. Identification of extensive genomic loss and gain by comparative genomic hybridisation in malignant astrocytoma in children and young adults. Genes Chromosomes & Cancer 31, 15-22. 2001.

Wasenius V-M, Jekunen A, Monni O, Joensuu H, Aebi S, Howell S, Knuutila S (1997) Comparative genomic hybridisation analysis of chromosomal changes occurring during development of acquired resistance to cisplatin in human ovarian carcinoma cells. Genes Chromosomes & Cancer 18:286-291

Wasson J, Saylors R, Zeltzer P, Friedman HS, Bigner S, Burger P, Bigner D, Look A, Douglass E, Brodeur G (1990) Oncogene amplification in pediatric brain tumours. Cancer Research 50:2987-2990

Watanabe K, Sato K, Biernat W, Tachibana O, von Ammon K, Ogata N, Yonekawa Y, Kleihues P, Ohgaki H (1997) Incidence and timing of p53 mutations during astrocytoma progression in patients with multiple biopsies. Clinical Cancer Research 3:523-530

Watanabe K, Tachibana O, Sato K, Yonekawa Y, Kleihues P, Ohgaki H (1996) Overexpression of the EGF receptor and *p53* mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. Brain Pathology 6:217-224

Weber-Hall S, Anderson J, McManus A, Abe S, Nojima Y, Pinkerton R, Pritchard-Jones K, Shipley J (1996) Gains, losses and amplification of genomic material in rhabdomyosarcoma analysed by comparative genomic hybridisation. Cancer Research 56:3220-3224

Weber R, Sabel M, Reifenberger J, Sommer C, Oberstrass J, Reifenberger G, Kiessling M, Cremer T (1996) Characterisation of genomic alterations associated with glioma progression by comparative genomic hybridisation. Oncogene 13:983-994

Wechsler D, Shelly C, Petroff C, Dang C (1997) *MXI1*, a putative tumour suppressor gene, suppresses growth of human glioblastoma cell lines. Cancer Research 57:4905-4912

Weggen S, Bayer T, von Deimling A, Reifenberger G, von Schweintz D, Wiestler O, Pietsch T (2000) Low frequency of SV40, JC and BK Polyomavirus sequences in human medulloblastomas, meningiomas and ependymomas. Brain Pathology 10:85-92

Weinstein R, Kuszak J, Kluskens L, Coon J (1990) P-Glycoproteins in pathology: The Multidrug resistance gene family in humans. Human Pathology 21:34-48

Weiss M, Hermsen M, Meijerm GA, van Grieken N, Baak J, Kuipers E, van Diest P (1999) Demystified..... Comparative genomic hybridisation. J Clin Pathol: Mol Pathol 52:243-251

Weith A, Brodeur G, Bruns G, Matise T, Mischke D, Nizetic D, Seldin M, van Roy N, Vance J (1996) Report of the second international workshop on human chromosome 1 mapping 1995. Cytogenetics & Cell Genetics 72:113-154

Weremowicz S, Kupsky W, Morton C, Fletcher J (1992) Cytogenetic evidence for a chromosome 22 tumour suppressor gene in ependymoma. Cancer, Genetics & Cytogenetics 61:193-196

Wernicke C, Thiel G, Lozanova T, Vogel S, Witkowski R (1997) Numerical aberrations of chromosomes 1,2 and 7 in astrocytomas studied by interphase cytogenetics. Genes Chromosomes & Cancer 19:6-13

Wessels P, Twijnstra A, Kessels A, Krijne-Kubat B, Theunissen P, Ummelen M, Ramaekers F, Hopman A (2002) Gain of chromosome, as detected by In Situ Hybridisation, strongly correlates with shorter survival in astrocytoma grade II. Genes Chromosomes & Cancer 33:279-284

Whitaker S, Bessell E, Ashley S, Bloom H, Bell B, Brada M (1991) Postoperative radiotherapy in the management of spinal cord ependymoma. Journal of Neurosurgery 74:720-728

White F, Anthony D, Yunis E, Tarbell N, Scott R, Schofield D (1995) Nonrandom chromosomal gains in pilocytic astrocytomas of childhood. Human Pathology 26:979-986

Whittle I, Gordon A, Misra B, Shaw J, Steers A (1989) Pleomorphic xanthoastrocytoma. Journal of Neurosurgery 70:463-468

Wiener J, Kerns B, Harvey E, Conaway M, Iglehart J, Berchuk A, Bast R (1994) Overexpression of the protein tyrosine phosphatase PTP1B in human breast cancer: Association with p185c-erbB2 protein expression. Journal of the National Cancer Institute 86:372-378

Wiest J (1997) Identification of a novel region of homozygous deletion on chromosome 9p in squamous cell carcinoma of the lung: The location of a putative tumour suppressor gene. Cancer Research 57:1-6

Wiestler, Lopze B, Crino P (1997) Tuberous sclerosis complex and giant cell astrocytoma. In: Kleihues P, Cavenee W (eds) Pathology and Genetics: Tumours of the Nervous System. International Agency for Research on Cancer, Lyon, France, pp 182-184

Wiestler, Schiffer D, Coons S, Prayson R, Rosenblum M (2001a) Anaplastic ependymoma. In: Kleihues P, Cavenee W (eds) Pathology and Genetics: Tumours of the Nervous System. IRAC, Lyon, pp 76-77

Wiestler, Schiffer D, Coons S, Prayson R, Rosenblum M (2001b) Ependymoma. In: Kleihues P, Cavenee W (eds) Pathology and Genetics: Tumours of the Nervous System. IRAC, Lyon,

Willert J, Daneshvar L, Sheffield V, Cogen P (1995) Deletion of chromosome arm 17p DNA sequences in pediatric high grade and juvenile pilocytic astrocytomas. Genes Chromosomes & Cancer 12:165-172

Williams C, Tsujii M, Reese J, Dey S, DuBois R (2000) Host cyclooxygenase-2 modulates carcinoma growth. Journal of Clinical Investigation 105:1589-1595

Winter S, Strik H, Rieger J, Beck J, Meyermann R, Weller M (2000) Glutathione S-transferase and drug sensitivity in malignant glioma. Journal of Neurological Sciences 179:115-121

Wolf M, Tarkkanen M, Hulsebos T, Larramendy M, Forus A, Myklebost O, Aaltonen L, Elomaa I, Knuutila S (1999) Characterisation of the 17p amplicon in human sarcomas: Microsatellite marker analysis. International Journal of Cancer 82:329-333

Wolff J, Molenkamp G, Westphal S, Pietsch T, Gnekow A, Kortmann R-D, Kuehl J (2000) Oral trofosfamide and etoposide in pediatric patients with glioblastoma multiforme. Cancer 89:2131-2137

Wong A, Ruppert J, Bigner S, Grzeschik C, Humphrey P, Bigner D, Vogelstein B (1992) Structural alterations of the epidermal growth factor receptor gene in human gliomas. Proceedings of the National Academy of Science USA 89:2965-2969

Yamada K, Kasama M, Kondo T, Shinoura N, Yoshioka M (1994) Chromosome studies in 70 brain tumours with special attention to sex chromosome loss and single autosomal trisomy. Cancer, Genetics & Cytogenetics 73:46-52

Yang X-Q, Imoto I, Fukada Y, Pimkhaokham A, Shimada Y, Imamura M, Sugano F, Nakamura Y, Inazawa J (2000) Identification of a novel gene, *GASC1*, within an amplicon at 9p23-24 frequently detected in esophageal cancer cell lines. Cancer Research 60:4735-4739

Yen C-C, Chen Y-J, Chen J-T, Hsia J-Y, Chen P-M, Liu J-H, Fan F, Chiou T-J, Wang W-S, Lin C-H (2001) Comparative genomic hybridisation of esophageal squamous cell carcinoma: Correlations between chromosomal aberrations and disease progression/prognosis. Cancer 92:2769-2777

Yin X-L, Hui AB-Y, Liong E, Ding M, Chang A, Ng H-K (2002) Genetic imbalances in pleomorphic xanthoastrocytoma detected by comparative genomic hybridisation and literature review. Cancer, Genetics & Cytogenetics 132:14-19

Yong W, Chou D, Ueki K, Harsh G, von Deimling A, Gusella J, Mohrenweiser H, Louis D (1995) Chromosome 19q deletions in human gliomas overlap telomeric to *D19S219* and may target a 425 kb regions centromeric to *D19S112*. Journal of Neuropathology & Experimental Neurology 54:622-626

Yu J, Lee K, Okamoto A, Reed E, Bostick-Bruton F, Michell K, Reed E (1997) A nucleotide polymorphism in ERCC1 in human ovarian cancer cell lines and tumor tissues. Mutation Research 382:13-20

Zauberman A, Flusberg D, Haupt Y, Barak Y, Oren M (1995) A functional p53-responsive intronic promoter is contained within the human mdm2 gene. Nucleic Acids Research 23:2584-2592

Zhan X, Bates B, Hu X, Goldfarb M (1988) The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. Molecular Cell Biology 8:3487-3495

Zhang Y, Xiong Y, Yarborough W (1998) ARF promotes MDM2 degradation and stabilizes p53: *ARF-INK4a* locus deletion impairs both the Rb and p53 tumour suppression pathways. Cell 92:725-734

Zhen H-N, Zhang X, Bu Z-W, Zhang Z-W, Huang W-J, Zhang P, Liang J-W, Wang X-L (1999) Expression of the simian virus 40 large tumour antigen (Tag) and formation of Tag-p53 and Tag-pRb complexes in human brain tumours. Cancer 86:2124-32

Zheng P-P, Pang J-S, Hui A, Ng H-K (2000) Comparative genomic hybridisation detects losses of chromosomes 22 and 16 as the most common recurrent genetic alterations in primary ependymomas. Cancer, Genetics & Cytogenetics 122:18-25

Zhou H, Kuang J, Zhong L, Kuo W-L, Gray J, Sahin A, Brinkley B, Sen S (1998) Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nature Genetics 20:189-193

Zhou X-P, Li Y-J, Hoang-Xuan K, Laurent-Puig P, Mokhtari K, Longy M, Sanson M, Delattre J-Y, Thomas G, Hamelin R (1999) Mutational analysis of the *PTEN* gene in gliomas: Molecular and pathological correlations. International Journal of Cancer 84:150-154

Zhuang Z, Park W-S, Pack S, Schmidt L, Vortmeyer A, Pak E (1998) Trisomy-7 harbouring non-random duplication of the mutant MET allele in hereditary papillary renal carcinoma. Nature Genetics 20:66-9

Zielenska M, Bayani J, Pandita A, Toledo S, Marrano P, Andrade J, Petrilli A, Thorner P, Sorensen P, Squire J (2001) Comparative genomic hybridisation analysis identifies gains of 1p35_36 and chromosome 19 in osteosarcoma. Cancer, Genetics & Cytogenetics 130:14-21

Zimmermann K, Sarbia M, Weber A-A, Borchard F, Gabbert H, Schrör K (1999) Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Research 59:198-204

Zitzelsberger H, Kulka U, Lehmann L, Walch A, Smida J, Aubele M, Lorch T, Hofler H, Bauchinger M, Werner M (1998) Genetic heterogeneity in a prostatic carcinoma and associated prostatic intraepithelial neoplasia as demonstrated by combined use of laser-microdissection,

degenerate oligonucleotide primed PCR and comparative genomic hybridisation. Virchows Archiv 433:297-304

Zurawel R, Allen C, Chiappa S, Cato W, Biegel J, Cogen P, de Sauvage F, Raffel C (2000) Analysis of *PTCH/SMO/SHH* pathway genes in medulloblastoma. Genes Chromosomes & Cancer 27:44-51

APPENDIX I

Clinical Protocols for the treatment of patients in this study

1. Baby Brain Protocol (UKCCSG CNS 9204)

For the treatment of patients less than 3 years of age with malignant brain tumours.

After surgery, patients received vincristine, carboplatin, methotrexate, cyclophosphamide and cisplatin over a 43 day cycle with a maximum of 7 cycles, a total treatment time of 379 days.

Dose schedule:	Vincristine	$1.5mg/m^2 \times 1$
	Carboplatin	$550mg/m^2 \times 1$
	Methotrexate	$8g/m^2$
	Cyclophosphamide	$1500mg/m^2 \times 1$
	Cisplatin	$40mg/m^2 \times 1$

2. Low Grade Glioma Study

For the treatment of patients with low grade glioma. After initial surgery some patients in this study group were on the "wait and see" arm of the trial. This meant that after surgery they were only treated with extra therapy if they had residual bulky disease, symptoms that were not relieved by surgery or if recurrence or progression of the tumour occurred.

Patients having treatment received carboplatin and vincristine in a two part protocol. The first part was a 10 week treatment with the administration of vincristine on the first day of each week and carboplatin in weeks 1, 4, 7 and 10. After a two week recovery period, vincristine and carboplatin were administered together in weeks 13, 17, 21, 25, 29, 33, 37, 41, 45, and 49.

Dose schedule:	Carboplatin	$550mg/m^2 \times 1$
	Vincristine	$1.5mg/m^2 \times 1$

The doses of vincristine and carboplatin were reduced by 33% in patients aged less than 6 months.

Appendix II
Preparation of Reagents

1: Tissue Culture Reagents

Transport media: 500ml Hams F10 media (Invitrogen Ltd) containing 20ml high dose antibiotic mix. 15ml media were pipetted into sterile universals and stored at 4°C. The universals were taken in batches of 6 to Cardiac Wing Theatres (GOS) and Maktoum Theatre Suite (Queen Square) and stored at 4°C until a sample was taken. The biopsy was placed in the transport media and taken back to the laboratory.

Antibiotic mix: 5ml Kanomycin (ICN Pharmaceuticals), 5 ml Amphotericin B (Sigma Aldrich), and 10ml Penicillin/Streptomycin (Invitrogen Ltd) were pipetted into sterile universals. The mix was stored at –20°C until required.

Complete Media: A 500ml bottle of FCS was allowed to thaw at room temperature. This was sufficient to make up 9x 500ml bottles of complete media. 9 bottles of Hams F-10 were labelled A-I and dated. 55ml of FCS was pipetted into each bottle to give a final concentration of 10%. 10ml of media was removed from each bottle and pipetted into a correspondingly labelled universal. The samples were incubated at 37°C for 3 days to check sterility. If any contamination was observed the corresponding bottle was discarded. The bottles of CM were stored at 4°C.

Collagenase (2000U/ml): The specific activity of the collagenase is stated on the data sheet. The collagenase was dissolved in sufficient HBSS to give the required concentration. When the collagenase was completely dissolved the solution was aliquoted into 30ml universal containers and centrifuged at 3000rpm for 15 minutes. The supernatants were then filtered once through a 0.45μm filter and once through a 0.2μm filter. The supernatants were then aliquoted into 1ml bijou bottles and stored at –20°C until use.

2: Reagents for DNA Extractions

20mg/ml Proteinase K: 1g of lyophilized Proteinase K was dissolved in 50ml sterile dH_2O. The solution was aliquoted into 1.5ml sterile eppendorf tubes and stored at –20°C.

Phosphate Buffered Saline (PBS): To make a 10X stock solution 80g NaCl, 2g KCl, 14.4g Na_2HPO_4 and 2.4g KH_2 PO_4 were made up to 1 litre in deionised water. The pH was adjusted to 7.4 with HCl. Diluting the stock solution 1 in 10 with deionised water made a 1X working solution. The solutions were stored at room temperature.

2.1 From Cell Cultures

C1 Buffer: 438.1g Sucrose (Sigma Aldrich), 4.06g $MgCl_2x6H_2O$ (Sigma Aldrich), and 4.84g Tris base (Invitrogen Ltd) were dissolved in 700ml dH_2O. 200ml 20% Triton X-100 solution (Sigma Aldrich) was added and the pH adjusted to 7.5 with HCl. The solution was allowed to cool to room temperature before a final pH adjustment was made and the volume made up to 1litre with dH_2O. The buffer was stored at 4°C.

G2 Buffer: 76.42g GuHCl (Sigma Aldrich), 11.17g Na_2-EDTAx2 H_2O (Sigma Aldrich), 3.633g Tris base were dissolved in 600ml dH_2O. 250ml 20% Tween-20 (Sigma Aldrich) and 50ml 10% Triton X-100 were added and the pH adjusted to 8.0 with NaOH. The solution was allowed to cool to room temperature before a final pH adjustment was made and the volume made up to 1litre with dH_2O.

QBT Buffer: 43.83g NaCl (Sigma Aldrich), 10.46g MOPS (free acid) (Sigma Aldrich) were dissolved in 800ml dH_2O. The pH was adjusted to 7.0 with NaOH. 150ml ethanol and 15ml of 10% Triton X-100 were added and the volume adjusted to 1 litre with dH_2O.

QC Buffer: 58.44g NaCl and 10.46g MOPS (free acid) were dissolved in 800ml dH_2O. The pH was adjusted to 7.0 with NaOH. 150ml ethanol was added before the volume was adjusted to 1litre with dH_2O.

QF Buffer: 73.05g NaCl and 6.055g Tris base were dissolved in 800ml dH_2O. The pH was adjusted to 8.5 with HCL. The solution was allowed to cool to room temperature before a final pH adjustment was made. 150ml ethanol was added and the volume adjusted to 1 litre with dH_2O.

TE Buffer: 1.21g Tris base and 0.372g EDTA were dissolved in 1 litre of dH_2O. The pH was adjusted to 8.0. The solution was then autoclaved and stored at room temperature.

2.2 From Biopsy material

Buffers AL, ATL, AW and AE are provided in the QiAmp extraction kit

2.3 From Paraffin embedded material

Buffers AL, ATL, AW and AE are provided in the QiAmp extraction kit

1M Sodium Thiocynate: MW = 81.04

1M = 81.04g/litre

20.26g Sodium Thiocynate were dissolved in 250ml dH_2O. The solution was autoclaved and stored at room temperature.

Lysis Buffer: 10mM Tris HCl, 400mM NaCl and 2mM Na_2.EDTA.

0.121g Tris base, 2.33g NaCl and 0.074g Na_2.EDTA were dissolved in 100ml dH_2O. The solution was autoclaved and stored at room temperature.

10% SDS: 100g SDS was dissolved in 900ml dH_2O. The solution was heated to 68°C to aid dissolution. The pH was adjusted to 7.2 with HCl. The volume was adjusted to 1 litre with dH_2O.

6M NaCl: MW = 58.44g

1M = 58.44g/1 litre

6M = 350.64g/1 litre

35.06g NaCl were dissolved in 100ml dH_2O. The solution was autoclaved and stored at room temperature.

3: Reagents for use with DyNAQuant Instrument

10x TNE Buffer: 12.11g Tris Base, 72g EDTA disodium salt and 116.89g Sodium Chloride were dissolved in 800ml dH_2O. The pH was adjusted to 7.4 with concentrated HCl. The volume was made upto 1 litre with dH_2O. The buffer was filtered and stored at 4°C

Hoechst 33258 dye (Amersham Pharmacia Biotech): 10mg H 33258 was added to 10ml distilled water to give a final concentration of 1mg/ml

Capillary Assay Solution: Low Range

(For DNA concentrations upto 10ng/μl)

2μl Hoechst 33258 stock solution and 100μl 10x TNE buffer were added to 898μl distilled filtered water.

Capillary Assay Solution: High Range

(For DNA concentrations from 10 to 100ng/μl)

20μl Hoechst 33258 stock solution and 100μl 10x TNE buffer were added to 880μl distilled filtered water

Calf Thymus DNA Standard: Prepared to a final concentration of 200ng/μl.

4: Reagents for CGH

20x SSC pH 5.3: 175.3g NaCl and 88.2g sodium citrate were dissolved in 800ml dH_2O. The pH was adjusted to 7.0 with NaOH. The volume was then adjusted to 1 litre with dH_2O.

Denaturation Solution: 49ml formamide, 7ml 20 X SSC and 14ml dH_2O were added to a glass coplin jar and mixed.

0.4x SSC/0.3% NP-40 wash solution: 20ml 20x SSC and 950ml dH_2O were mixed together and 3ml NP-40 added. The pH was adjusted to 7.0-7.5 with NaOH before the solution was made up to 1litre with dH_2O.

2x SSC/0.1% NP-40 wash solution: 100ml 20x SSC and 850ml dH_2O were mixed together. 1ml NP-40 was added. The pH was adjusted to 7.0-7.5 with NaOH before the solution was made up to 1litre with dH_2O.

0.2M SpectrumGreen dUTP: 10μl 1mMSpectrumGreen dUTP was added to 40μl nuclease free water.

0.1M dTTP: 10μl 0.3mM dTTP was added to 20μl nuclease free water.

0.1M dNTP: 40μl each 0.3M dCTP, 0.3M dGTP and 0.3M dATP were mixed together in a 0.5ml eppendorf tube.

50X TAE Buffer: 242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (pH8.0) were made upto 1 litre with deionised water. For a 1X working solution the stock solution was diluted 1 in 50 with deionised water. Both solutions were stored at room temperature.

Hybridisation buffer: 10% dextran sulphate, 2xSSC, 50% formamide, 1% Tween 20, pH 7.0. 1g dextran sulphate, 1ml 20X SSC, 5ml formamide, 100µl Tween 20 were made up to 10ml with deionised water. The solution was mixed well and the pH adjusted to 7.0. The solution was then stored in 500µl aliquots in 1.5ml eppendorf tubes at –20°C.

70% Alcohol: 70ml ethanol added to 30ml dH_2O

95% Alcohol: 95ml ethanol added to 5ml dH_2O

5: Reagents for Y-PCR

10mM dNTP: 50µl each of 100mM dATP, dGTP, dTTP and dCTP were added to 300µl sterile dH_2O and stored at –20°C

Primers: a working solution of 20pmol was prepared by adding 20µl of 0.5mM stock to 480µl sterile dH_2O and stored at –20°C

10x buffer: provided with *Taq* Polymerase from Promega

25mM $MgCl_2$: provided with *Taq* Polymerase from Promega

6: Reagents for the extraction of RNA

Buffers were all provided in the Qiagen RNA buffer kit

7: Reagents for Gel electrophoresis of RNA

DEPC Water: A 0.1% solution of diethyl pyrocarbonate (Sigma Aldrich) was prepared in distilled water, stirred overnight at room temperature and then autoclaved.

10X Sodium Phosphate buffer (pH7.0): For 100mM stock solution prepare 500ml of 100mM sodium phosphate (dibasic) and 500ml of sodium phosphate (monobasic). 7.1g of sodium phosphate (dibasic) was dissolved in 450ml of distilled water in a 1 litre graduated cylinder and made up to 500ml with distilled water. 6.9g of sodium phosphate (monobasic) were dissolved in 450ml of distilled water in a 500ml graduated cylinder and made upto 500ml with distilled water. Using a pH meter the sodium phosphate (dibasic) was titrated with the sodium phosphate (monobasic) until the pH reached 7.0. The 10X buffer was then sterilised by autoclaving and stored at room temperature.

1X Sodium Phosphate buffer: For 10mM working solution 1 volume of the 10X stock was added to 9 volumes of DEPC-treated water.

8: Reagents for MDR1 RT-PCR

Solution Q, buffer and enzyme mix were provided in the Qiagen One step RT-PCR kit.

10x dNTP: 5μl of each dNTP (100mM stock solution) were added to 480μl dH_2O

9: Reagents for DOP-PCR

1mM Stock Solution UNI primer: The weight of the lyophilized primer is given on the data sheet. The primer was reconstituted in sterile deionised water:

MW= 6775.8g

1M = 6775.8g/litre

1mM = 6.7758g/litre = 6.75μg/μl

Weight of primer = 789.4μg

$$1\text{mM} = \frac{789.4}{6.75}$$

= 117μl deionised water

Adding 5μl stock solution to 495μl deionised water made a 10μM working solution.

Adding 1μl stock solution to 499μl deionised water made a 2μM working solution.

200μM dNTP: 1μl of each dNTP (100mM stock solution) were added to 496μl deionised water

600μM dNTP: 3μl of each dNTP (100mM stock solution) were added to 488μl deionised water

2mM dNTP: 10μl of each dNTP (100mM stock solution) were added to 460μl deionised water

10x dNTP: 5μl of each dNTP (100mM stock solution) were added to 480μl dH_2O

4U/μl Thermosequenase: 1μl thermosequenase 32U/μl stock solution + 7μl thermosequenase dilution buffer.

10 x High salt buffer:	200mM TrisHCl (pH 9.2)
	600mM KCl
	20mM $MgCl_2$
10 x Low salt buffer:	100mM TrisHCl (pH 8.4)
	100mM KCl
	15mM $MgCl_2$
10 x labelling dNTP:	2mM dATP
	2mM dCTP
	2mM dGTP
	0.5mM dTTP

10: Reagents for Kreatech ULS

Buffers PN, PE and EB were provided in the Qiagen QIAquick nucleotide removal kit.

Labelling buffer is provided with the ULS kits.

MATERIAL/REAGENT	SUPPLIER
Tissue culture	
Kanomycin (5000g/ml)	ICN Biochemicals, Chineham Business Park, Crockford Lane, Basingstoke, Hampshire
Hams F10 nutrient mixture	Invitrogen Ltd., 3 Fountain Drive, Inchinan, Renfrewshire PA9 9FR
Foetal Calf serum	Invitrogen Ltd.
Hanks balanced salt solution (HBSS)	Invitrogen Ltd.
Penicillin & Streptomycin	Invitrogen
Tissue Culture flasks	Triple Red Ltd., Unit C4, Station Yard, Thame, Oxfordshire OX9 3UH
Trypsin EDTA	Sigma Aldrich, Fancy Road, Poole, Dorset BH12 4QH
Dimethyl sulphoxide (DMSO)	Sigma Aldrich
Amphotericin B	Sigma Aldrich
Collagenase type 1A	Sigma Aldrich
1ml cryovial	Scientific Laboratory Supplies, Wilford Industrial Estate, Nottingham NG11 7EP
30ml Universals	Scientific Laboratory Supplies
Sterile pipettes	Scientific Laboratory Supplies
Scalpel blades	Scientific Laboratory Supplies
Petri dishes	Scientific Laboratory Supplies
Isoton II	Beckman Coulter Ltd., Kingsmead Business Park, High Wycombe, HP11 1JU
Glass pasteurs	Fisher Scientific UK, Bishop Meadow Road, Loughborough LE11 5RG
DNA Extraction	
Genomic tip 100/G	Qiagen Ltd., Boundary Court, Crawley, West Sussex RH10 2AX
QiAmp DNA mini kit	Qiagen Ltd.
Qiagen-tip 100	Qiagen Ltd.
DNA/RNA buffer kit	Qiagen Ltd.
50ml polypropylene conical tubes	Helena Biosciences, Colima Avenue, Sunderland Enterprise Park, Sunderland
Proteinase K	ICN Biochemicals,
Isopropanol	Sigma Aldrich

1.5ml eppendorf tubes	Alpha Laboratories, Eastleigh, Hants SO5 4NU
Xylene	Fisher Scientific UK
Sodium thiocynate	Sigma Aldrich
Lauryl sulfate (SDS)	Sigma Aldrich
RNAse	Sigma Aldrich
Sodium Chloride	Sigma Aldrich
Fluorometric quantification of DNA	
DyNAQuant 200 fluorometer	Amersham Bioscience, Amersham Place, Little Chalfont, Bucks, HP7 9NA
DQ 130 Capillary cuvette kit	Amersham Bioscience
Capillary tubes	Amersham Bioscience
Hoechst 33258 dye	Amersham Bioscience
Calf thymus DNA	Sigma Aldrich
Comparative Genomic Hybridisation	
Formamide	Sigma Aldrich
2mM SpectrumGreen dUTP	Vysis, Abbott Laboratories, Abbott House, Norden Road, Maidenhead, SL6 4XL
0.3mM dTTP	Vysis
0.3mM dCTP	Vysis
0.3mM dATP	Vysis
0.3mM dGTP	Vysis
CGH nick translation kit	Vysis
DNA size marker lambda DNA/*Eco*911	Helena Biosciences
CGH metaphase target slides	Vysis
SpectrumRed reference DNA	Vysis
Human Cot-1 DNA	Vysis
DAPI II	Vysis
QUIPS CGH analysis system	Vysis
Sodium Acetate	Sigma Aldrich
Thermal Cycler	Techne, Duxford, Cambridge CB2 4PZ
Ethidium Bromide	Sigma Aldrich
Loading dye	Helena Biosciences
0.5ml PCR tubes	Sigma Aldrich
Coverslips	Scientific Laboratory Supplies
Agarose	Helena Biosciences
Polymerase Chain Reaction	
Primers	Amersham Bioscience

Taq polymerase	Promega , Delta House, Enterprise Road, Chilworth Research Centre, Southampton SO9 1BG
PCR buffer	Promega
Ultrapure dNTP set	Amersham Bioscience
Filter pipette tips	Fisher Scientific
GeneRuler 100bl DNA ladder	Helena Biosciences
Thermal Cycler	GRI Ltd., Gene House, Queensborough Lane, Braintree, Essex CM7 8TF
RNA work	
Sodium phosphate (dibasic)	Sigma Aldrich
Sodium phosphate (monobasic)	Sigma Aldrich
Diethyl pyrocarbonate	Sigma Aldrich
0.24 – 9.5 Kb RNA ladder	Invitrogen Ltd.
OneStep RT-PCR kit	Qiagen Ltd.
Scan-It gel scanning software	Silk Scientific Corporation
Flatbed scanner	Epson
DOP-PCR	
AmpliTaq DNA Polymerase	Applied Biosystems, Birchwood Science Park North, Warrington WA3 7PB
ThermoSequenase	Amersham Bioscience
12-dUTP FITC	Amersham Bioscience
Universal Linkage System	
d-Green nucleic acid labelling kit	Kreatech Diagnostics, PO Box 12756, 1100AT Amsterdam, The Netherlands
Rhodamine nucleic acid labelling kit	Kreatech Diagnostics
QIAquick nucleotide removal kit	Qiagen Ltd.

APPENDIX III
STATISTICAL ANALYSIS

	Primary tumours		Recurrent tumours		
Alteration	**with**	**without**	**with**	**without**	**p-value**
Gain 1q	7	30	4	10	0.467
Gain 2q	5	32	5	9	0.113
Gain 4q	8	29	4	10	0.714
Gain 5q	6	31	3	11	0.692
Gain 6q	8	29	2	12	0.706
Gain 7p	2	35	3	11	0.119
Gain 7q	2	35	4	10	**0.041**
Gain 8q	3	34	3	11	0.327
Gain 9p	5	32	3	11	0.667
Gain 9q	1	36	1	13	0.477
Gain 11p	2	35	1	13	1
Gain 11q	2	35	0	14	1
Gain 12q	2	35	1	13	1
Gain 13	7	30	5	9	0.217
Gain 18q	3	34	1	13	1
Loss 1p	7	30	5	9	0.271
Loss 6q	0	37	2	12	0.0714
Loss 9p	2	35	0	14	1
Loss 9q	3	34	2	12	0.606
Loss 10p	0	37	1	13	0.274
Loss 10q	1	36	1	13	0.477
Loss 12q	3	34	5	9	**0.027**
Loss 16p	5	32	1	13	1
Loss 16q	3	34	2	12	0.606
Loss 17p	2	35	2	12	0.3
Loss 17q	3	34	2	12	0.606
Loss 18q	0	37	1	13	0.274
Loss 19p	8	29	3	11	1
Loss 19q	10	17	4	10	0.733
Loss 20p	1	36	1	13	0.477
Loss 20q	4	33	3	11	0.376
Loss 22	6	31	6	8	0.066
Aberrations	16	11	9	5	0.22

Statistical analysis of the occurrence of individual CNAs in primary versus recurrent ependymoma. P-values in bold types are statistically significant at the 95% confidence level.

	Benign		Anaplastic		
Alteration	**with**	**without**	**with**	**without**	**p-value**
Gain 1p	2	41	0	8	1
Gain 1q	10	33	1	7	0.668
Gain 2p	3	40	1	7	0.506
Gain 2q	9	34	1	7	1
Gain 4q	12	31	0	8	0.173
Gain 5q	9	34	0	8	0.322
Gain 6q	10	33	0	8	0.329
Gain 7p	3	40	1	7	0.506
Gain 7q	5	38	2	6	0.3
Gain 8q	6	37	0	8	0.572
Gain 9p	8	35	0	8	0.327
Gain 11q	3	40	0	8	1
Gain 12q	3	40	0	8	1
Gain 13	12	31	0	8	0.173
Gain 18q	2	41	1	7	0.407
Loss 1p	11	32	0	8	0.175
Loss 6q	2	41	0	8	1
Loss 8p	1	42	0	8	1
Loss 9p	2	41	0	8	1
Loss 9q	5	38	0	8	0.579
Loss 10p	1	42	0	8	1
Loss 10q	2	41	0	8	1
Loss 11q	1	42	0	8	1
Loss 12q	8	35	0	8	0.327
Loss 16p	10	33	0	8	0.329
Loss 16q	5	38	0	8	0.579
Loss 17p	4	39	0	8	1
Loss 17q	5	38	0	8	0.579
Loss 18q	1	42	0	8	1
Loss 19p	12	31	0	8	0.173
Loss 19q	14	29	0	8	0.088
Loss 20p	2	41	0	8	1
Loss 20q	7	36	0	8	0.578
Loss 22	11	32	1	7	0.661
Aberrations	23	20	2	6	0.248

Statistical analysis of the occurrence of individual CNAs in benign versus anaplastic ependymoma. P-values in bold types are statistically significant.

	<3 years		>3 years		
Alteration	with	without	with	without	p-value
Gain 1p	1	20	1	29	1
Gain 1q	2	19	9	21	0.097
Gain 2p	0	21	4	26	0.133
Gain 2q	2	19	8	22	0.166
Gain 3q	0	21	1	29	1
Gain 4q	3	18	9	21	0.315
Gain 5q	3	18	6	24	0.719
Gain 6q	4	17	6	24	
Gain 7p	0	21	4	24	0.133
Gain 7q	2	19	5	25	1
Gain 8q	1	20	5	25	0.38
Gain 9p	3	18	5	25	1
Gain 11q	1	20	2	28	1
Gain 12q	2	19	1	29	0.561
Gain 13	2	19	10	20	0.091
Gain 18q	2	19	1	29	0.561
Loss 1p	3	18	8	22	0.49
Loss 6q	2	19	0	30	0.164
Loss 8p	0	21	1	29	1
Loss 9p	0	21	2	28	0.505
Loss 9q	1	20	4	26	0.39
Loss 10p	0	21	1	29	1
Loss 10q	1	20	1	29	1
Loss 11q	0	21	1	29	1
Loss 12q	2	19	6	24	0.444
Loss 16p	2	19	8	22	0.166
Loss 16q	1	20	4	26	0.39
Loss 17p	2	19	2	28	1
Loss 17q	2	19	3	27	1
Loss 18q	1	20	0	30	0.411
Loss 19p	5	26	7	23	0.533
Loss 19q	5	26	9	21	0.235
Loss 20p	0	21	2	28	0.505
Loss 20q	2	19	5	25	0.685
Loss 22	3	18	9	21	0.315
Aberrations	6	15	9	21	0.258

Statistical analysis of the ocurrence of individual CNAs in ependymoma patients aged 3 years and younger compared to patients aged over 3 years.

	Posterior Fossa		Supratentorial		
Alteration	with	without	with	without	p-value
Gain 1p	2	39	0	10	1
Gain 1q	10	31	1	9	0.428
Gain 2p	3	38	1	9	1
Gain 2q	9	32	1	9	0.663
Gain 4q	10	31	2	8	1
Gain 5q	9	32	0	10	0.176
Gain 6q	9	32	1	9	0.663
Gain 7p	3	38	1	9	1
Gain 7q	5	36	2	8	0.611
Gain 8q	5	36	1	9	1
Gain 9p	7	34	1	9	1
Gain 11q	3	38	0	10	1
Gain 12q	3	38	0	10	1
Gain 13	11	30	1	9	0.417
Gain 18q	3	38	0	10	1
Loss 1p	9	32	2	8	1
Loss 6q	2	39	0	10	1
Loss 8p	1	40	0	10	1
Loss 9p	2	39	0	10	1
Loss 9q	5	36	0	10	0.568
Loss 10p	1	40	0	10	1
Loss 10q	2	39	0	10	1
Loss 11q	1	40	0	10	1
Loss 12q	8	33	0	10	0.329
Loss 16p	9	32	1	9	0.663
Loss 16q	5	36	0	10	0.568
Loss 17p	4	37	0	10	0.573
Loss 17q	5	36	0	10	0.568
Loss 18q	1	40	0	10	1
Loss 19p	11	30	1	9	0.417
Loss 19q	13	28	1	9	0.249
Loss 20p	2	39	0	10	1
Loss 20q	7	34	0	10	0.32
Loss 22	11	30	1	9	0.417
Aberrations	21	20	4	6	0.498

Statistical analysis of the occurance of CNAs in posterior fossa ependymoma compared to supratentorial ependymoma.

	deceased		alive		
Alteration	**with**	**without**	**with**	**without**	**p-value**
Gain 1q	5	14	7	22	0.288
Gain 2p	1	18	3	26	0.657
Gain 2q	3	16	7	22	0.447
Gain 4p	2	17	2	27	0.968
Gain 4q	4	15	7	22	0.358
Gain 5q	2	17	6	23	0.747
Gain 6q	2	17	7	22	0.194
Gain 7p	2	17	3	26	0.637
Gain 7q	2	17	4	25	0.853
Gain 8q	3	16	3	26	0.822
Gain 9p	2	17	6	23	0.283
Gain 11q	0	19	2	27	0.189
Gain 12q	1	18	2	27	0.799
Gain 13	3	16	9	20	0.095
Gain 18q	0	19	4	25	0.159
Loss 1p	4	15	8	21	0.353
Loss 6q	1	18	1	28	0.681
Loss 9q	2	17	3	26	0.593
Loss 12q	3	16	5	24	0.232
Loss 16p	4	15	5	24	0.23
Loss 16q	1	18	3	26	0.075
Loss 17	2	17	3	26	0.728
Loss 19	2	17	10	19	**0.037**
Loss 20q	2	17	4	25	0.5688
Loss 22	3	16	8	21	0.2041
Aberrations	8	11	15	14	0.566

Statistical analysis of the association between copy number aberrations and survival in patients with ependymoma. P-values in bold type are statistically significant at the 95% confidence level.

	No recurrence		With recurrence		
Alteration	**with**	**without**	**with**	**without**	**p-value**
CNAs	8	12	5	8	1
death	1	19	8	5	**0.0007**
Gain 1q	4	16	3	10	1
Gain 2q	1	19	3	10	0.2757
Gain 4q	4	16	3	10	1
Gain 5q	3	17	2	11	1
Gain 6q	5	15	2	11	0.6756
Gain 7q	0	20	2	11	0.1477
Gain 8q	1	19	1	12	1
Gain 9p	1	19	3	10	0.2757
Gain 11	1	19	1	12	1
Gain 12q	0	20	2	11	0.1477
Gain 13	4	16	3	10	1
Gain 18q	1	19	2	11	0.5473
Loss 1p	4	16	3	10	1
Loss 9p	2	18	0	13	0.5076
Loss 12q	2	18	1	12	1
Loss 16p	3	17	2	11	1
Loss 16q	0	20	3	10	**0.0524**
Loss 17p	0	20	2	11	0.1477
Loss 17q	1	19	2	11	0.5473
Loss 19p	4	16	3	10	1
Loss 19q	5	15	3	10	1
Loss 20q	2	18	1	12	1
Loss 22	4	16	1	12	0.6253

Statistical analysis of the occurrence of copy number aberrations in ependymoma that have shown no clinical recurrence compared to those tumours where recurrence has occurred. P-values in bold type are statisically significant at the 95% confidence level.

Aberration	No. with	No. without	p- value
Gain 1q	6	27	0.4889
Gain 2q	4	29	0.2086
Gain 4q	7	26	0.453
Gain 5q	5	28	0.2981
Gain 6q	7	26	0.1732
Gain 9p	4	29	0.2086
Gain 13q	7	26	0.112
Loss 1p	7	26	0.453
Loss 9p	2	31	0.0724
Loss 12q	3	30	0.439
Loss 16p	5	28	0.669
Loss 16q	3	30	0.17
Loss 17	3	30	0.439
Loss 19p	7	26	0.139
Loss 19q	7	26	0.112
Loss 22	5	28	0.279

Statistical analysis of the association of CNAs
with survival in ependymoma patients with and without tumour recurrence

	Pilocytic Astrocytoma		Diffuse Astrocytoma		Anaplastic Astrocytoma		Glioblastoma		
Alteration	**No with**	**No without**	**No with**	**No without**	**No with**	**No without**	**No with**	**No without**	**p-value**
Gain 1p	0	29	2	18	0	5	0	10	0.2086
Gain 2q	0	29	2	18	0	5	1	9	0.1632
Gain 3q	0	29	0	20	0	5	1	9	0.1395
Gain 4q	1	28	1	19	1	4	2	8	0.2529
Gain 5q	0	29	1	19	0	5	1	9	0.4088
Gain 6q	0	29	1	19	1	4	2	8	0.0776
Gain 7q	2	27	2	18	0	5	1	9	0.8833
Gain 8q	0	29	1	19	0	5	0	10	0.5251
Gain 9p	0	29	1	19	0	5	0	10	0.5251
Gain 9q	0	29	1	19	0	5	0	10	0.5251
Gain 12p	0	29	1	19	0	5	0	10	0.5251
Gain 12q	0	29	1	19	0	5	0	10	0.4088
Gain 13	0	29	1	19	1	4	0	10	0.1026
Gain 18	0	29	0	20	0	5	1	9	0.1395
Gain 19	0	29	2	18	0	5	0	10	0.1395
Gain 20q	0	29	2	18	0	5	0	10	0.1395
Loss 1p	0	29	1	19	0	5	1	9	0.4088
Loss 4	0	29	1	19	0	5	0	10	0.5251
Loss 6q	0	29	1	19	0	5	0	10	0.5251
Loss 9p	0	29	2	18	0	5	0	10	0.2086
Loss 10q	0	29	1	19	0	5	0	10	0.5251
Loss 13	0	29	1	19	0	5	0	10	0.5251
Loss 14q	0	29	1	19	0	5	0	10	0.5251
Loss 15	0	29	1	19	0	5	0	10	0.5251
Loss 16p	0	29	2	18	0	5	1	9	0.3121
Loss 16q	0	29	0	20	0	5	1	10	0.1395
Loss 17	0	29	1	19	0	5	0	10	0.5251
Loss 18	0	29	1	19	0	5	0	10	0.5251
Loss 19	0	29	2	18	0	5	0	10	0.2086
Loss 20q	0	29	0	20	1	4	0	10	**0.0074**
Loss 22	0	29	1	19	0	5	0	10	0.5251
Loss X	0	29	1	19	0	5	0	10	0.5251
Amplicons	1	28	3	17	0	5	1	9	0.4416
CNAs	3	26	6	14	2	3	2	8	0.247

Statistical analysis of CNAs in paediatric astrocytoma using Fishers exact test
Values in bold type are statistically significant at 95% confidence level

Alteration	p -value
Gain 2q	**0.0017**
Gain 4q	0.3113
Gain 5q	**0.0498**
Gain 6q	0.1111
Gain 7	**0.042**
Gain 8q	0.6917
Loss 1p	0.6589
Loss 16p	**0.0092**
Loss 19	0.2496
Tumour grade	**0.0002**
Patient age	0.6587
Patient sex	0.5831
Extent of resection	0.3413
Treatment regimen	**0.0021**
CNAs	0.242

Statistical analysis of survival in astrocytoma patients
Figures in bold type are statistically significant at the 95% confidence level

APPENDIX IV
PUBLICATIONS & PRESENTATIONS

Publications

S J Ward & T J Warr (1998) Detection of genetic changes in paediatric ependymoma by comparative genomic hybridisation. J Neuro-Oncol **39**: 164

T J Warr, **S J Ward**, P E Finn, J L Darling & D G T Thomas (1999) Identification of chromosomal gains and losses in paediatric ependymoma & medulloblastoma. Neuro-Oncology **1**: S79

S J Ward, B Harding, P Wilkins, W Harkness, R Hayward, J L Darling, D G T Thomas & T J Warr (2001) Gain of 1q and loss of 22 are the most common changes detected by comparative genomic hybridisation in paediatric ependymoma. Genes Chromosomes & Cancer **32**: 59-66

S J Ward, R Hayward, W Harkness, K Phipps, D Thompson, B Harding, P Wilkins, J Darling, D Thomas, T Warr (2003) Correlation of copy number aberrations with clinicopathological criteria in paediatric glial tumours Neuro-Oncology **5**: Issue 1: 194

S J Ward, R Hayward, W Harkness, K Phipps, D Thompson, B Harding, P Wilkins, J Darling, D Thomas, T Warr (2003) In preparation

Presentations

Third Congress of the European Association for Neuro-Oncology, 13th-16th Sepetember 1998, Versailles, France (**Poster: Detection of genetic changes in paediatric ependymoma by comparative genomic hybridisation**).

19th Annual Meeting of the British Neuro-Oncology Group, 24th & 25th June 1999, John McIntyre Centre, Pollock Halls, University of Edinburgh **(Poster: Detection of Novel genetic alterations in paediatric ependymoma by comparative genomic hybridisation).**

20th Annual Meeting of the British Neuro-Oncology Group, 7th & 8th September 2000, Chamberlain Tower Hotel, Birmingham **(Oral: CGH Analysis of paediatric ependymoma).**

Queen Square Symposium, Institute of Neurology, London 13th March 2001 **(Poster: CGH Analysis of Paediatric Ependymoma)**

21st Annual meeting of the British Neuro-Oncology Group 21st – 23rd June 2001, Academic Medical Center, Amsterdam **(Oral: Correlation of chromosomal gains and losses with clinicopathological factors in paediatric astrocytoma and ependymoma).**

10th International Symposium on Paediatric Neuro-Oncology 9th – 12th June 2002, Hilton Metropole Hotel, London **(Oral: Correlation of copy number aberrations with clinicopathological criteria in paediatric glial tumours).**

ACKNOWLEDGMENTS

Firstly I would like to thank my supervisor, Dr Tracy Warr, for her help and guidance throughout the laboratory work and writing of this thesis. At least it's the last one for a while!

I would also like to thank my other colleagues, Dr Blanca Suarez-Merino and Dr Roy Poh, for their encouragement during the last daunting months of writing. I would especially like to thank Tracey Collins for sharing a very cluttered office with me, and being a listening ear on many a fraught occasion.

A big thank you to the Samantha Dickson Research Trust for providing the funding that allowed this research to take place.

Thank you to my friends at Welwyn Evangelical Church, especially Jon, Daniella & Ben Sheehan for keeping me sane and reminding me there's life beyond a Ph.D. Your support has been greatly appreciated!!

Lastly to my husband Andy... what can I say, but thanks for everything! I couldn't have done it without you.