MOLECULAR GENETIC STUDIES
IN
FAMILIAL AND SPORADIC COLORECTAL NEOPLASIA

by

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Abstract

Colorectal carcinogenesis is a multistep process classically characterised by at least four independent genetic events including loss of constitutional heterozygosity on chromosomes 5, 17 and 18 and point mutations of the K-ras cellular protooncogene. Because of the ready availabilty of post-operative material, both premalignant and malignant, adenomatous polyposis coli (APC) provides an ideal source and model for the study of the molecular genetic events involved in colorectal tumour progression. This study has, therefore, involved a detailed investigation of these phenomena and an attempt to correlate them with respect to the stage of tumour development.

Loss of heterozygosity in tumorigenesis is thought to signify involvement of tumour suppressor sequences at those loci and was therefore investigated using polymorphic DNA probes specific for the regions of the genome previously implicated in progression towards the malignant phenotype. Included in this survey was investigation of the status of the tumour suppressor genes p53 and DCC and that of the putative tumour suppressor gene MCC. The frequency of losses seen was generally in agreement with the results previously published with chromosome 17 and 18 losses being much more frequent in carcinomas than in adenomas. The frequency of K-ras point mutations was approximately seven-fold lower in adenomas than in carcinomas signifying that it is an early event in colorectal carcinogenesis but that it is not the initiating one. The nature of K-ras mutations was also studied by direct sequencing of the first exon and revealed the most common base change to be a G to A transition at position 2 of codon 12 representing the substitution of aspartic acid for glycine.

To date little evidence has been presented for the occurrence of chromosome 5 allele loss in APC-derived adenomas. This study illustrates that such loss, though infrequent in the general APC population, is significantly higher in a subset of patients who seemingly represent "new mutations" in that they have no prior family

history of the disease. Using a panel of locus-specific hypervariable minisatellite probes the possibility of non-paternity was ruled out. These patients, therefore, represent true "new mutations" and appear to exhibit a more aggressive form of the disease characterised by earlier onset of malignancy. These observations are possibly indicative of genetic heterogeneity at the APC locus.

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For W. J. Williams

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Page

1. INTRODUCTION

1 Introduction

1.1 Epidemiology of Colorectal Cancer

Colorectal cancer is very much a disease of the developed rather than the developing world. It is a major cause of death in North America and Western Europe (Lambert, 1982) which suggests an environmental role in its aetiology. In Britain it is the second most common lethal malignancy (Thornton et al., 1988) with around 25000 new cases annually of which 19000 will prove terminal (Northover, 1989). In 1976 it was estimated that colorectal cancer was responsible for 12 - 15% of all U.S. cancer deaths (American Cancer Society, 1977) and although detection and treatment has improved over the past few decades little impact has been made on the overall mortality rate (Hardcastle, 1989).

Geographical distribution studies particularly amongst migrant populations have proved invaluable in establishing aetiological factors associated with the disease. For example, studies show that Japanese migrants to Hawaii and California have an increased incidence of colorectal cancer compared with their non-migratory counterparts in Japan. Interestingly their risk of stomach cancer decreases. Similar colon cancer incidences have been observed in Polish migrants to the USA and Australia (Lambert, 1982). Also some religious groups such as Mormons and Seventh Day Adventists noted for their low meat diet and abstinence from alcohol have a significantly lower incidence of the disease (Wynder and Reddy, 1974). Several hypotheses have been proposed to explain these observations mostly on the basis of dietary differences.

1.2 The Role of Diet

Dietary content, especially starch and fibre which escape digestion in the small intestine, is a major determinant of colonic function in humans. These complex carbohydrates are the principle substrates for the various fermentation reactions carried out by the gut flora. This fermentation leads to an overall lowering of caecal pH and the production of short chain fatty acids such as butyric acid which may protect against dysplastic changes in the colonic epithelium (Cummings and Bingham, 1988). It is

thought that both tumour induction (through mutagenic changes) and tumour promotion may be susceptible to dietary influence.

The possible relationship between diet and colonic neoplasia was first reported by Higginson and Oettle (1960) and by Brenner (1964) amongst the Bantu people of South Africa. Their low incidence of large bowel cancer coupled with their high fibre intake suggested some protective influence of their diet. In 1971, Burkitt proposed that dietary fibre might serve to reduce the risk of bowel cancer through its capacity to regulate speed of transit as some faecal components have been shown to elicit mutagenic effects (Bruce et al., 1977). High levels of dietary fats may also increase the concentration of bile acids and their subsequent metabolism to carcinogens. Hill et al. (1971, 1975) found that the typical western diet produced a higher faecal concentration of neutral and acid steroids (derived from cholesterol and bile salts respectively) which may enhance carcinogenesis. Dietary fibre may also serve as a protective measure by absorbing steroids thereby reducing their contact time with the mucosal surface.

Faecal pH may also have a role to play - Patients with colorectal cancer have been found to have a higher than average pH (MacDonald et al., 1978) whereas the lower pH which results from a high fibre intake may inhibit bile acid degradation and reduce absorption into epithelial cells. Aside from fibre content, high intakes of fat are thought to be correlated with an increased risk of colorectal cancer (Cummings and Bingham, 1988) as is a high alcohol intake (Hirayama, 1989).

1.3 Heredity and Colon Cancer

Many cancers possess hereditary as well as sporadic manifestations. Although diet and environment appear to play an important aetiological role in colon cancer a fraction of cases (~5%) is the result of a clear genetic predisposition. These cases are characterised by an early age of onset compared with the sporadic form which is generally regarded as a disease of old age. Inherited disorders associated with the development of colorectal carcinoma broadly fall into two categories, as illustrated in table 1.1 below.

Table 1.1 Inherited syndromes associated with colorectal carcinoma

	ADENOMATOUS	HAMARTOMATOUS
POLYPOSIS	adenomatous polyposis (APC; FPC; FAP) Gardner's syndrome (Turcot's)	Peutz-Jegher's Juvenile polyposis Ruvalcaba-Myhre syndrome (Gorlin's)
NON-POLYPOSIS	Turcot's syndrome Muir-Torre syndrome Hereditary non-polyposis colorectal cancer: - site specific colon cancer (Lynch I) - cancer family syndrome (Lynch II)	Cowden's disease Gorlin's syndrome

(Classification adapted from Murday and Slack, 1988)

Adenomatous polyposis coli and Gardner's syndrome will be described in detail in later sections but a brief description of the other above mentioned disorders is necessary here to put them into context

1.3.1 Adenomatous Conditions

1.3.1.1 Turcot's syndrome

As in all colon cancer susceptibility syndromes this disease is rare but, unlike the others, inherited in a recessive manner (Turcot et al., 1959). It is characterised by adenomas of the bowel, tumours of the central nervous system, liver hyperplasia, basal cell naevi and carcinomas. It may be associated with adenomatous polyposis of the colon but the adenomas when present tend to be far fewer in number than in traditional APC (<100). Malignancies tend to have an early age of onset and few patients survive into the third decade (Murday and Slack, 1989).

1.3.1.2 Muir-Torre syndrome

This condition was first described by Muir et al. (1967) and comprises multiple carcinomas of the larynx and facial tumours including keratocanthomas and sebaceous adenomas. The familial, and probably dominant, nature of the condition was further investigated by Reiffers et al. (1976). A pedigree with similar characteristics was reported by Anderson (1980) in which there was also a high frequency of tumours similar to those seen in cancer family syndrome suggesting that one gene may be responsible for both conditions (Lynch et al., 1985).

1.3.1.3. Hereditary non-polyposis colorectal cancer

This umbrella description covers both aspects of Lynch syndrome. Cancer family syndrome (Lynch syndrome II) is an autosomally dominant condition first described by Lynch and Krush (1967). It exhibits early onset proximal colon cancer and a high incidence of other extracolonic adenocarcinomas such as those of the breast and endometrium (Lynch and Krush, 1973). Lynch syndrome I (site-specific colon cancer)

essentially possesses the same features except for the extracolonic manifestations (Woolf et al., 1955). A high incidence of adenomas is common but profuse polyposis is absent. In both cases the average age of malignancy is around 45 years (Lynch et al., 1977).

1.3.2 Hamartomatous Conditions

1.3.2.1 Peutz-Jegher's syndrome

In 1896 Hutchinson described a patient with mucocutaneous pigmentation who died of an intussusception. Similar pigmentation together with intestinal polyposis was observed in a kindred by Peutz (1921) and in 1949 Jegher and his colleagues reported a series of similar cases. Again this is a dominantly inherited autosomal condition with a high degree of penetrance and is associated with hamartomatous polyposis of the bowel. These hamartomas are not generally considered to be neoplastic (unlike adenomas) but cause recurrent intussusception and obstruction of the small intestine. The overall risk of colorectal cancer is low but there is a high risk of other malignancies (Giardello et al., 1987) especially of the stomach and duodenum (Bussey, 1975). A recent survey of 72 such patients at St. Marks Hospital (Spigelman and Phillips, 1989) revealed malignancies in 16 patients. These were mostly gastrointestinal but other tumours encountered included carcinomas of the ovary, fallopian tube, lung and thyroid as well as a basal cell carcinoma. All the gastrointestinal tumours proved fatal with an average age of mortality of 36 years.

1.3.2.2 Juvenile polyposis

Juvenile polyps are characterised by their non-neoplastic hamartomatous pathology and are found mainly in the colon and rectum or occasionally in the duodenum (Erbe, 1976). The main features are anaemia (due to continual rectal bleeding), diarrhoea leading in some cases to severe malnutrition, and intussusception. Congenital abnormalities can also occur. The syndrome usually presents with rectal bleeding in the second decade and the sporadic type of polyp may occur in as many as 1% of children (Jass et al., 1989). The inherited syndrome (again dominant) is much rarer and was

originally not thought to be associated with an increased risk of colon cancer (Veale et al., 1966). However such cases are documented (Smilow et al.,1966; Stemper, Kent and Summers, 1975; Restrepo et al., 1978; Goodman, Yardley and Milligan, 1979; Grigioni et al., 1981; Rozen and Barak, 1982; Jarrinen and Fransilla, 1984). Dysplasia in such cases occurs in two forms:

(i) as an adenomatous focus within a juvenile polyp

(ii) as an adenoma showing no residual hamartomatous features.

Based on these observations, Jass et al. (1988) suggest that such malignancies arise either (a) through conversion of a pre-existing hamartoma to a premalignant adenoma or (b) through proliferation of *de novo* adenomas.

1.3.2.3 Ruvalcaba-Myhre syndrome

This is a variant form of juvenile polyposis and was also reported by Erbe (1976) in two unrelated males with severe mental retardation, macrocephaly, freckling on the penis, seizures and diabetes (Smith, 1958; Ruvalcaba et al., 1980). Again a dominant mode of inheritance seems likely (DiLiberti et al., 1983) suggested by the case of a 7 year old boy whose mother was found to possess similar facial features and a hamartomatous polyp.

1.3.2.4 Gorlin's syndrome

Juvenile polyps have also been reported in Gorlin's syndrome (Swartz, 1978) another dominantly inherited condition. Colorectal cancer is rarely synonymous with this disorder but other malignancies such as medulloblastoma occur together with a high incidence of basal cell naevi which have a tendency to become cancerous.

1.3.2.5 Cowden's syndrome (Multiple hamartoma syndrome)

This is yet another example of a dominantly inherited condition in which sparse juvenile polyps are found in association with mucocutaneous lesions (Lloyd and Dennis, 1963; Tyrreson and Doyle, 1981; Haggit and Reid, 1986). Adenomas and colorectal cancer may occur in this condition although the greatest risk posed is that of

malignancies of the breast and thyroid (Gentry et al., 1974; Burnett et al.,1975). There may also be other abnormalities including macrocephaly, fibromas, angiomas, lipomas and disorders of the nervous system.

Apart from the above well-documented syndromes (and APC to be discussed later) there are cases in which adenomas and colorectal cancer appear to run in families but follow no strict pattern of Mendelian inheritance possibly due to interaction of hereditary and environmental factors. One study of a 500-member kindred suggested a dominant pattern of inheritance (Burt et al., 1985). Further similar studies by the same group showed that such inheritance was quite common and as such could account for a large proportion of clinically observed neoplasms (Cannon-Albright et al., 1988).

1.4 Adenomatous Polyposis Coli (APC)

Throughout this review the above name will be used to describe the condition which has previously been called both familial polyposis coli (FPC) and familial adenomatous polyposis (FAP).

1.4.1 APC - An Historical Review

According to Bulow (1987), the first reported case was published by Menzel in 1721. Dukes (1930) reported that it was first recognised as a familial and potentially malignant condition by Harrison-Cripps in 1882 when described in a brother and sister although previous accounts had been presented by Covisart (1847) and by Chargelaigue (1859). Similarly Bickersteth (1890) identified the condition in a mother and son. The risk of colorectal cancer ascribed to the disease was established by Smith in 1887. In 1925-6 Dukes described the various stages of polyposis and its relationship to cancer and his review of 1952 cited pedigrees investigated by many earlier workers in the field. Other reports by Lockhart-Mummery (1934) and Gardner (1951) concluded that it was a disease which manifested itself mainly between puberty and early adulthood.

The establishment of the St. Mark's Hospital polyposis register did much to enhance knowledge and understanding of the disease. It was initiated in 1925 by Lockhart-Mummery with three families and by the mid-1970s had grown to encompass over 200 families containing more than 600 individuals (Bussey, 1975; Bussey and Morson, 1978). A polyposis family is defined as one in which at least one member suffers from multiple (>100) adenomatous polyps of the large intestine (Bussey and Morson, 1978). Recently a similar register has been established in northern England (Chapman et al, 1989).

1.4.2 Characteristics of APC

Pedigree analysis of affected families (Dukes, 1952; Veale, 1965) have shown that adenomatous polyposis coli (APC) is an autosomally dominant inherited condition with a high degree of penetrance and that it predisposes the sufferer to the development of large bowel cancer. The disease is characterised by the appearance usually during the 2nd or 3rd decade of life of hundreds or even thousands of benign adenomas which are neoplastic growth extensions of the intestinal epithelium (figure 1.1). If left untreated one or more of these polyps progress to form malignant adenocarcinomas usually by the 4th or 5th decade of life. Onset of the disease rarely occurs before the age of 14 with a median age of adenoma development of 16 years (Bulow, 1986) although diagnosis may not occur until much later when symptoms such as rectal bleeding and diarrhoea present. As in other autosomal dominant conditions there is an even distribution between the sexes (Bussey, 1975; Bulow, 1986). Bussey (1975) reported that the density of adenomas increases from the ascending colon to the rectum (figure 1.2). The number of adenomas present generally varies between 100 (by definition) and 5000, with an average of just over 1000 (Bussey, 1975). Occasionally adenomas are found higher up in the gastrointestinal tract (Bulow, 1987) and it is thought that these may contribute to the periampullary carcinoma sometimes seen in the disease (Guyton and Schreiber, 1985; Bulow, 1987).

The majority of reports of APC have originated in the U.K., U.S.A. and northern Europe i.e. in caucasian populations. However the St. Mark's registry has records of

several families of other racial origins and the disease has been reported in other countries including South Africa, Korea and Japan. It seems likely therefore that the incidence is fairly constant throughout all races (Bussey, 1975).

The frequency of the disease has proved difficult to ascertain and various estimates have been arrived at using different criteria of analysis e.g. 1 in 29000 (Neel, 1954); 1 in 8300 (Reed and Neel, 1955); 1 in 23790 (Veale, 1965); 1 in 6850 (Pierce, 1968). More recently Alm and Liznierski (1975) used Swedish data to establish a frequency of 1 in 7646. It seems likely therefore that the actual frequency is of the order of 1 in 5000 to 1 in 10000 live births.

1.4.2.1 Gardner's syndrome

In 1951 Gardner reported a familial syndrome consisting of the triad of colonic polyposis, multiple osteomas (especially of the mandible) and soft tissue tumours of the body surface. The disease was christened Gardner's syndrome but there is sufficient reason to believe that it merely represents another manifestation of APC and results from the same basic genetic defect. Gardner and Plenk (1952) concluded that the mode of inheritance of osteomas associated with polyposis could be explained either by the action of a single pleiotropic gene or by two closely linked genes. Gardner and Richards (1953) took this argument one step further and suggested that all three types of lesion were inherited via the same mutated gene. In association with these extracolonic manifestations large fibrous collagen-rich tissue masses called desmoid tumours have been found in a number of patients (Smith, 1958,1959; Gardner 1962; Bulow, 1987). These tumours are not malignant but often occur in or around abdominal incision scars of patients and cause problems by physical obstruction rather than by any pathological effect inherent in the tumours themselves. Smith (1959) concluded that this syndrome was a "full-blown manifestation of a spectrum of pathologic changes which would affect in varying number and combination any patient with multiple polyposes" and that desmoids may also possibly be explained in the same hereditary basis.

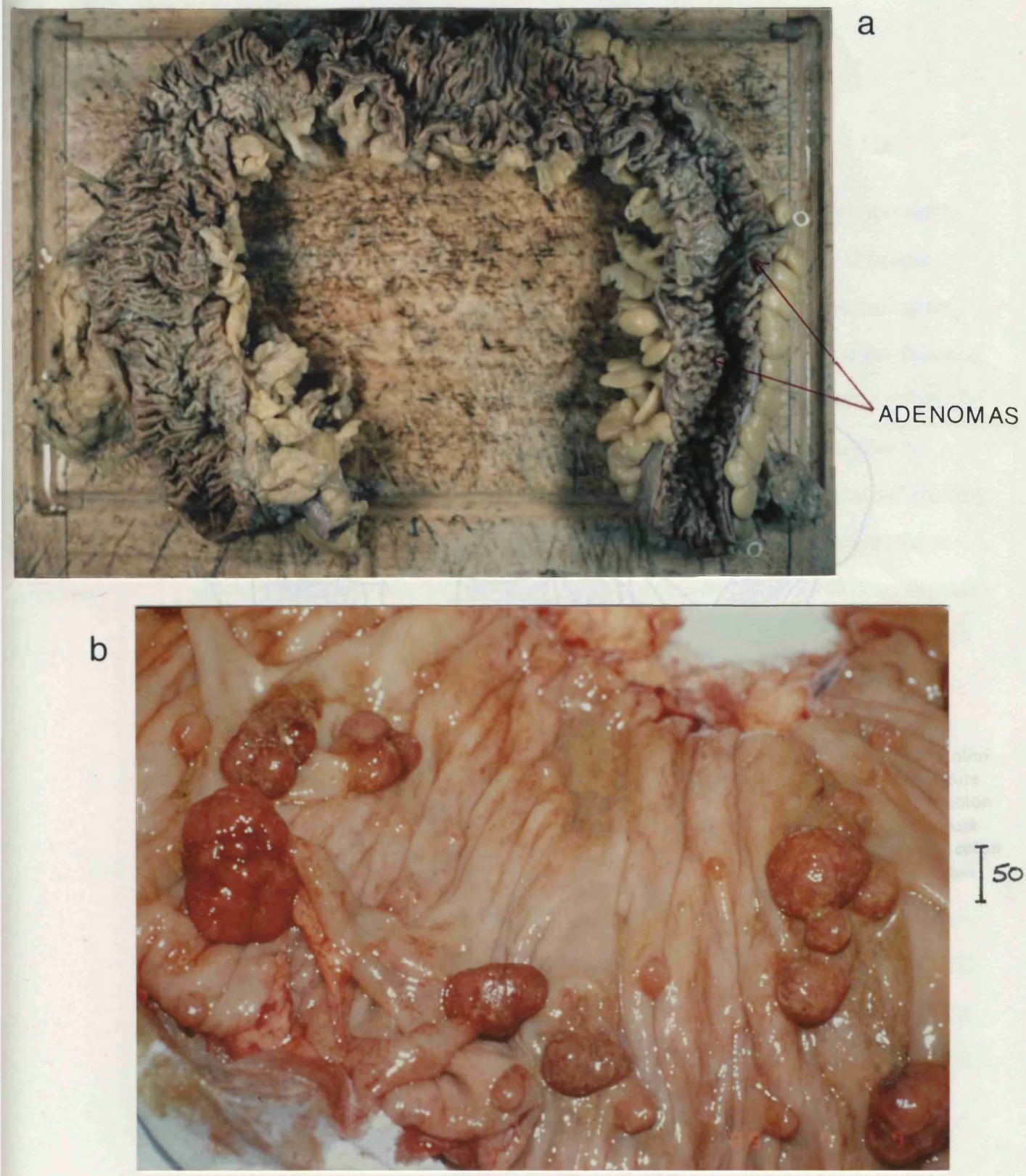


Figure 1.1.(a) Colon of patient 139 following removal by proctocolectomy and formalin fixation. **(b)** Close-up view of APC adenomas in the surgically removed colon of patient 136.

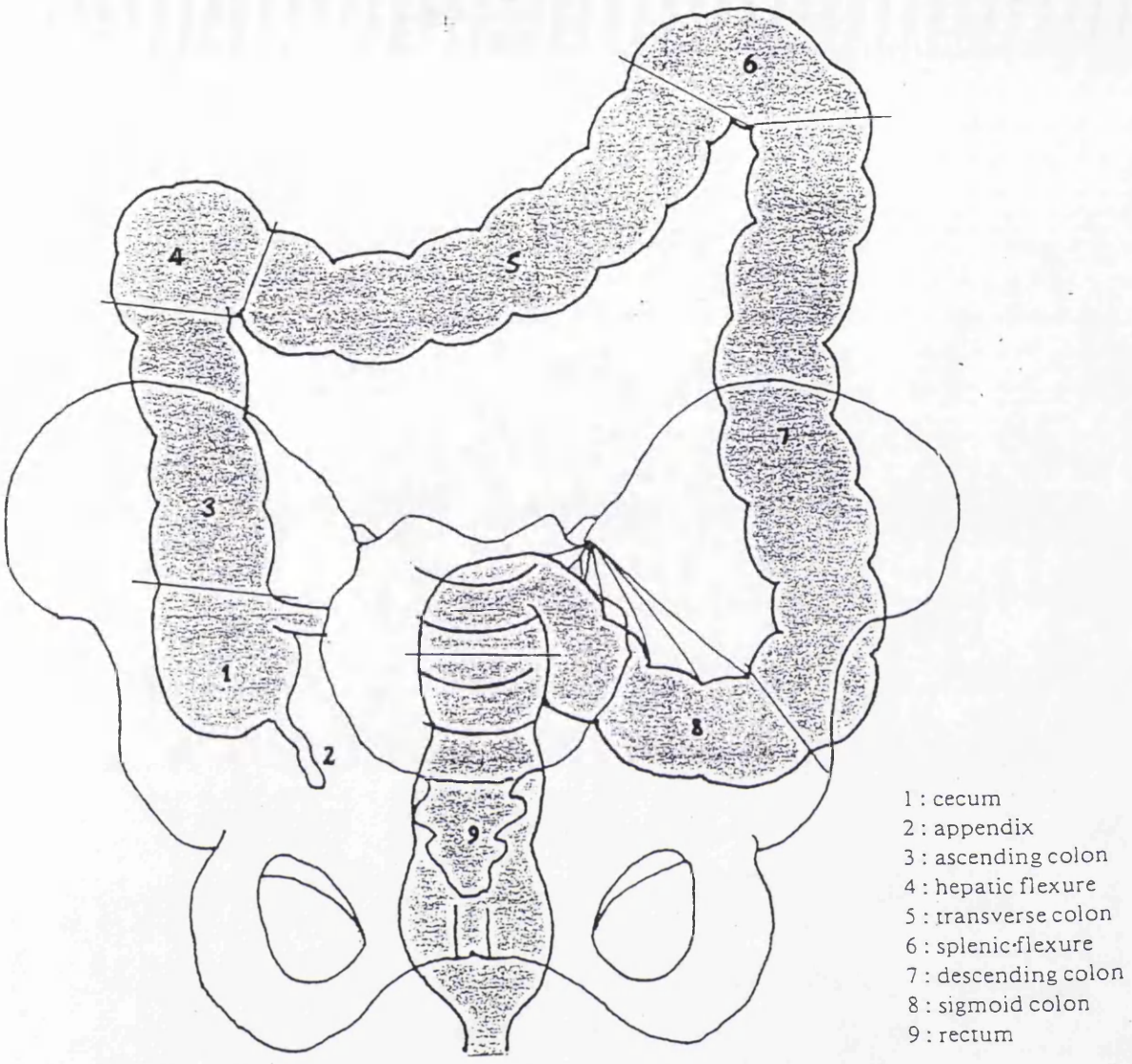


Figure 1.2. Diagrammatic representation illustrating the subdivision of the colorectal region. (from Hermanek et al., 1983)

1.4.3 Management of APC

1.4.3.1 Diagnosis

Diagnosis in families known to harbour the defective gene usually occurs prior to the development of symptoms. Detection of adenomas by regular sigmoidoscopic examination from an early age remains the surest method of diagnosis backed up by colonoscopy and barium enema (Bussey and Morson, 1978). However if the disease is the result of a new mutation it is likely to remain undetected until symptoms present. So far there is no sure way of predicting which family members are affected prior to adenoma development. Blood groups (Veale, 1958), intracellular epithelial cell granules (Birbeck and Dukes, 1963) and fingerprint analysis have all proved unsuccessful as predictive genetic markers. A report by Utsinomiya and Nakamura in 1975 highlighted a high incidence of subclinical mandibular osteomas in APC patients which are visible on X-ray but this does not appear to be routinely applicable as a means of diagnosis.

More recently limited predictive testing based on the unusually high incidence of congenital hypertrophy of the retinal pigment epithelium (CHRPE) in individuals with Gardner's syndrome (Blair and Trempe, 1980; Traboulsi et al., 1987) and APC (Diaz-Lopez and Menzo, 1988) has been successfully carried out in APC families (Chapman et al., 1989). Lewis et al. (1984) illustrated the presence of CHRPEs in 3 such families whereas they are only expected in 5% of the general population. However not all APC families appear to posses the trait (Lyons et al., 1988) but in those that do it is thought to be a reliable means of diagnosis. The study by Chapman et al. (1989) found that all carriers of the *APC* gene previously identified had such retinal lesions ranging in number from 2 to >40 whereas none of the control subjects possessed more than 2. They suggested that the small number of APC pedigrees who are negative for the eye signs (Traboulsi et al., 1988) may comprise a distinct genetic sub-group. This method of simple ocular examination has the advantage that it is far less invasive than the yearly bowel examination which suspected patients would normally undergo and hence trauma to the patient is drastically reduced. It is hoped that such ocular fundoscopy

combined with pedigree analysis and genetic linkage data may in the near future prove sufficiently accurate in its identification of non-carriers to remove the need for three decades of endoscopy (Chapman et al., 1989).

1.4.3.2 Treatment of APC.

Surgery remains the only effective treatment, the aim being to remove the adenomas thus preventing their progression to malignancy. Total proctocolectomy i.e. the excision of the colon and rectum achieves this but most surgeons prefer the less drastic removal of the colon followed by ileo-rectal anastomosis. The remaining rectal polyps are destroyed as far as possible but periodic examination of the retained rectum is essential although spontaneous regression of rectal polyps has been observed following the removal of the colon (Bussey, 1975). It must be pointed out,however, that removal of the colon does not completely alleviate cancer risk associated with adenomas present higher up in the gastrointestinal tract but in general they are far fewer in number and therefore represent a much lower risk of malignant progression.

1.4.4 The Adenoma - Carcinoma Sequence.

1.4.4.1 Classification of carcinomas

It is generally agreed that the majority of carcinomas of the colon and rectum arise in pre-existing benign adenomas (Morson and Bussey, 1970). Approximately 85-90 % of colorectal cancers are adenocarcinomas containing small amounts of detectable mucus. About 10% of colorectal cancers are mucinous adenocarcinomas with excessive extra-cellular mucus. The remainder (<5%) are made up of carcinomas of the signet ring cell, squamous, adenosquamous and undifferentiated types (Hermanek et al., 1983).

The classification of cancer staging was first initiated by Dukes in 1932 who subdivided colorectal cancers into 3 groups on the basis of their extent of spread (fig. 1.3. - from Hermanek et al., 1983).

In addition to the Dukes stage (A,B or C) , at St. Marks Hospital in London intestinal cancers are also subdivided into three categories on the basis of their histology (Bussey, 1975):

(i) low grade malignancy (well-differentiated)

(ii) average grade malignancy (moderately differentiated)

(iii) high grade malignancy (poorly differentiated).

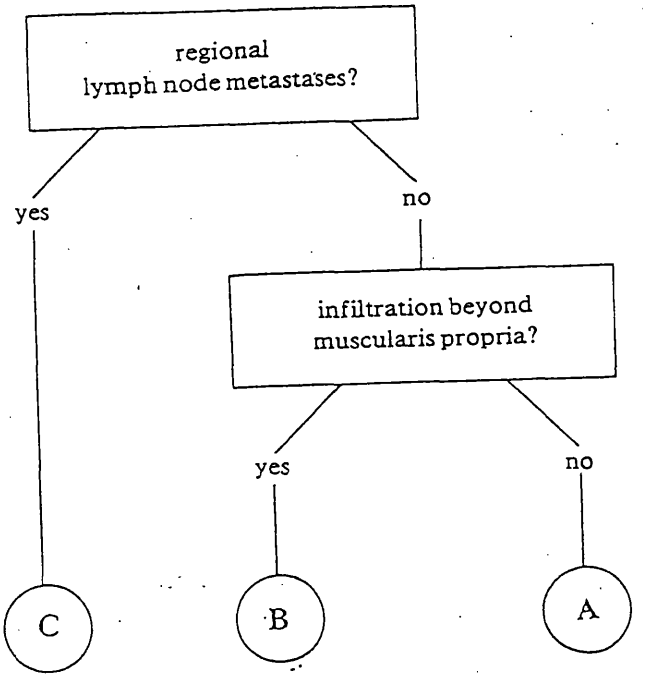


Figure 1.3. Dukes' classification of colorectal carcinomas. (from Hermanek et al., 1983)

1.4.4.2. Classification of adenomas.

The term adenoma is applied to any benign neoplastic lesion arising from the mucus-secreting cells of the intestinal mucosa (Bussey, 1975). They can be subdivided into 3 histological types (Muto et al., 1975).

(i) tubular adenoma (adenomatous polyp) ~ 75%

(ii) villous adenoma ~10%

(iii) tubulo-villous adenoma ~ 15%

They can also be subdivided on the basis of their shape into sessile, semi-sessile or pedunculated. Those of the sessile, villous type appear to have a greater malignant potential which maybe due to their greater area of contact with the mucosal surface. Adenomas from polyposis and non-polyposis patients appear to share the same structure and APC patients therefore provide a useful source for the study of early polyp formation (Bussey and Morson, 1978). Various studies have been carried out which implicate adenomas in the precursive stages of most colorectal malignancies. In particular, a study by Muto et al.(1975) examined the relative malignant potentials of tubular and villous adenomas. Both macro- and microscopic examination of a series of colon tumours (polyps and cancers) revealed areas of contiguous benign and malignant material suggesting that these cancers arose from a previously existing benign tumour. The frequency with which the associated benign tumour was found varied considerably:-

- 60% if the cancer invasiveness extends only to the submucosa
- 20% if the cancer is limited to the bowel wall
- 7% if the cancer invades the bowel wall and the extramural tissue

Similar results have been obtained by other workers and suggests that as the cancer spreads it destroys the remainder of the benign tumour. Muto et al. (1975) also found pure villous adenomas to have a higher malignant potential than either of the other types. This difference is difficult to explain on a histological basis as the cytological features are essentially the same in all three classes of adenoma the classification merely reflecting differences in tissue architecture. The risk of cancer also increases with increasing size of the adenoma and an increase in degree of epithelial atypia is also

associated with increased malignant potential. It has been suggested (Hill et al., 1978) that the major factor in determining the incidence of carcinoma is that affecting the growth of adenomas rather than that which initiates malignant change. In practice, the majority of polyps found in APC patients are of the tubular type but some have a villous component. Pure villous adenomas are very rare. There is histological evidence to suggest that the cancers seen in APC arise from adenomatous polyps and that the morphology of the adenoma-carcinoma sequence in polyposis is the same as for sporadic cases. There is no hypothetical mechanism for colorectal cancer arising *de novo* i.e. without a premalignant adenomatous stage. APC therefore provides an ideal human model for the study of the cellular transitions involved in large bowel carcinogenesis.

1.4.4.3 Formation of adenomas

Adenomas arise from the crypts of Lieberkuhn as a result of a shift in the proliferative compartment of the crypt (Lipkin, 1988) from the base of the crypt to the top although mitotic activity may be seen at all levels . In general cellular regeneration and proliferation arise in the basal third of the crypt (which contains mostly columnar cells); differentiation/ migration occurs in the upper two-thirds; and extrusion occurs at the surface where occasional necrobiotic (dead) cells are found (figure 1.4).

1.4.4.4 Clonality of adenomas

There has been much work done to establish the clonal origins of colorectal adenomas. A study of Gardner's syndrome patients by Hsu et al. (1983) investigated X-chromosome inactivation in relation to G-6-PD isoenzymes. This technique was employed in female patients who were heterozygous for the two isoenzymes. The G6PD gene is carried on the X chromosome and in a given cell population some inactivated X chromosomes will carry the A isoform and others the B isoform. The two isozymes can be distinguished electrophoretically due to migratory differences.

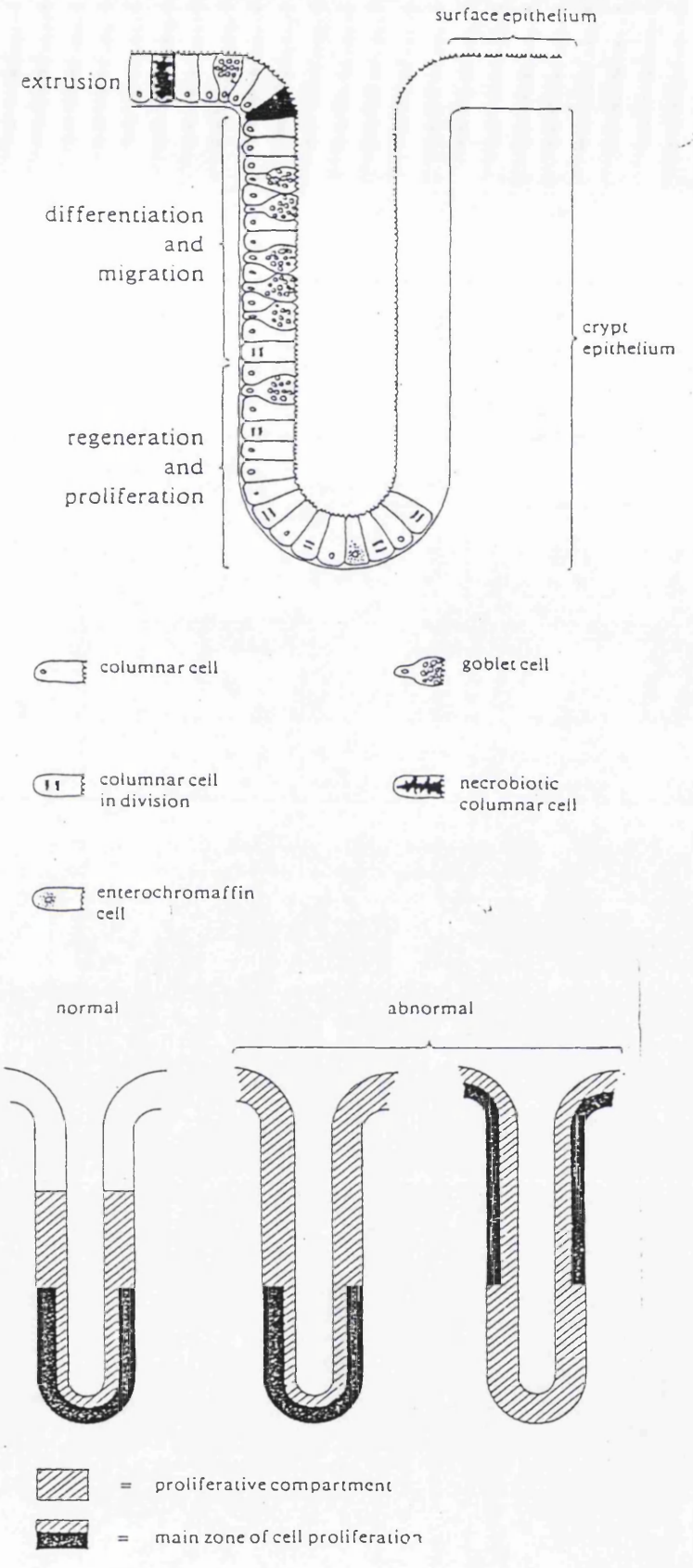


Figure 1.4. **(a)** Cellular types and organisation in the Crypts of Lieberkuhn.

(b) Shift in cell proliferation in abnormal crypts.(from Hermanek et al., 1983)

Tumours which originate from a single precursor cell (i.e. are clonal) will therefore only express one allele whereas those which express both alleles are regarded as being derived from more than one cell and are therefore non-clonal. The study by Hsu et al. reached the conclusion that the polyps were multi-clonal. This hypothesis was more recently brought into doubt by another similar study this time carried out at the DNA level (Fearon et al., 1987) in which X-linked restriction fragment length polymorphisms were examined to establish the clonal origin of polyps from APC patients. The general consensus is that each adenoma is derived from a single crypt and is therefore monoclonal since the colonic crypts are known to be maintained by a single stem cell (Griffiths et al., 1988).

1.5 The Theory of Tumour Suppression

1.5.1 The Two-hit Hypothesis

This hypothesis was first proffered by Knudson (1971) to explain events involved in the genesis of both sporadic and inherited forms of the childhood cancer retinoblastoma. The theory suggests that two separate mutational events at the same gene locus are required for carcinogenesis. In the familial form the first mutation is dominantly inherited via the germline and the second arises spontaneously at the somatic level. In the sporadic form both events occur somatically. These allelic mutational events would therefore result in a complete loss of gene function at this locus. Knudson also suggested (1985) that every form of cancer has a rare inherited counterpart and that the two-hit hypothesis could also be applied to other inherited forms of cancer susceptibility.

Subsequent work by Ohno (1971) suggested that chromosome loss may play a role in tumorigenesis and Comings (1973) theorised that regulatory loci whose products control proto-oncogene expression may act as targets for loss of function carcinogenic mutations.

Work by Harris et al.(1969) and by Klein et al. (1971) in which fusion of tumorigenic malignant cells to non-tumorigenic cells produced non-malignant fusion hybrids suggests that the non-malignant cells express "tumour-suppressor" genes which result in the non-tumorigenic phenotype of the hybrid. Recessive loss of function mutations have since been implicated in several human and animal tumours (Knudson, 1985) and is not restricted to mammalian tumours since they have been reported in drosophila (Gateff 1978; 1982) and in the fish genus Xiphophorus (Anders, 1983; Anders et al.,1985). Such genes have been referred to as "antioncogenes" (Green and Wyke, 1985; Knudson, 1985) although tumour suppressor is now the preferred term.

1.5.1.1 Retinoblastoma

The first hurdle to be overcome in isolating the gene responsible for retinoblastoma was the identification of its genetic locus. It was found that a small percentage of cases contained a chromosomal deletion around band 13q14 (Knudson et al., 1976; Francke and Jung, 1976; Yunis and Ramsay, 1978; Strong et al., 1981). Deletion analysis and family studies (Sparkes et al., 1980,1983) demonstrated linkage between the esterase D locus (ES-D) and the retinoblastoma locus (designated RB-1). Additional evidence for inheritance via a recessive gene comes from a patient with a 50% esterase D activity in the normal cells but none in the tumour. Two stem cell lines were identified in the tumour each with only one chromosome 13 but with no detectable deletion in the remaining chromosome (Benedict et al., 1983) i.e. the loss of gene function at this locus was due to an extremely small deletion or point mutation. The unmasking of such a recessive mutant allele could occur either by total loss of the wild-type chromosome resulting in hemizygosity or by conversion to homozygosity for the mutant chromosome e.g. by mitotic non-disjunction leading to duplication of the mutant allele (Cavanee et al., 1983).

Molecular analysis of DNA from retinoblastoma patients using the technique of restriction fragment length polymorphism has proved fruitful in revealing submicroscopic deletions of chromosome 13 (Cavanee et al., 1983; Dryja et al., 1984;

Dryja et al., 1986a). Similar studies (Hansen et al., 1985) have produced the same results for retinoblastoma patients suffering from subsequent osteosarcomas and Dryja et al (1986b) found homozygosity at loci on chromosome 13q in osteosarcoma patients with no history of retinoblastoma. The retinoblastoma gene (RB) was finally identified and cloned by Friend et al. (1986) and subsequently by Lee et al. (1987). Identification of the mutations involved has been carried out (Dunn et al., 1988) and the ultimate demonstration of its tumour suppressor capabilities was exhibited by the elegant work of Huang et al. (1988) in which the cloned RB gene was transfected into retinoblastoma and osteosarcoma cell lines in which the endogenous gene was known to have been inactivated. Transfection resulted in suppression of the neoplastic phenotype.

Loss of function at the RB gene locus has since been implicated in a variety of tumours including those of the lung and breast suggesting a more general role in tumour suppression. (Lundberg et al., 1987; Yokota et al., 1987).

1.5.1.2 Wilms' tumour

Another well-characterised cancer-predisposition syndrome in which Knudson's hypothesis seems to apply is Wilms' tumour, an embryonal malignancy of the kidney which occurs in both inherited and sporadic forms (Knudson and Strong, 1972). The autosomal dominant and heritable form of the disease is often associated with other specific congenital malformations such as aniridia, genito-urinary abnormalities and mental retardation (the so-called WAGR complex).Constitutional chromosomal deletions of 11p13 have been demonstrated (Orkin et al.,1984; Fearon et al., 1984) suggesting that loss of information within the 11p13 band is responsible for predisposition to the disease. This idea is supported by other studies of allelic loss (Koufos et al.,1984; Fearon et al., 1985) whereas work by Reeve et al. (1984) suggests the involvement of the H-ras gene (located close by at 11p14.1) demonstrating loss of one of the two constitutional alleles. Other molecular data also show that development of homozygosity at 11p15 is common to all three tumour types characteristic of the Beckwith-Wiederman syndrome i.e. hepatoblastoma, rhabdomyosarcoma and Wilms' tumour (Koufos et al., 1985). Somatic loss of loci on

11p resulting in homo- or hemizygosity have also been demonstrated in adult bladder cancer whereas retinoblastoma and colon cancer showed no such loss (Fearon et al., 1985). However recent linkage analysis of a family segregating for Wilm's failed to demonstrate linkage at either 11p13 or 11p15 suggesting that development of the disease may be dependent on more than two mutational events - one each at 11p13 and 11p15 and another at an unidentified locus thus calling into question the applicability of Knudson's hypothesis to this disease (Grundy et al., 1988). Recently, however, a candidate gene has been cloned independently by 2 groups (Rose et al., 1990; Call et al., 1990; Gessler et al., 1990) and mapped to band p13 of chromosome 11. The gene appears to code for a zinc-finger protein possibly with some role in transcriptional regulation. Demonstration of deletions of this genomic region in Wilms' tumours of patients with the WAGR syndrome and in some sporadic Wilms' patients indicates the involvement of this gene in at least a subset of tumours but demonstration of the presence of mutations in the tumour RNA transcripts has yet to be published.

The isolation of this gene is yet another vindication of the reverse genetics strategy indicating the usefulness of allele loss determination as a marker for the isolation of tumour suppressor sequences.

One molecular study of WT by Schroeder et al.(1987) does not quite fit predictions of the two-hit theory. In all 5 sporadic Wilms' tumours looked at the maternal chromosome 11 had been lost whereas maternal and paternal allele loss would occur with equal frequencies if the events were entirely random. Data suggests that loss of the maternal allele is non-random and that the initial mutation occurs in the paternal allele which is retained. Such a phenomenon is known as genomic imprinting.

1.5.1.3 The role of genomic imprinting

Expression of genes may vary depending on whether they are inherited from the mother or the father and it has been proposed that the maternal genome largely determines embryonic development whereas the paternal genes are important for extraembryonic development (Monk et al., 1987). In the mouse both sets of chromosomes are necessary for complete development. It has been observed that

neither androgenic (diploid paternal) or parthenogenetic (diploid maternal) embryos develop to term and suggest that maternal and paternal genomes have different inherent and epigenetic information imprinted upon their chromosomes which results in different developmental programmes (Reik, 1989). It has been suggested that differential methylation of particular regions could be responsible for imprinting of homologous chromosomes according to their parental origin (Reik, 1989).

Another avenue of research, on hyatidiform moles (Wake et al., 1984), has shown that they develop as a result of elimination of the maternal genome from a fertilised egg and many of these moles progress to choriocarcinomas. Therefore it is possible that some of these imprinted genes may determine malignancy in some cell types (Wilkins, 1988). One hypothesis has been put forward to explain the occurrence of Wilms' tumour on the basis of imprinting in which both parental Wilms' genes (Wg) are expressed in the embryo and in the normal situation the product of these genes regulate another gene close by called the transforming gene (Wilkins, 1988). If one of the Wg genes is inactivated (either through an inherited mutation or a somatic one) the Tr gene is still suppressed by the remaining allele. However, if the second Wg gene is lost the Tr genes are released from their suppressed state and expressed at a high level but only if it is unmethylated. It is therefore thought possible that methylation of the maternal transforming gene inactivates it so only if the paternal allele is retained and the maternal one lost is the Tr gene expressed. This hypothesis may explain the observation of non-random loss of maternally derived material in Wilms' tumour but it remains to be seen whether it will have a general applicability in the study of other carcinogenic mechanisms. Also in light of the recent isolation of a candidate Wilms' gene it will be of great interest to see whether this theory stands up to empirical scrutiny.

1.5.1.4 Other hereditary cancers

Since the first demonstrations of allele loss at loci implicated in retinoblastoma and Wilms' tumour, similar research has been conducted on a wide range of human tumours and various chromosomes implicated as illustrated in table 1.2.

Table 1.2.Chromosomes implicated in human tumours by allele loss

Chromosome	Tumour	References
1	endocrine neoplasia	Mathew et al. 1987
2	uveal melanoma	Mukai and Dryja 1986
3p	renal cell carcinoma	Zbar et al. 1987
	small cell lung carcinoma	Kok et al. 1987
	lung adenocarcinoma	Yokota et al. 1987
5q	colorectal carcinoma	reviewed later
11p	Wilms' tumour	Fearon et al 1984; Orkin et al.1984
	hepatocellular carcinoma	Rogler et al 1985
	rhabdomyosarcoma	Scrable et al. 1987
13q	retinoblastoma	Cavanee et al. 1983
	ductal breast carcinoma	Lundberg et al. 1987
	small cell lung carcinoma	Yokota et al. 1987
15	acute lymphoblastic leukaemia	Stamberg et al. 1986
17p	small cell lung carcinoma	Yokota et al. 1987
	colorectal carcinoma	reviewed later
	astrocytoma	Fults et al., 1989
	osteosarcoma	Toguchida et al., 1989
	breast carcinoma	Coles et al., 1990; Devilee et al., 1990
18q	colorectal carcinoma	reviewed later
22	acoustic neuromas	Seizinger et al. 1986
	colorectal carcinoma	reviewed later

Green and Wyke (1985) suggest that such deletions result in the loss of negative regulatory elements (tumour suppressor sequences) and either allows the expression of proto-oncogenes or encourages their mutation and may apply to a wide range of human solid tumours (Atkin et al.,1985). It maybe that the chromosomes that are involved in more than one tumour type (table 1.2) harbour more general tumour suppressor sequences able to induce more than one type of cancer perhaps in cooperation with other events. It may be the case that allele loss is more important than inactivation of the dominant (protective) allele by point mutation or intragenic alteration (Yandell et al., 1986). It seems therefore that allele loss is an important aetiological factor in many forms of carcinogenesis and may play a role in the development of APC and colorectal cancer in general.

1.6 Recent work in colorectal neoplasia

1.6.1 Karyotypic studies

In order to apply the molecular techniques used previously in retinoblastoma and Wilms' tumour to the possible identification of submicroscopic deletions in colorectal carcinoma and also in adenomas (e.g. by RFLP analysis) we first need to obtain an idea of where to look. As the human genome is so large the implication of one or two chromosomes of interest would greatly reduce the time needed to determine deletions which may be of importance in the initiation or promotion of colorectal cancer, particularly with respect to the characterisation of tumour suppressor sequences. At one time it was thought that APC may be linked to the HLA system on human chromosome 6 (Vargish, 1975) and evidence of rearrangements of HLA class I genes has been seen in colorectal polyps and cancers (Bar-Eli et al., 1988) but more recent work has concentrated on those chromosomes implicated in karyotypic studies.

Many karyotypic studies have been carried out on adenocarcinomas of the colon and rectum and some on adenomas although the results have not been entirely conclusive. One problem encountered is the difficulty of obtaining good chromosome

spreads from solid tumours and also there tends to be widespread constitutional chromosome instability in familial cases (Delhanty et al., 1983; Rider et al., 1986). However certain chromosomes have been implicated in colon cancer particularly chromosomes 17 and 18 (Muleris et al., 1985; 1986). They found consistent deletions of the short arm of chromosome 17 often present as iso17q (i.e. duplication of the long arm) together with systematic loss of the whole of one copy of chromosome 18. Frequent deletions of 1p were also observed. Similar losses have been seen in other adenocarcinomas (Martin et al., 1979; Reichmann et al.,1981; Ferti-Passantopolou et al., 1986) though always together with other abnormalities. A chromosome translocation t(Xp:17p) has been demonstrated in a cell line derived from an APC cancer (Paraskeva et al., 1984).

Figure 1.5. shows the results of a study by Reichmann et al., (1985a) which demonstrates the involvement of karyotypically detectable chromosome 17 deletions in a large proportion of colorectal cancers and also the high frequency of chromosome 5 deletions. As can be seen other chromosomes involved in this study include 1, 8 and 9.

Some workers have observed that adenocarcinomas of the descending colon appear to be subject to a greater degree of chromosomal rearrangement than those of the ascending colon (Couturier-Turpin et al., 1982; Reichmann et al., 1985a) although the clinical significance of this observation remains to be established.

Two karyotypic studies of adenomas (Reichmann et al.,1982; 1985b) have revealed no particular chromosome anomalies. Although the results obtained in the various studies are by no means conclusive certain trends occur especially in the case of chromosomes 1, 5, 17 and 18. An hypothetical explanation of the involvement of 17 and 18 has been proposed by Dutrillaux and Muleris (1986) on the basis of a decrease in the *de novo* pathway of thymidine biosynthesis and an increase in the salvage pathway but this remains to be confirmed. One study, however, by Sakamoto et al (1985) has found evidence of increased thymidine kinase activity in colorectal polyps and carcinomas but whether or not this is associated with the chromosomal events discussed above remains to be seen.

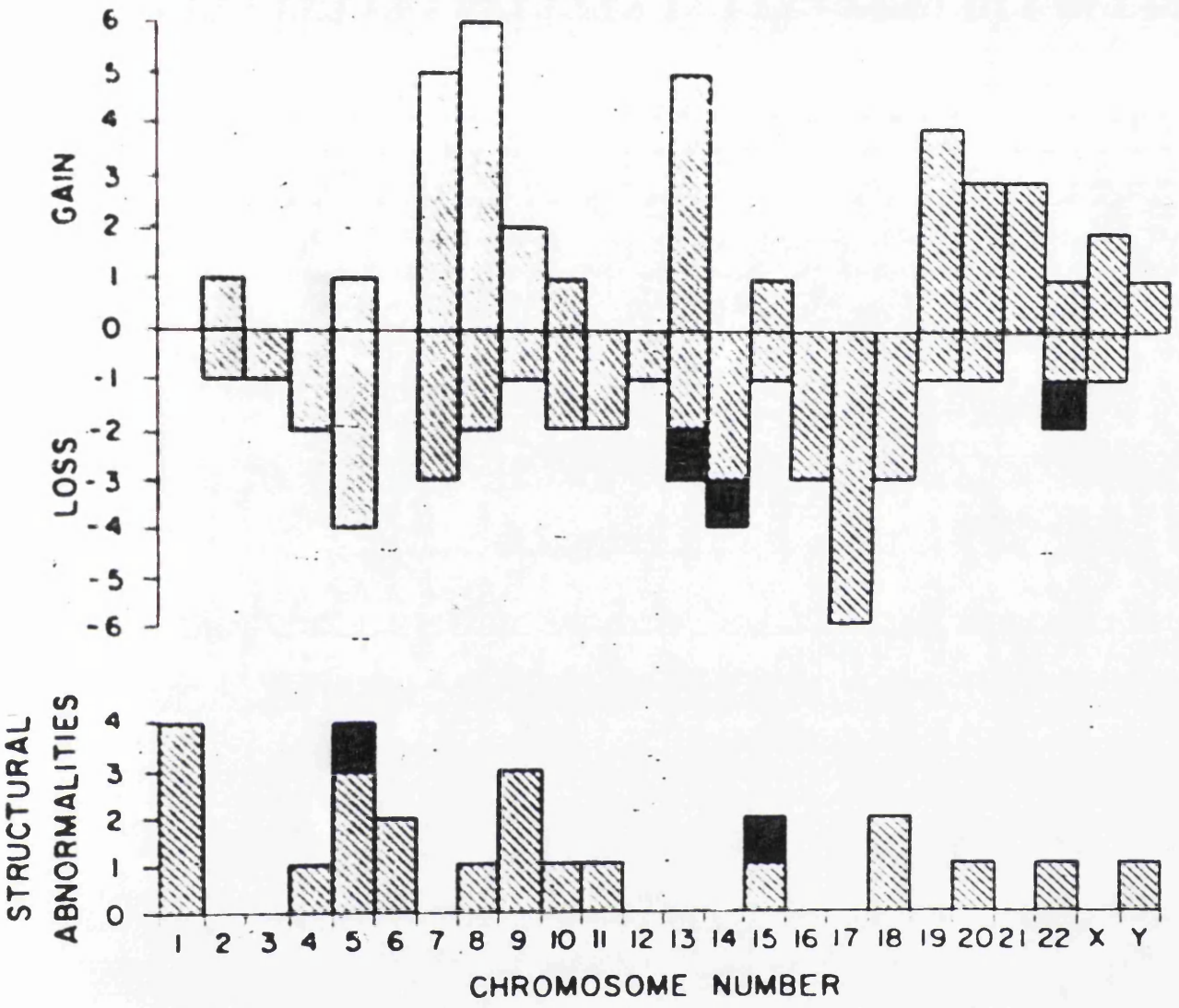


Figure 1.5. Chromosomes implicated in colorectal tumorigenesis by virtue of loss, gain or the presence of structural abnormalities (after Reichmann et al., 1985a)

■ Both chromosome copies involved

1.6.2 Molecular Genetic Studies

1.6.2.1 Linkage analysis

Evidence of linkage of a disease locus to a particular chromosomal region is an essential first step in the isolation of any gene responsible for an inherited defect particularly when the gene function and hence the underlying biochemical defect is unknown. The successful implementation of this "reverse genetics" strategy has been exemplified recently by the isolation and characterisation of the gene responsible for cystic fibrosis (Kerem et al., 1989). It is hoped that similar progress will eventually be made in the isolation of the APC gene.

Little karyotypic data is available from APC patients but cultured skin fibroblasts from one such patient were shown to possess a t(5:17) translocation (Delhanty et al., 1983) which was either present constitutionally or occurred during early passage. This observation may or may not be of significance with respect to the aetiology of adenomatous polyposis and colon cancer but its possible involvement was strengthened by reports linking the APC gene to a polymorphic DNA marker at a locus on chromosome 5. This work was first reported by Bodmer et al. (1987) who utilised the observation of a visible interstitial deletion of chromosome 5q in a mentally retarded patient who also suffered from Gardner's syndrome (Herrera et al., 1986). Restriction fragment length polymorphism (RFLP) analysis using several chromosome 5 DNA markers was used to establish linkage. One of these markers, C11p11 (D5S71) reveals two alleles on Taq 1 digestion of genomic DNA and exhibited tight linkage with APC. *In situ* hybridisation to metaphase chromosome spreads revealed C11p11 to lie in the 5q21-22 region. These results were subsequently confirmed by Leppert et al. (1987). Linkage analysis carried out in our laboratory also confirmed linkage of APC to this region but also detected the presence of an obligate recombinant in one of our informative families, the first report of recombination between the APC and C11p11 loci (Aldred et al., 1988). Following the establishment of linkage, another chromosome 5 marker, π227 (D5S37) which had been mapped to 5q21 (Stewart et al., 1987) was shown also to be linked to APC although not as closely as C11p11 (Meera-Khan et

al.,1988; Burn et al., 1991; Dunlop et al., 1989) and originally thought to lie about 5Mb centromeric to the disease locus with C11p11 telomeric (Meera-Khan et al., 1988). More recently, however, it has been established (Dunlop et al, 1989) that both C11p11 and π 227 lie approximately 5 and 10 cM respectively centromeric to the APC gene.

Further linkage analysis done using a clone originally isolated by Nakamura et al. (1988) showed it to be even more closely linked to the APC locus than C11p11. This marker, YN5.48 (D5S81) is thought to lie on the telomeric side of the disease gene (Dunlop et al., 1989). Work by Varesco et al. (1989) analysed material from two mentally retarded brothers with APC both of whom had a visible chromosomal deletion in the 5q21-22 region (Hockey et al., 1989). They succeeded in producing a somatic cell hybrid with the deleted chromosome 5 as the only human component and showed that neither C11p11 or π227 mapped within the deletion. They finally isolated a clone ECB.27 (D5S73) which lies within the deletion and exhibits a DNA polymorphism on BglII digestion of genomic DNA and is therefore useful for further linkage analysis. More recently another APC patient with a small interstitial chromosome 5 deletion just detectable karyotypically has been identified. Analyses show that ECB27 lies outside the deleted region in this patient (Cross et al., 1991) and further investigations of the nature of this deletion are underway.

Recent work in our laboratory (Burn et al., 1991) has established evidence for linkage of APC with C11p11, π227 and with the eye defect CHRPE in conjunction with workers at the Northern Polyposis Registry and further linkage to the YN5.48 and ECB.27 has been established (Cachon-Gonzalez et al., 1991). The diagnostic applicability of such linkage techniques has recently been proven in a Dutch study (Tops et al., 1989). The most recent linkage map of the region surrounding the *APC* gene has been formulated using six linked markers in nine families, including two closely-linked markers recently isolated, EF5.44 and L5.62, along with the four previously mentioned (Dunlop et al., 1990), giving the order shown in figure 1.6. Another linked marker, KK5.33 (D5S85) proximal to *APC* and probably distal to ECB27 has also recently proved useful in family studies (Friedl et al., 1991).

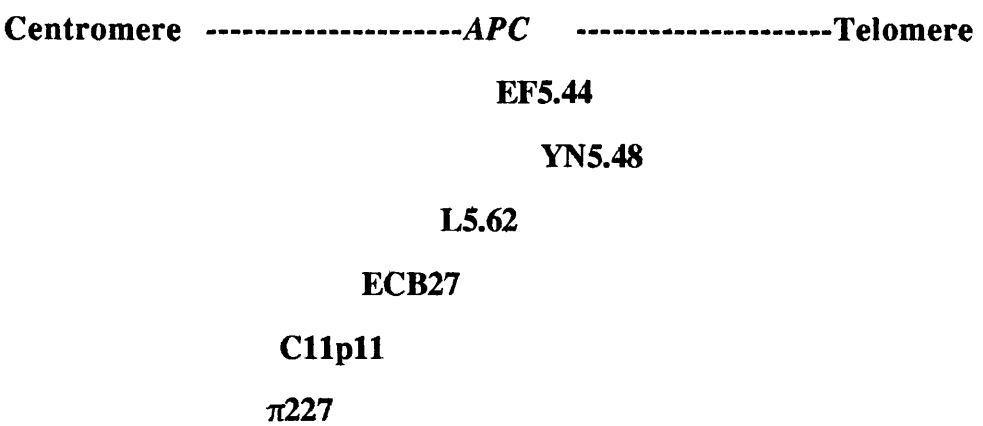


Figure 1.6. Relative positions of linkage markers around the *APC* locus (Dunlop et al., 1990).

1.6.2.2 Allele loss studies in APC and colon cancer

Recognition of linkage of the APC gene to a locus on chromosome 5 has resulted in many subsequent studies demonstrating loss of alleles using various polymorphic DNA markers both on chromosome 5 and at other sites in the genome suggesting the involvement of several genes. The rationale behind this investigative strategy, as with retinoblastoma and Wilm's tumour, is that if Knudson's hypothesis holds true for APC and colorectal carcinoma, the initial mutational event be it inherited or sporadic, will be followed by loss of the corresponding allele. Demonstration of the involvement of chromosome 5 in such a hypothetical sequence of events was supported by the study of Solomon et al.(1987) in which two polymorphic DNA markers , λMS8 (D5S81, 5q35-qter) and L1.4 (D5S4,5pter-p15) were used to demonstrate loss of one allele in a range of cancer and normal DNA pairs. 7/44 sporadic colon cancer DNA samples in which the normal DNA was informative (i.e. heterozygous) for one or both of the markers showed clear loss of one allele whilst another 3 showed a marked reduction in intensity of one of the alleles, the residual faint band presumably due either to contamination of the tumour with normal stromal DNA or to the presence of more than one cellular clone within the sample. Investigation of DNA from 11 APC adenomas resulted in no

demonstrable loss of alleles. Subsequent studies have confirmed the high frequency of loss using chromosome 5 markers in cancers (Okamoto et al., 1988; Law et al., 1988; Vogelstein et al., 1988; Wildrick et al., 1988; Rees et al., 1989; Dunlop et al., 1989) and more limited loss has been demonstrated in both sporadic and familial adenomas (Vogelstein et al., 1988; Rees et al., 1989).

Okamoto et al (1988) used 3 chromosome 5 markers , C11p11, D5S2 (5pter-q35) and the *fms* oncogene (5q34-35) to demonstrate allele loss in 3/5 APC carcinomas but not in the corresponding adenomas from these patients. Similarly, out of 8 informative sporadic cancers , 3 showed evidence for complete loss or reduction in intensity of one allele. Other chromosome regions were also looked at and losses of between 21 and 29% were demonstrated with markers on chromosomes 6, 12, 15 and 22. The most marked changes were seen with the chromosome 22 markers where 5/14 (36 %) informative APC carcinomas and 4/19 (21 %) informative sporadic cancers exhibited allele loss when compared with the corresponding normal DNA samples. Such reduction to homozygosity was only seen in one adenoma in which loss of alleles was seen at two loci, 6q15-q24 and also at 12q14-qter. The high frequency of loss seen at chromosome 22 loci may represent a similar situation to that seen in type II multiple endocrine neoplasia in which allele loss is seen on chromosome 1 even though the gene is known to reside on chromosome 10 (Okamoto et al., 1988). Further evidence for the involvement of chromosome 5 is shown by loss of alleles at the glucocorticoid receptor gene locus (5q11-13) (Wildrick and Boman, 1988) in the tumour DNA of 4/11 informative cancers. A more recent study (Okamoto et al., 1990) examined more closely the precise mechanism of chromosome 5 loss in colorectal tumours and desmoids. Loss was detected in 47% of APC carcinomas and 53% sporadic carcinomas. 1/5 desmoids and 4/19 adenomas from Gardners syndrome patients and 1/17 APC adenomas also exhibited differential loss of various chromosome 5 markers. The results point to interstitial deletion or mitotic recombination as the main mechanisms of loss and the loss seen in the desmoid tumour suggests involvement of the same region in the development of extracolonic manifestations.

One of the main problems encountered in these studies is the possibility of contamination of the tumour samples with normal stromal tissue. This was overcome by the microdissection of homogeneous "islands of malignancy" (Law et al., 1988). This resulted in most cases in preparations which were 70 - 90 % homogeneously malignant. It has been suggested that allele loss determination is made difficult if the proportion of non-neoplastic cells in the tumour exceeds 30% (Vogelstein et al., 1988). In this study, loci on chromosomes 1, 12, 13, 17, 18 and 22 were examined for evidence of loss. No loss was seen in 42 adenomas derived from APC and Lynch syndrome patients except one (Lynch) on chromosome 5. Of the carcinomas looked at, 56 % of 34 informative demonstrated loss on chromosome 17 and 52 % on chromosome 18. Other chromosomal markers used showed infrequent loss and in contrast to the study by Okamoto et al. (1988), no chromosome 22 changes were seen. In the 6 cases where chromosome 5 loss was seen, 17 and 18 were also involved suggesting that these may represent later changes in colorectal carcinogenesis (Law et al., 1988) and that the mutation of the APC gene leads to polyposis but other events are necessary for complete malignant conversion.

The involvement of chromosomes 17 and 18 at the molecular level has been confirmed (Monpezat et al., 1988) by the same group who first implicated them on the basis of karyotypic examination (Muleris et al., 1985; 1986). Densitometric measurements were used to establish allelic imbalance in 75% (9/12) of carcinomas informative for the chromosome 17 locus D17S1 and in 85% (12/14) informative for D18S1 . To overcome the problem of normal tissue infiltration of tumour samples they used flow-sorted polyploid nuclei from 5 carcinomas and 3/4 of these showed complete loss of one allele on chromosome 17 and 4/5 on chromosome 18. (Based on the data obtained following cell sorting, it appears that the under-represented allele in the cases showing incomplete loss, merely represents normal contamination of the tumours .) No conclusive losses were seen in these 5 tumours using markers on chromosomes 11, 13 or 22 although small allelic imbalances were seen in some cases. Rare loss of heterozygosity has been reported for the H-ras locus on chromosome 11 and also for

the myb locus on chromosome 6 (Alexander et al., 1986; Yokota et al.,1987; Yuasa et al., 1986b).

By far the most comprehensive series of investigations into molecular events controlling tumour initiation and progression have been those carried out by Vogelstein et al. (1988; 1989). The first of these studies examined allelic changes on chromosomes 5, 17 and 18 together with K-ras activation (which will be reviewed in greater detail later) with the specific purpose of establishing a chronological sequence of events involved in colorectal neoplasia. For this purpose the adenomas used in the study were divided into three categories:

APC	Class I	40 adenomas (7 patients): small with low-grade dysplasia
Sporadic	Class II	19 adenomas - no evidence of malignancy
	Class III	21 adenomas with areas of invasive carcinoma

In the class III adenomas, cryostat sectioning was carried out to separate contiguous areas of benign and malignant growth. The three classes, therefore, represented different stages of neoplasia with the size and villous component generally increasing from I to III. Table 1.3. summarises the results found in this study.

In the case of deletions of chromosome 5, the critical region (5q21-23) was defined by analysing the region of overlap between the various tumours which exhibited loss. For this they used 5 of the 30 tumours with 5q allelic deletions which had mitotic recombinations or deletions which differentially affected the markers used. Similarly those tumours showing loss of chromosome 18 were used to establish a common region of overlap at 18q21.3-qter and this observation has led directly to the isolation and characterisation of a candidate tumour suppressor gene with homology to a neural cell adhesion molecule (Fearon et al., 1990) possibly with a role in cell-cell interaction. Similar frequencies of 5q allelic deletions and ras mutations in adenomas and carcinomas point to these changes occurring early in the tumorigenic pathway whereas the 17p deletions are much more frequently seen in carcinomas and are

therefore likely to occur later. Deletions of 18q were uncommon in early adenomas although occurred in about half of adenomas of class II or III. The hypothetical sequence of events suggested by Fearon and Vogelstein (1990) and by Stanbridge (1990) is as shown in figure 1.7.

Table 1.3. Frequency of ras mutations and allele loss in adenomas and carcinomas (Vogelstein et al., 1988)

	% Allele loss (no. analysed)			% *ras* mutations (no.analysed)	
	Chromosome			K-*ras*	N-*ras*
	5q	17p	18q		
ADENOMAS					
Class I	0(34)	6(32)	13(23)	12(40)	0(40)
Class II	29(17)	6(18)	11(18)	37(19)	5(19)
Class III	29(14)	24(17)	47(17)	57(21)	0(21)
CARCINOMAS	36(58)	75(60)	75(56)	41(92)	5(92)

It seems therefore that colorectal carcinogenesis is not a simple two-step process but in fact is multi-factorial and several steps are involved in the development of a cancer (Okamoto et al., 1988).

In order to establish the variation of allele loss seen in colorectal carcinomas, Vogelstein et al. (1989) examined 56 paired normal and tumour samples using polymorphic markers from every non-acrocentric autosomal arm. They coined the name "alleotype"for this type of analysis and again adopted the cryostat sectioning method to reduce the risk of normal stromal contamination. The only chromosome arms not covered by this study were the acrocentric ones (13p, 14p, 15p, 21p and 22p) as only the ribosomal genes are thought to reside in these regions. Again allele loss of markers from 17p and 18q were seen in 75% of cases with losses on 1p, 4p, 5q, 6p, 6q, 8p, 9q, 18p and 22q varying between 25 and 50 %. Losses at other regions were much more infrequent. It appears that most of the losses represent subchromosomal events

rather than whole chromosome loss as in 65% of cases in which allelic deletion was seen, it occurred only on one of the chromosomal arms and therefore points to interstitial deletions being mainly responsible for the phenomenon, an observation backed up by the work of Dunlop et al. (1989). Statistical analysis of allele loss has been carried out (Vogelstein et al., 1989) by measuring the fractional allelic loss which is determined as shown below:

$$\text{Fractional allelic loss} = \frac{\text{no. of chromosome arms on which loss observed}}{\text{total no. of chromosome arms informative in patient}}$$

The median FAL in the 56 tumours looked at was 0.2 i.e. 20% of the chromosome arms exhibited loss. On this basis the patients were divided into two groups

Group I FAL < 0.2

Group II FAL > 0.2

Both these groups were followed for ~ 38 months and were matched for age, sex and extent of invasiveness. The frequency of ras mutations was identical in the two groups. Statistical analysis showed that group II patients were more likely to suffer recurrent disease (as well as distant metastases) and were more likely to die with, or from, the cancer. They suggest that such alleotypic studies may identify patients who are more likely to respond to treatment. Thus allele loss determination may not only be scientifically interesting but may have a real clinical application.

It must be stressed that even though there may be a large number of tumour suppressor genes present in the human genome the deleted loci observed in the studies discussed may not all necessarily highlight the presence of such genes. Some may merely be the result of aberrant mitoses, perhaps due to the level of general chromosome instability observed in colorectal cancers (Reichmann et al., 1980). However, because such deletions are irreversible they persist as the cell proliferates and eventually become the predominant clone.

Various other studies have been done (Fey et al., 1989; Sasaki et al., 1989) and estimate losses on 5q at 29% and 24% respectively using the probe λMS8 although these figures are probably underestimates as the probe used is some distance from the disease locus and as suggested previously the main source of allelic deletions are small

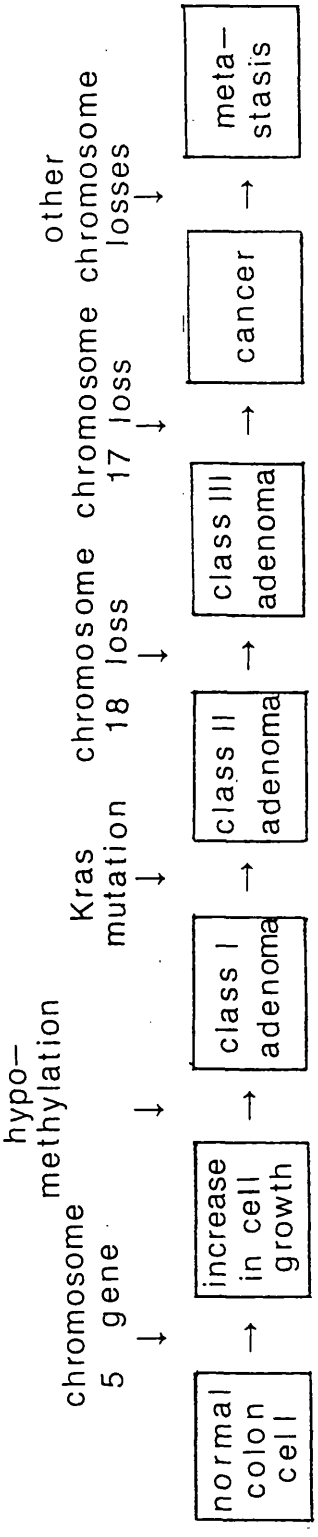


Figure 1.7. Proposed sequence of events involved in colorectal tumour progression (Fearon and Vogelstein, 1990; Stanbridge, 1990).

interstitial deletions (Vogelstein et al., 1989). Figures for losses on chromosome 17 seem to vary enormously ranging from 69% (Fey et al., 1989) to 31% in APC-derived carcinomas (Sasaki et al., 1989). The figure produced by Fey et al. (1989) correlates well with that previously quoted (Vogelstein et al., 1988; Law et al., 1988) and this difference in results may reflect different racial origins of colorectal carcinoma as the results obtained by Sasaki et al. (1989) agree with those of Okamoto et al. (1988) especially with respect to the high frequency of chromosome 22 deletions observed in both Japanese studies - 35% and 36% respectively. Although chromosome 18 losses were seen in 40% of cases looked at by Sasaki et al. (1989) but none were seen in the previous Japanese study but this may merely reflect differences in the choice of markers. Sasaki et al. (1989) also looked at a number of non-polyposis colon cancers and in this study the highest percentage of losses seen were those on chromosome 5 (32%) with lower frequencies on chromosomes 14, 17 and 8 so again this points to a slightly different tumorigenic mechanism in these patients. Sasaki et al. (1989) also suggest that their figures may be an underestimate as they did not carry out cryostat sectioning of their tumour samples and also in the case of chromosome 5, the probe used was the *APC*-linked C11p11 (D5S71) which is homozygous in a large proportion of the population (Aldred et al., 1988) whereas in more recent studies (Ashton-Rickardt et al., 1989) more highly polymorphic probes have been used which result in a much higher frequency of loss being demonstrated.

One observation supports the hypothesis (Vogelstein et al., 1988) that loss of heterozygosity on chromosome 17 is a secondary event. In one of the Japanese patients (Sasaki et al., 1989), one carcinoma and one adenoma exhibited allele loss on chromosome 17 but a different homologous chromosome was lost in each case suggesting that mutation of one allele and deletion of the other occurred independently in two distinct tumours. The authors argue that if the tumour-suppressor gene at this locus acts in a dose-dependent manner loss of either allele will have the same effect if one normal allele is retained. However, recent work on the putative tumour-suppressor gene p53 (17p13) points to the normal allele being lost and the retained allele being mutated. (discussed in detail later).

Several pieces of evidence have suggested the possibility that distal and proximal colorectal tumours may have different aetiologies and hence differ in their respective tumorigenic mechanisms. Delattre et al. (1989) classified their respective tumours into proximal (caecum to splenic flexure); distal (splenic flexure to end of sigmoid) and rectal in order to establish whether there was any difference between them. They suggest three main types of genetic alterations occur in colorectal cancer

(i) change in DNA content

(ii) specific allelic deletions involving chromosomes 5, 17 and 18

(iii) ras oncogene activation

Comparison of allelic deletions on 17p resulted in a much smaller frequency of loss in the proximal cancers (30%) compared with the distal or rectal ones (74%). Similarly, a lower frequency of loss of 18q sequences is seen in the proximal tumours (30%) than in either the distal (85%) or rectal (64%) tumours. Observed allele loss on 5q was also less frequent in the proximal tumours - 11% - compared with around 45% and 41% for the others. Hyperdiploidy was observed in 20% of proximal tumours and 66 and 56 of distal and rectal tumours respectively. No statistically significant difference in K-ras activation was seen in the different tumour sub-groups. The authors therefore suggest that there may be an association between chromosome losses on 17p and 18q and the generation of hyperdiploidy. The differing frequencies of observed genetic changes between the proximal and distal / rectal tumours may therefore define two independent pathways of colorectal tumorigenesis. A recent study has demonstrated suppression of tumour formation by the introduction of the 5q31-ter region and the region surrounding the *APC* gene into a colon cancer line resulting in drastically reduced colony formation in soft agar (Yanagowa and Sasazuki, 1990). Similar results were obtained in an independent study by the separate introduction of both a normal chromosome 5 and a normal 18 (Tanaka et al., 1991). In the latter study, a distinctive change in cellular morphology was seen together with suppression of tumorigenicity supporting the presence of tumour suppressor gene sequences on both these chromosomes.

1.6.3 Possible role of the *APC* gene

The likelihood that the normal role of the *APC* gene is one of prevention of epithelial hyperproliferation is supported by the frequent observation of skin tags in patients with colorectal adenomas (Kune et al., 1985) and also by the higher than average occurrence of mandibular osteomas both in APC patients and in patients with non-familial colorectal cancer (Sondergaard et al., 1985) and suggests that the defective gene may be responsible for the pleiotropic effects on skin and colonic epithelium (Dunlop, 1990). The implication of chromosome 5q loss in sporadic colorectal cancers (Solomon et al., 1987) as well as in the aetiology of APC (Bodmer et al., 1987) is suggestive of the possibility that the *APC* gene may be involved in colorectal carcinogenesis in a broader sense perhaps due to the presence of different mutant alleles which in some familial cases are responsible for the extracolonic manifestations of the disease.

It has been proposed that inherited heterozygosity (i.e. in which one allele is mutated) for a gene such as the *APC* gene could give rise to proliferative abnormalities (such as the localised effects seen in the polyps) via a threshold effect involving negative control of growth factor production (Bodmer et al., 1987). In its normal role, the *APC* gene product maintains a certain level in the cell otherwise excessive localised growth ensues although this normal level is subject to random fluctuations. The deficient heterozygote may be unable to prevent these random fluctuations from frequently falling below a particular threshold hence giving rise to localised growths i.e. adenomas. A similar theory , although one which does not involve such random fluctuations is that the *APC* gene acts as a negative regulator of the widespread epithelial hyperproliferation which precedes adenoma formation in APC patients such that inactivation of one allele results in decreased expression of the protein product even if the other allele is intact (Vogelstein et al., 1988). Further inactivation of the remaining allele would magnify the proliferative effect but is not necessary for it. Both theories essentially agree in the role of the protein product but differ in their interpretation of the fine control of cellular proliferation. Therefore there seems no doubt that the

fundamental role of the gene product is in the prevention of localised epithelial hyperproliferation but for now, at least, its exact nature and mode of action eludes us.

One possible explanation for the observed differences in allele loss between sporadic and hereditary adenomas is the effect of the cellular microenvironment and the observation that in sporadic patients, an adenoma precursor cell heterozygous for a mutation at the *APC* locus may be prevented from clonal expansion by the suppressive effect of the cells surrounding it. The effect of this would make extra changes (e.g. the action of a tumour promotor or another genetic event) necessary to allow adenoma formation. This would not be true of an adenoma arising in an APC patient which would be free of any such suppressive effects since the patient is constitutively heterozygous at this locus (Paraskeva and Williams, 1990; Paraskeva et al., 1990).

Recent linkage data from a large Utah kindred containing two APC sufferers and a high degree of predisposition to development of adenomas and colon cancer but in which the number of adenomas was low (<40) suggests that the *APC* gene may also be responsible for a range of more subtle colorectal cancer-susceptibility syndromes (Leppert et al., 1990).

Recently a candidate *APC* gene mapping to 5q21 has been isolated (Kinzler et al., 1991) and has been designated MCC (mutated in colorectal cancer). Isolation of this clone resulted from using a cosmid (5.71) to screen a panel of 150 colorectal cancers one of which contained a novel 11kb EcoR1 fragment in addition to the normal 20kb fragment seen in the corresponding normal DNA. The fragment was isolated and found to represent a novel rearrangement in this region bringing together sequences that were usually at least 100kb apart. Using two subclones of 5.71 and a combination of cross-species hybridisation and an exon-connection pcr strategy, two putative exons were identified and used to isolate cDNA clones covering 4181 bp of MCC cDNA with an ORF of 2511bp. Searching of a sequence database revealed a 19aa region of similarity between MCC and the G-protein coupled M3 muscarinic acetylcholine receptor of humans and pigs but it is not yet known whether this is significant but it is possible that the role of the *APC* gene may be similar to another G-protein coupled tumour suppressor NF-1 which has been shown to interact with $p21^{ras}$ (Martin et al., 1990).

Indeed NF-1 has a similar aetiology to APC with the initial formation of benign tumours (neurofibromas) and an increased risk of malignant progression to neurofibrosarcomas later, presumably due to subsequent genetic events.

1.6.4 Hypomethylation studies

One mechanism which might allow differences in gene expression between the adenomatous epithelium of APC patients is DNA methylation. One comprehensive study of changes in methylation patterns between benign and malignant colorectal neoplasms has been carried out (Goelz et al., 1985) in which the methylation patterns of 10 genes on 6 different chromosomes were examined They chose genes which were unlikely to be expressed in normal colon (and therefore likely to be highly methylated) and compared patterns in normal and tumour DNA pairs. The samples were analysed using the restriction enzymes Hpa II and Hha I which are methylation sensitive. The MspI isoschizomer was used as a control for Hpa II digestion as it has the same recognition site (C^CGG) but will cut whatever the methylation state of the cytosine residues whereas Hpa II requires the second cytosine to be methylated. Of the ten genes looked at, 8 exhibited decreased methylation in the neoplastic tissue DNA especially, γ-crystallin, growth hormone, α-chorionic gonadotrophin and γ globin. Four were hypomethylated to a lesser degree : insulin, melanocortin, β-chorionic gonadotrophin and platelet-derived growth factor. Two showed no change in methylation patterns : α-fetoprotein and parathyroid hormone. The four genes which were frequently hypomethylated were used further to probe 23 neoplastic DNA samples. The crystallin gene was shown to be substantially undermethylated in all polyps and cancers although the extent of hypomethylation was subject to considerable variation but there was no difference in the degree of methylation between adenomas and cancers which suggests that this change precedes malignancy. As a control, a satellite probe was used to look for evidence of changes in the methylation state of repetitive sequences but none was found so it appears that the changes seen do not occur at random. Methylation studies were carried out in two Gardner's syndrome patients and revealed changes in methylation patterns even in very small adenomas when compared to the normal colonic

epithelium even though the normal tissue exhibited substantial hyperplasia. The authors argue that this suggests that the methylation changes occur specifically between the stages of hyperplasia and benign neoplasia and could represent a key event in the initiation of ectopic expression of genes important in neoplastic growth which gives these cells a selective growth advantage. More recently, another study discovered a reduction in 5-methyl-cytosine content in neoplastic colon cells when compared with the normal colon counterpart (Feinberg et al., 1988a).

1.7 The role of oncogenes in colorectal carcinogenesis

Up to now, the only cellular mechanism discussed with respect to carcinogenesis has been that of the inactivation of putative tumour suppressor sequences resulting in the loss of an essential regulatory protein product. This mechanism is exemplified by the retinoblastoma gene - RB1 (Lee et al., 1987). However it is essential to realise that this is not the only type of gene associated with the tumorigenic process. The other well-documented class, the proto-oncogenes, are activated in a dominant rather than a recessive manner and their altered protein products play a direct role in the malignant process (Cooper and Lane, 1984). A study of 26 cellular oncogenes has shown that 19 of these map to one of the 83 bands in the genome known to be involved in cancer-specific rearrangements (Heim and Mitelman, 1987). The cellular tumour antigen, p53 has also been implicated in the process of transformation in a variety of tumours including those of the colon and breast (Crawford et al., 1984) and will be discussed in detail in a later section.

Vogelstein's study of allele loss and K-*ras* activation in colorectal tumours (Vogelstein et al., 1988) previously reviewed suggested a degree of interplay between the two tumorigenic mechanisms but the exact chronological order of events remains to be established although one hypothetical sequence has been suggested (Vogelstein, 1990; Stanbridge, 1990) (figure 1.7). Investigations of K-*ras* activation have revealed mutations in at least 40% of colorectal cancers (Bos et al., 1987; Forrester et al., 1987) and these are reviewed in detail in the next section. However, other studies have

revealed the involvement of a wide range of oncogenes in colorectal carcinogenesis. Amplification of the c-*myb* oncogene has been shown in two cell lines derived from a colon carcinoma (Alitalo et al., 1984) although in general amplification of cellular proto-oncogenes is not thought to be a common mechanism of increased expression in colorectal cancers (Masuda et al., 1987). Levels of c-*fos*, c-*myc*, H-*ras* and K-*ras* mRNAs were all found to be elevated in two colon adenocarcinomas (Slamon et al., 1984) but recent work on c-*fos* suggests no difference in the respective levels of its gene product in normal and tumour DNAs (Elvin et al., 1986). Premalignant adenomatous tissue from an APC patient has also been shown to have transforming growth factor activity which stimulates anchorage-independent growth (a common property of many malignant cells) in rat fibroblasts and may be involved in early adenoma-carcinoma changes (Wigley et al., 1986). Increase in pp60 c-*src* protein kinase activity was demonstrated in 21/21 human colon carcinoma lines and 15/15 colon carcinomas (Bolen et al., 1987). D'Emilia et al. (1989) also suggest a role for the EGF-like p185 product of the c-*erb*B-2 oncogene in the early stages of colonic neoplasia as evidenced by increased expression in a minority of adenomas and carcinomas although it is not thought to be an initiating event. Amplification of c-*myc* in colon carcinoma cell lines has been reported (Lin et al., 1985) as has overexpression of c-*myc* mRNA (Erisman et al., 1985; Rothberg et al., 1985; Stewart et al., 1986) and increased expression in colonic polyps may implicate it in the early evolution of colonic tumours (Stewart et al., 1986). The involvement of c-*myc* has also been proposed on the basis of a study by Rothberg et al. (1985) to define two distinct sub-sets of colorectal carcinoma in that its overexpression seems to relate to those cancers arising in APC patients which are usually found in the distal colon whereas low c-*myc* expression is common in hereditary colon cancers of the non-polyposis type which are more frequently situated in the proximal colon (Rothberg et al., 1985). It is postulated that this theory can be extended to the sporadic counterparts of these hereditary cancers (Rothberg et al., 1985). This hypothesis is further supported by a study which correlates deregulation of c-*myc* expression with allele loss on chromosome 5q (Erisman et al., 1989) suggesting that the *APC* gene product may, therefore, function

as a trans-acting regulator of c-*myc* expression and tumours which exhibit allele loss at the *APC* locus represent a distinct genetic sub-group although this theory has been called into question by a more recent study (Maestro et al.,1991).

The cooperation between *ras* and *myc* gene products has been demonstrated in which it appears that the *myc* product is responsible for cellular immortalisation whilst the *ras* products elicit the transformation process (Land et al., 1983a and b). Again this may have implications for different genetic sub-groups of colorectal tumours but whatever individual changes are present it seems certain that some degree of interplay between several oncogenes and/or tumour suppressor genes is essential for complete malignant transformation.

1.7.1 The *ras* Genes and Human Malignancy

The *ras* genes represent a family of three closely related cellular proto-oncogenes which appear to play significant roles in a number of human malignancies (Popescu et al., 1985).. The three genes are N-*ras* on chromosome 1, H-*ras* on chromosome 11 and K-*ras* on chromosome 12. All three genes encode related 21 kD proteins which are located on the inner surface of the plasma membrane where they bind and hydrolyse GTP by interaction with the GTPase activity protein or GAP (Marshall, 1988). and are thought to represent part of a much larger "superfamily" of proteins present in a wide diversity of organisms (Chardin, 1988) For example, yeast and mammalian *ras* gene products show significant sequence homology and functional similarity (Temeles et al., 1985) and a normal mammalian *ras* gene has been shown to complement yeasts mutant in their intrinsic *ras* genes to enable them to retain viability (DeFeo-Jones et al., 1985). Their structural similarity with the G-proteins suggests that the normal role of the p21 product is in the signal transduction pathway (Trahey and McCormick, 1987) allowing message relay of external stimuli possibly via growth factors or the like (Bos, 1989) so that stimulation leads to activation, transduction of the signal to an effector molecule and subsequent inactivation (Bos, 1989). The oncogenic action of the *ras* gene products is elicited via one of two mechanisms :

(i) by amplification or increased expression of the normal gene product (Gallick et al., 1984) although this is an infrequent cause of malignancy (Marshall, 1988)

(ii) by point mutational activation usually at one of three "hotspot" codons - codons 12 or 13 in exon 1 or codons 59/61 in exon 2 resulting in amino acid substitution (see later references).

It is thought that the second of these mechanisms results in a decrease in GTPase activity by disruption of GTP binding (Marshall, 1988). Such disruption is elicited by the mutated proteins being locked into the "on" or GTP-bound state leading to a constant stimulation of growth or differentiation (Milburn et al., 1990). An early study of the mutated H-*ras* protein present in the T24 bladder carcinoma cell line indicated that the mutant protein had significantly reduced GTPase activity and that comparative microinjection of both normal and mutant proteins into quiescent rodent fibroblasts resulted in a much greater alteration in cell morphology and stimulation of DNA synthesis and cell division in the case of the mutant protein (Sweet et al., 1984).

Although the precise nature of the *ras* signal transduction pathway has not yet been fully resolved, evidence suggests that unlike yeast, adenylate cyclase is not the effector molecule (Beckner et al., 1985). GAP represents one possible effector molecule in the signal transduction cascade , although it is probably not the only one (Bourne et al., 1990). Exactly what stimulus elicits *ras* activation in mammalian cells is unclear although in yeast response to nutrient conditions and environmental stress clearly plays a part (Broach, 1991).

The active form of $p21^{ras}$ appears to be the GTP-bound state and it is thought that mutations in loop 1 of the protein (amino acids 1-15) which is the GDP-binding domain result in a change in the highly constrained conformation thereby eliciting an inhibitory effect on intrinsic GTP-ase activity (Marshall, 1988). By virtue of the fact that amino acid 61 is in contact with loop 1 mutations at this codon will have a similar effect. Three dimensional modelling of the p21 protein has confirmed that the glycine normally present at codon 12 cannot be substituted by any other amino acid without significantly altering the nucleotide binding capacity of the protein (Wierenga and Hol, 1983). Introduction of oncogenic $p21^{ras}$ into quiescent fibroblast lines stimulated DNA

synthesis and morphological transformation but the exact mechanism by which this occurs is unclear. It is known that introduction of the mutant protein into quiescent 3T3 cells activates protein kinase C and that this is essential for p21 induced DNA synthesis by in turn activating other cellular oncogenes such as *fos* and *myc* . Morphological transformation maybe achieved by activation of *raf* and *mos* via another intermediate (Marshall, 1991).

Activated *ras* genes have been detected in several types of human malignancy suggesting that it represents a general tumorigenic event present in 10 - 15 % of human cancers (Barbacid, 1987). However this theory is put in doubt by the observation that K-*ras* activation is detectable in at least 90% of pancreatic carcinomas (Almoguera et al., 1988) but *ras* mutations are very rare in carcinomas of the breast (Kraus et al., 1984) suggesting that they are specific to certain types of cancers and this is supported by the fact that different *ras* mutations are characteristic of different cancers. Haemopoetic malignancies, for example, tend to be associated with mutations of the N-*ras* gene (codons 12 and 13) and these mutations are particularly involved in acute lymphocytic leukaemia (18%) when compared with non-Hodgkin lymphoma or chronic lymphocytic leukaemia (Neri et al., 1988) whilst presence of both N- and K-*ras* mutations has been demonstrated in childhood acute myeloid leukaemia (Vogelstein et al., 1990) and in male germ cell tumours (Samaniego et al., 1990) . In contrast, no N-*ras* mutations were found in a large study of colorectal tumours in which K-*ras* mutations were common (Farr et al., 1988b) but activation of N-*ras* has been shown in acute myeloid leukaemia (Bos et al., 1985, 1987; Farr et al., 1988a).

Point mutations in the H-*ras* gene (codon 12) have been reported in a gastrocarcinoma cell line (Deng et al., 1987) and further analysis has revealed similar mutations in 2/3 cell lines and 5/18 solid tumours (Deng, 1988). It is also responsible for the transforming features of the T24 human bladder carcinoma cell line (Reddy et al., 1982; Parada et al., 1982).

Mutations in the K-*ras* gene have been reported in various lung and colon carcinoma cell lines leading to structural alterations in their protein products (Capon et al., 1983; Der and Cooper, 1983) whereas increased p21 expression has been reported

in lung and colon cancers (Gallick et al., 1985), a gastric carcinoma (Bos et al., 1986) and in premalignant and malignant colorectal tumours (Spandidos and Kerr, 1984). Such studies have revealed a high prevalence of K-*ras* activation in colorectal tumours and suggest that this is of most significance with respect to this type of cancer. The reason for such bias in the occurrence of different *ras* mutations in different cancer types is unknown since there is no known functional difference in the three proteins but it could be the result of a physiological difference or of differential expression in the tissues affected by the tumour (Marshall, 1988).

1.7.2 K-*ras* Studies in Colorectal Neoplasia

The human K-*ras2* proto-oncogene is located on chromosome 12 (Sakaguchi et al., 1983) and activation of the gene is thought to be an early event in colorectal tumorigenesis (Kerr, 1989) based on studies of adenomas and carcinomas in APC patients (Farr et al., 1988b). Point mutation at codon 12 in the first exon is by far the most common mutational event (Bos et al., 1987; Forrester et al., 1987; Farr et al., 1989; Burmer and Loeb, 1989; Burmer et al., 1990; Sasaki et al., 1990; Oliva et al., 1990). K-*ras* mutation has previously been shown to be associated with early events in leukaemia (Liu et al., 1987). Increased expression of the gene product has also been linked to increased dysplasia in colonic epithelium as evidenced by immunohistochemical staining (Bradfield et al., 1986). The colon carcinoma cell line KMS-4 derived from an APC patient has a mutated K-*ras* gene with a single base change at codon 12 which results in the substitution of a cysteine for the normal glycine residue (Yuasa et al., 1986a).

Many of the early studies of K-*ras* activation in tumours were transfection studies involving calcium phosphate precipitation of the sample DNA onto NIH3T3 mouse fibroblasts. This technique is extremely time-consuming and has raised questions about the integrity of the transfected DNA (Forrester et al., 1987; Bos et al., 1987). Recent studies have therefore concentrated on molecular genetic techniques, specifically RNAse mismatch cleavage (Forrester et al., 1987) and oligomer hybridisation analysis (Bos et al., 1987) to reveal a high level of K-*ras* activation in colorectal tumours.

Forrester et al. (1987) used a technique based on the ability of RNAse A to recognise and cleave mismatches in RNA:RNA duplexes. The RNA extracted from the sample is hybridised to labelled antisense RNA probes for the K-*ras* gene synthesised by *in vitro* transcription from a SP6 promoter. Digestion by RNAse A will occur if there are single base mismatches present in the duplex molecule and the digestion products can be analysed by gel electrophoresis. The size of the bands observed indicates the position of the mutation. Of a panel of 66 primary colorectal tumours, 26 (39%) were positive in the mismatch assay and all the detectable mutations were at codon 12 in the first coding exon of the K-*ras* gene. In contrast, only 20% proved positive in the NIH3T3 transfection assays. Of the samples which contained codon 12 mutations, 8/26 were at the first base and 18 at the second. There was found to be no significant correlation between the mutations and the cancer staging but activation was detected in 7/8 adenomas and the other proved positive in the transfection assay suggesting the presence of a mutation at another position. One villous adenoma exhibited simultaneous activation of both K- and N-*ras* genes but this is generally thought to be a rare occurrence. It is thought that the *ras* gene products confer a selective growth advantage on the cells and that the observation of activation in adenomas point to these mutations being early events in the tumorigenic mechanism (Forrester et al., 1987).

Bos et al. (1987) questioned the sensitivity of the transformation technique with respect to the detection of mutations due to the large size of the K-*ras* locus and also to the fact that extracting DNA from primary tumours often results in slight degradation so that genes of > 45 000 base pairs such as the K-*ras* gene (Capon et al., 1983; Shimizu et al., 1983) rarely remain intact. They overcame this problem by using the polymerase chain reaction to specifically amplify selected regions of the three *ras* genes which included the mutation hotspots previously described. The amplified DNA was dotted onto nylon membranes and hybridised to individual allele-specific oligonucleotide probes representing the various point mutations possible. In order to reduce possible contamination of the tumours with normal DNA all the tumours were examined histologically. Using this method 35% of tumours looked at contained K-*ras* mutations the majority of which were at the second base of codon 12 resulting in the substitution

of aspartic acid and valine for glycine. Five tumours were shown histologically to contain residual regions of adenomatous growth and four of these were mutated in the adenomatous regions also. There are two possible explanations for this observation:

(i) ras mutation may be a frequent event in adenomas only a small number of which progress to malignancy

(ii) ras mutations may occur infrequently but bestow a high probability of progression to malignancy

The second of these possibilities is supported by the study of Farr et al. (1988b) which examined 75 APC adenomas from 29 patients. Using a similar technique to that previously described (Bos et al., 1987) they looked for the presence of mutations in all three *ras* genes. Only 7% proved positive - all mutations in codon 12 of the K-*ras* gene. Three of these adenomas were from the same patient whereas a further one from the same patient was negative. The mutated samples represented substitutions of aspartic acid, valine and cysteine for the normal glycine. Two cell lines, one adenomatous (PC/AA) , and one carcinomatous (PC/JW) were also positive for K-*ras* mutations as was a sporadic villous adenoma-derived line whereas a tubular adenoma-derived line proved negative. Another similar study also revealed codon 12 and 13 mutations in 36% of APC carcinomas compared with 12% of adenomas. No codon 61 mutations were detected (Sasaki et al., 1990). These results all point to *ras* activation being an early event (if not the initiating one) in colonic tumorigenesis and this is further supported by animal studies (Balmain, 1985; Leon et al., 1988). *Ras* activation may result, therefore, in an increase in epithelial atypia and a gradual transition from the benign to the malignant state (Muto et al., 1985). Farr et al. (1988b) believe that these mutations occur infrequently in adenomas and although not the initiating event , such adenomas have a high probability of malignant progression. This view is substantiated by the levels of K-*ras* activation found in the study by Vogelstein et al. (1988). Only 12%of class I APC-derived adenomas were mutated compared with 42 and 57% of class II and III adenomas respectively and in 47% of carcinomas which is in general agreement with figures previously described (Bos et al., 1987; Forrester et al., 1987). In order to increase the sensitivity of the detection technique, Burmer and Loeb (1989)

used a combination of histological enrichment, cell-sorting, the polymerase chain reaction and direct sequencing of K-*ras* exon 1 region in paraffin-embedded tissue sections and found mutations at the first position of codon 12 in 65% of adenomas and 75% carcinomas respectively. The detection of such changes in both diploid and aneuploid cells point to their preceding changes in cell ploidy and the occasional finding of mutations in regions of normal tissue adjacent to malignant material is once more indicative of its being a relatively early event in the carcinogenic process. The authors suggest that direct sequencing of the amplified region gives a much more accurate and unambiguous interpretation of K-*ras* mutations compared with allele-specific oligonucleotide hybridisation and furthermore their results were confirmed by restriction endonuclease digest.

Other methods used to investigate these mutations include that of single-strand confirmation polymorphism (SSCP) analysis (Orita et al., 1989) used to detect a codon 12 mutation in the SW480 colon cancer cell line which was then confirmed by direct sequencing. Another method especially applicable to tumours in which only a small clone of cells possesses a mutation was demonstrated by Haliassos et al. (1989) using the PCR to introduce an artificial restriction site into the amplified product which would not cut if a mutation was present. In this way the authors estimate 20% more mutations can be detected.

One recent study of K-*ras* mutations in liver tumours resulted in their detection in 5/9 cholangiocarcinomas (a relatively rare tumour derived from epithelial cells) whereas no such mutations were found in hepatocellular carcinomas (derived from hepatocytes) suggesting that K-*ras* activation is particularly characteristic of tumours of epithelial origin (Sasaki et al., 1990).

Investigation of other types of colorectal dysplasia such as ulcerative colitis and cancers arising from it have resulted in little evidence for K-*ras* activation (Meltzer et al., 1990; Burmer et al., 1990). In the latter study, only 1/28 cancers proved positive compared with 11/21 sporadic carcinomas suggesting a different aetiology in these tumours.

Whatever the frequency of K-*ras* mutations in colorectal neoplasms, it seem certain to be an early event in tumorigenesis,an observation supported by studies of mammary carcinomas in rats exposed to nitrosomethylurea at birth in which mutations could be detected after 2 weeks and at least 2 months prior to the onset of neoplasia (Kumar et al., 1990). The nature of the gene product may result in a selective growth advantage by functioning as a "go-between" passing on messages from external growth factors (Barbacid, 1987). Once mutated it appears to remain overactive and as a consequence may have a destabilising effect on the genome (Kerr, 1989) perhaps increasing the likelihood of further mutational events such as those involving chromosomes 17 and 18. In light of the evidence for homology between the recently isolated neurofibromatosis type 1 (NF-1) tumour suppressor gene (Viskochil et al., 1990; Cawthon et al., 1990) and the IRA-1 and IRA-2 negative regulators of yeast p21 which share functional similarity with mammalian GAP (Buchberg et al., 1990) the possibility remains that tumour suppressor proteins play a role in the regulation of $p21^{ras}$ which may have important implications for the carcinogenic mechanisms which lead to malignancy.

1.7.3 Role of p53 in Colorectal Tumorigenesis

As mentioned previously the cellular protein p53 has been implicated in the process of transformation in a variety of human tumours including colorectal and mammary tumours (Crawford et al., 1984) and leukaemias (Koeffler et al., 1985; Prokocimer et al., 1986; Ahuja et al., 1989) and in the transformation of normal embryonic cells (Eliyahu et al., 1984). The p53 gene has been localised to the short arm of chromosome 17 (Benchimol et al., 1985; Muller et al., 1986), more specifically to band p13 (Isobe et al., 1986; McBride et al., 1986) and a cDNA clone has been obtained (Harlow et al., 1985). It has been suggested that cooperation exists between p53 and the products of the *ras* genes with respect to cellular transformation (Parada et al., 1984) and that p53 elicits cellular immortalisation (Jenkins et al., 1984). This situation is similar to the relationship between the *ras* and *myc* gene products proposed by Land et al. (1983a and b). Cooperation between SV40 large T antigen and p53 has also been demonstrated

with respect to the maintenance of cellular transformation (Deppert et al., 1989). Overexpression of p53 and its possible relationship to K-*ras* mutations has been investigated but the authors conclude that these occur as independent events (Laurent-Puig et al., 1990)..

Recent observations (Vogelstein et al., 1988) indicate the involvement of the 17p13 region in around 75% of colorectal cancers and this deleted region has since been more precisely defined as 17p12-p13.3 (Baker et al., 1989). As indicated above, the p53 gene has been mapped to this region which points to its possible involvement in the carcinogenic process. To further investigate this possibility, Baker et al. (1989) used cDNA probes for the p53 gene to examine DNA from 50 colorectal cancers and 32 cell lines. No rearrangements of the region were obvious on Southern blot analysis of Eco RI and Bam HI digested genomic DNA. Furthermore, no large scale genetic alterations in the surrounding regions were detectable on pulse-field gels. Similarly northern analysis of tumour mRNAs gave no indication of an abnormal transcript size but in 4 tumours relatively little expression was apparent. Of these, two were studied further. At the DNA level both had lost a 17p13 allele but expressed normal levels of a normal size transcript. Sequencing of the remaining alleles in both cases revealed the presence of point mutations both of which led to amino acid substitutions - valine to alanine at codon 143 in one case and arginine to histidine at codon 175 in the other. Both mutations occurred in highly conserved regions of the p53 gene (Nigro et al., 1989) highlighting their functional significance and both were confirmed by polymerase chain reaction-mediated amplification and subsequent restriction endonuclease digestion. Similar mutations have been detected in breast cancer lines (Bartek et al., 1990) and germline transmission of a mutation has been demonstrated in 4 members of a family with Li-Fraumeni syndrome (Srivastava et al., 1990) although such mutations do not apparently account for familial breast cancer (Prosser et al., 1991). Further evidence in breast cancer suggests the involvement of two genes on 17p (Coles et al., 1990) so possibly there is interaction between mutated p53 and some other protein.

Involvement of the p53 protein and the *ras* gene products may echo the *in vitro* situation described by Eliyahu et al. (1984) and Parada et al. (1984) in which mutant

murine p53 cooperates with mutant *ras* to elicit the transformation of rodent embryo cells. The more virulent nature of mutant p53 is demonstrated by a cotransfection study of p53 and an activated *ras* gene which resulted in reduced numbers of foci when the wild-type p53 gene was used (Finlay et al., 1989). Transfection studies on two colorectal cancer lines indicate that wild type p53 protein suppresses cell growth but showed less stringent effects when expressed in an adenoma line (Baker et al., 1990a).

A possible role of the normal p53 protein is that it interacts with other cellular macromolecules to suppress neoplastic growth. Decreased expression or mutation of the gene would therefore prevent this interaction (Baker et al., 1989). The authors also suggest that the mutant p53 will compete with the normal product in the heterozygote but a more pronounced effect will occur if the normal allele is removed and they hypothesise that mutation of one p53 allele may occur at one step in the tumorigenic pathway and loss of the corresponding allele at another perhaps near the adenoma-carcinoma transition point as 17p deletions are so common in colorectal cancers but relatively rare in adenomas (Vogelstein et al., 1988). It seems likely, therefore, that p53 is involved in later stages of malignancy possibly with respect to tumour progression rather than initiation (Menon et al., 1990) and the occurrence of p53 mutations late in colon carcinogenesis has recently been demonstrated (Baker et al., 1990b). Mutations were relatively rare at the adenomatous stage regardless of tumour size and were infrequently seen in tumours retaining both copies of 17p (17%). In sharp contrast, tumours which had lost one copy of 17p usually had a mutation in the remaining allele suggesting that occurrence of a mutation resulted in the rapid loss of the other allele and that these events occur near the transition between benign and malignant growth.

Investigation of p53 gene status in neurofibromatosis type 1 demonstrates a predisposition due to the NF-1 gene on 17q but suggests that tumour progression is due to p53 mutation and subsequent loss of the normal p53 at a later stage (Menon et al., 1990). In this study, 5/6 neurofibrosarcomas showed loss of alleles on chromosome 17 especially in the region 17p13. Of these, two had lost a normal p53 allele with the remaining one being mutated in the conserved region. No loss of this 17p13 region was apparent in the benign neurofibromas.

It seems certain therefore,that p53 is involved in a wide range of human malignancies, possibly in cooperation with other cellular proteins, via mutations in highly conserved regions of the gene leading to a mutated protein with a much longer cellular half-life which accounts for the higher levels of expression detected in tumour cells (Laurent-Puig et al., 1990), as the wild type protein is almost undetectable by conventional immunochemical assays (Lane and Benchimol, 1990). It is thought that in the normal situation, p53 protein exists in an oligomeric form so the presence of one mutant copy may inactivate the growth-suppressor capabilities of the normal allele by complexing with it and thereby eliciting a transforming effect (Vogelstein, 1990). Subsequent loss of the normal allele then releases the cell completely from normal p53 control, and it seems that this is a necessary precursor to complete malignant transformation.

1.8. Experimental Objectives

The aims of this project were essentially threefold:

i) to evaluate the importance of allele loss with respect to colorectal tumorigenesis and to attempt to explain the most likely mechanisms by which such loss occurs

ii) to assess the importance of mutational activation of the K-*ras* gene and the interaction of such mutations with other events in the adenoma-carcinoma sequence

iii) to investigate the nature of putative "new" mutations at the *APC* locus and possibly to correlate these with the severity of disease manifestation in these patients.

2. MATERIALS & METHODS

2. Materials & Methods

2.1. Materials

2.1.1. Chemicals

Unless otherwise stated general laboratory chemicals were obtained from Merck Ltd., Poole, Dorset. All buffers comprised "Analar" or biochemical grade reagents and, where appropriate, were sterilised by autoclaving at 15 lbs psi 121°C for 30 minutes.

2.1.2. Enzymes

Restriction endonucleases were obtained from BRL UK Ltd., with the exception of Bst X1 and Bcl I which came from NBL Ltd.

Taq polymerase was supplied by Perkin Elmer Cetus or Promega. T4 polynucleotide kinase came from Amersham International. Klenow fragment (large fragment polymerase DNA I) was obtained from BRL.

2.1.3. Radionuclides

α^{32}P dCTP and γ^{32}P dATP were obtained from Amersham International, Amersham, U.K.

2.1.4. Tissues

Blood and tissue samples were obtained from a variety of sources: St. Marks' Hospital, London; Ashington General Hospital, Northumberland; The Royal Victoria Infirmary, Newcastle-Upon-Tyne, Salisbury General Infirmary, Darlington Memorial hospital and The Royal Naval Hospital, Plymouth. Blood samples were received fresh as were the majority of tissue samples which had been placed in phosphate buffered saline. Some tissue samples were flash frozen in liquid nitrogen and transported in dry ice.

2.1.5. Cell Lines

For details of cell lines used and references see Appendix 12

2.1.6. DNA Probes

These were also obtained from various sources and arrived either as purified plasmid DNA or as agar stabs (Appendix 11). Agar stabs were grown up immediately and a large scale plasmid preparation carried out. Those received as plasmid DNA were transformed into a suitable E. coli strain prior to culture.

2.2. General Methods

2.2.1. DNA Extraction

DNA was prepared using a standard phenol chloroform extraction method (Maniatis et al 1982) the details of which are given below.

2.2.1.1. From Solid Tissue

Frozen tissue was placed in a petri dish, a drop of sterile STE buffer (appendix 1) added, and the tissue chopped up as small as possible using a pair of clean scalpels. This was then transferred to a universal containing 10ml STE to which was added 100 µl 25mg $^{-1}$ Proteinase K (fungal, Sigma) + 500µl 10% SDS.The mixture was incubated overnight at 37°C, or at 60°C for 3 hours.When digestion was complete the mixture was extracted twice for 10 minutes with phenol and once with chloroform (appendix 2) each time taking the aqueous (top) layer after separating the phases by spinning at 2500 rpm in a bench-top centrifuge.To the final aqueous phase was added 2 volumes ice-cold "Aristar" absolute ethanol and the mixture incubated for >30 minutes at -20°C to precipitate the DNA. The DNA precipitate was hooked out and dissolved in TE buffer (appendix 1) and then reprecipitated by the addition of 1/2 volume 7.5M ammonium acetate followed by 2 volumes absolute ethanol. On precipitation, the DNA was again hooked out and redissolved in an appropriate volume of TE buffer. The concentration of the DNA was established by measuring the optical density at 260nm. If the piece of tissue was extremely small, the whole prep. was scaled down to a volume of 500µl and carried out in a microcentrifuge tube.

2.2.1.2. From Blood

Blood samples in EDTA or heparin tubes were allowed to stand overnight and spun at 2500 rpm. The serum (top layer) was removed and the white cells which lie at the interface taken. The red cells which remain were washed with 0.9% saline, respun and any further white cells collected. The white cells were washed with sterile deionised water to lyse any contaminating red cells, pelleted by spinning at 2500 rpm and the supernatant discarded. The cells were then resuspended in 10ml STE buffer and DNA extraction carried out as for the solid tissue method.

2.2.1.3. From Cultivated Cells

The cells were washed briefly in Hank's balanced salt solution and then trypsinised by incubating in versene (appendix 2) for 5 minutes at 37°C. The cells were transferred to a conical centrifuge tube and spun down to remove the medium (1500 rpm for 5 minutes). The pellet was washed with 0.9% saline, respun and then resuspended in 10ml STE buffer and the extraction continued as for the solid tissue method above.

2.2.1.4. From Desmoid Tumours

These are largely comprised of collagen and even a seemingly large tumour may yield a small amount of DNA as very few cells are present. The high collagen content means that a pretreatment with a protease such as Pronase and a further treatment with collagenase must be carried out before proteinase K digestion and SDS-mediated lysis can take place. The tumour was chopped up with a scalpel as far as possible and treated with 0.1% Pronase (Sigma protease type XIV) in phosphate-buffered saline at 37°C for 3 hours. 5ml of 2.5Mg ml-1 collagenase (Worthington) in Hank's BSS was added and incubated for a further 3 hours. The mixture was spun down and the cells resuspended in 500µl STE and treated as for a small scale tissue DNA extraction.

2.2.2. Probe Preparation

2.2.2.1. Preparation of Competent Cells (Berger and Kimmel, 1987)

A fresh overnight culture of an appropriate E. coli strain (DH1 or HB101) was grown in 10ml L-Broth (appendix 3). The cells were diluted into 200ml of fresh sterile L-Broth and grown at 37°C with agitation to ensure thorough aeration until an absorbence reading of 0.4 - 0.5 at 550 nm was obtained.The suspension was immediately chilled by swirling in an ice-water bath and then centrifuged at 4°C.The supernatant was discarded, the pellets resuspended in 100ml ice-cold sterile 100 mM $CaCl_2$ and the mixture was homogenised by sucking up and down in a cold pipette. The solution was incubated on ice for 30 minutes with occasional swirling and then pelleted as before.The cells were gently resuspended in 10ml ice-cold 100mM $CaCl_2$ to which was added 15% v/v glycerol. They were then split into 0.2ml aliquots in sterile eppendorf tubes and kept on ice at 4°C for 12 - 24 hours (this extra step is essential for high efficiency).The aliquots were flash frozen in liquid nitrogen and stored at -70°C. Using this method competency is maintained for several months.

2.2.2.2. Plasmid Transformation

A 0.2ml aliquot of frozen competent E. coli cells was thawed slowly on ice. To this was added 50 - 100 ng plasmid DNA and the mixture gently agitated and incubated on ice for 30 minutes.The cells were heat-shocked for 5 minutes at 43°C. 0.4ml sterile L-Broth was added and the mixture incubated shaking at 37°C for 1 hour. 200µl was then plated out using the appropriate antibiotic and the plates were incubated overnight at 37°C. A positive colony was then picked and a large scale plasmid preparation carried out.

2.2.2.3. Large Scale Plasmid Preparation (Alkali lysis method)

A 10ml aliquot of L-Broth was inoculated and grown up during the day without antibiotic selection. This culture was used to prepare several 1ml glycerol stocks (15 % v/v glycerol) for long term storage at -70°C and the rest used to seed 2x200ml flasks of

L-Broth. The cultures were grown overnight with aeration and harvested the following day at 6000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellets resuspended in a total volume of 10ml lysosyme buffer (solution I). When homogeneous 50mg of solid lysozyme (Sigma) was added, mixed and left at room temperature for 5 minutes. To this was added 20ml freshly prepared solution II and the mixture incubated 10 minutes on ice. 15ml ice-cold solution III was added and again the mixture left for 10 minutes at room temperature. Following lysis, centrifugation was carried out at 10000rpm for 15 minutes at 4°C. The plasmid containing supernatant retained and the pellets containing cell debris discarded. Details of solutions I, II and III are given in appendix 4.

The volume of supernatant was measured and 0.6 volumes of propan-2-ol was added to precipitate the DNA. Following incubation at room temperature for 10 minutes, the precipitate was pelleted at 10000 rpm for 15 minutes at room temperature, drained and then washed with 70% ethanol.The pellet was drained thoroughly and freeze dried for 5 minutes. It was then resuspended by vigorous vortexing in 11ml TE and purified by CsCl gradient ultracentrifugation.

2.2.2.4. Plasmid Purification by CsCl Gradient Centrifugation

As explained above the plasmid pellets were suspended in 11ml TE buffer. To this suspension was added :

0.46ml 0.2M K_2HPO_4 pH 7.5

12g solid CsCl

1.2ml 10mg ml^{-1} ethidium bromide

The solution was mixed thoroughly and pipetted into Sorvall polyallomer ultracentrifuge tubes. A sufficient amount of paraffin oil wad added to prevent leakage and the tubes sealed with a Sorvall "ultracrimp" tube sealer. The tubes were spun in a Sorvall ultracentrifuge at 45000 rpm at room temperature for 17 - 18 hours.

On removal the tubes were examined under UV light and the plasmid band removed using a syringe.The ethidium bromide was removed by repeated extraction with CsCl-saturated isoamyl alcohol and the CsCl then removed by dialysis in Sartorius collodian

bags (presoaked in deionised H_2O) against 2 changes of distilled water, preferably overnight.The plasmid DNA was precipitated by addition of 1/2 volume 7.5M ammonium acetate and 2 volumes of absolute ethanol. The pellet was washed in 70% ethanol to remove any traces of salt, freeze dried and resuspended in an appropriate volume of TE. Plasmid DNA was routinely stored at -20°C. Using this method the yield of plasmid DNA varied between 250 and 800µg.

2.2.2.5. Preparation of Inserts

The concentration of plasmid DNA prepared as above was calculated by measuring its optical density at 260 nm. 20µg was digested with 100U of the appropriate restriction enzyme (appendix 11) and electrophoresed on a 1% agarose gel containing 100µg ml^{-1} ethidium bromide to separate vector and insert fragments. The gel was viewed under UV light at 354nm and the required fragment isolated. This was then spun through a plug of siliconised glass wool in a microcentrifuge tube according to the method of Heery et al. (1990).The yield of insert was estimated by running an aliquot on a 1% minigel containing ethidium bromide (100µg ml-1) against.known amounts of a standard λ phage DNA sample.

2.2.3. Restriction Digestion of Genomic DNA Samples

In general restriction digest mixtures were set up as follows :

5µg genomic DNA (in TE)

* 2µl 0.1M spermidine (Sigma)

* 4.5 µl appropriate reaction buffer (10x working strength)

* 4 - 5U restriction enzyme per µg DNA

sterile ddH_2O to 45µl

* Except for Msp1 digests (4µl 0.0.1M spermidine pH8, 10U enzyme per µg DNA incubated overnight at room temp.)

The mixture was spun for a few seconds in a microcentrifuge and incubated at 37°C (65°C - Taq1, 45°C Bst XI) for 3 - 4 hours.

Following digestion, 4.5 µl sucrose blue loading buffer was added to stop the reaction and the digests run at 20 - 25V for 8 - 20 hours on 0.6 - 1.0% agarose gel in

TAE running buffer (appendix 1). Both the gel and the buffer contain ethidium bromide at a concentration of 100µg ml^{-1}. Digested DNA samples were run alongside an appropriate molecular weight marker such as Hind III - cut λ DNA or the yeast derived 1kb ladder. The gel was visualised under short wave UV and photographed using an orange filter.

2.2.4. DNA Transfer (Southern Blotting)

The gel was capillary blotted onto Genescreen Plus hybridisation membrane (NEN, Dupont) or onto Hybond N+ (Amersham) according to manufacturers specifications. For details of solutions used see appendix 5.

2.2.5. Radiolabelling of DNA Probes for Southern Hybridisation

Probes for Southern hybridisation consisted of purified insert DNA labelled to high specificity by the random hexanucleotide priming method (Feinberg and Vogelstein 1983) and $\alpha^{32}P$ dCTP (3000 Ci $mmol^{-1}$).

50 - 100ng of insert DNA was boiled for 10 minutes to melt the duplex as it must be single stranded for good results. The labelling mixture comprised the following :

11 µl labelling solution buffer (appendix 6)

1 µl 10mgml^{-1} BSA

1 - 6µl DNA

Volume made up to 20 µl with sterile distilled water.

The mixture was quenched on ice for 2 - 10 minutes to anneal the random hexanucleotides. 2 - 3U (0.5µl) Klenow (BRL) was added along with 30 - 50 µCi $\alpha^{32}P$ dCTP. The tube was spun for several seconds to mix thoroughly and incubated for 3 - 16 hours at room temperature in a lead pot.To remove the unincorporated labelled nucleotide, the reaction mixture was expanded to 100µl with 3xSSC and spun through a G-50 sephadex column (Pharmacia).

2.2.6. Southern Hybridisation

Filters were generally prehybridised in the appropriate mix (appendix 5) for at least 1 hour before addition of the radiolabelled probe DNA. To the probe labelled as above

was added sufficient sheared salmon sperm DNA (Sigma type III) (10mgml^{-1}) to give a concentration of 100 µgml^{-1} in the final reaction mix. The probe and salmon sperm DNA were boiled for 5 minutes and quenched on ice for 5 minutes and then added to the prehybridisation mixture. Hybridisation was carried out at the same temperature for 12 - 24 hours and the probe solution was then poured off and retained for future use.

2.2.6.1. Filter Wash - Genescreen Plus

The membrane was washed to a stringency of 2xSSC (appendix 1) at room temperature with regular changes for up to 30 minutes and if necessary a further 2x20 minute wash in 2xSSC, 0.1%SDS at 65°C was carried out. Occasionally, a further wash at room temperature in 0.1xSSC was necessary to reduce severe background. The filter was then blotted dry, wrapped in cling film and autoradiographed at -70°C using preflashed Fuji RX-L Xray or Amersham Hyperfilm MD for between 1 and 14 days.

2.2.6.2. Filter Wash - Hybond N+

Filters were washed in 2xSSPE (appendix 1), 0.1%SDS at room temperature, then for 15 minutes in 1xSSPE,0.1% SDS at 65 °C. If required, a further more stringent wash in 0.1xSSPE, 0.1% SDS was carried out at 65°C. Autoradiography was as described in section 2.2.6.1.

2.2.7. The Polymerase Chain Reaction - General Method (Saiki et al., 1988)

When using this method, it was essential that Gilsen tips and microcentrifuge tubes were prepared, autoclaved and handled wearing gloves so that any extraneous DNA contamination was minimised. All reactions were carried out using a Hybaid automated heating block.

The reaction mix was set up as follows :

1xPCR buffer (appendix 7)

200µM each dNTP

500ng-1 µg genomic DNA

50pMol each oligonucleotide primer

H_2O to final volume of 100µl.

The mix was spun for a few seconds to mix. The cycles used were obviously primer-dependent but followed the general pattern set out below.

An initial 5 second denaturation step at 95°C was carried out. 2 - 3U Taq polymerase was added along with 100µl paraffin to prevent evaporation, and the cycles started (30 cycles were generally used)

1) Denaturation at 94°C 30 seconds

2) Annealing at 12°C below T_d of primers

3) Elongation at 70 - 72°C for 30 - 60 seconds.

Upon completion, the paraffin was carefully removed and a 10µl aliquot of each reaction was run on a 1.5% minigel in TBE buffer (appendix 1) at 50V for 30 - 60 minutes to visualise the fragments.

2.2.7.1. K-*ras* Amplification

21mers specific for exon 1 of the K-*ras* gene were used to amplify the region surrounding codons 12 and 13. The sequence of the primers (K1 and K2) used is given in appendix 8 and amplification results in a 111bp product the normal sequence of which is shown in appendix 9.1.

1µg of each genomic DNA sample to be analysed was amplified for 30 cycles:

94°C 30 seconds

48°C 30 seconds

72°C 30 seconds

A similar amplification procedure was initially carried out on a panel of colon cancer cell lines in which a modified primer K1A (appendix 9.2) was used in conjunction with K2. This primer contained a single base mismatch which resulted in the introduction of an artificial MspI restriction site (C^CGG) if the sample contains a wild type codon 12. The product was then digested with MspI (following purification using a Mermaid kit supplied by Stratech Scientific) and any mutant codon-containing samples remain undigested while normal samples digest to give 79bp and a 21bp

fragment which could be visualised and photographed under UV following electrophoresis on an ethidium bromide containing 2.5% agarose minigel.

2.2.8. DNA Dot Blot Procedure

DNA samples were amplified for 30 cycles with ras specific primers as in PCR protocol (section 2.2.7 1.). 10µl aliquots of its PCR products were taken and brought to a final concentration of 0.4M NaOH in a total volume of 50µl i.e.

10µl PCR product

10µl 2M NaOH

5µl 0.25M EDTA pH8

25µl ddH_2O

25µl of the final mixture was dotted in duplicate onto dry Hybond N+ hybridisation membrane (Amersham) using a BRL dot blot manifold. The samples were allowed to sit for 30 minutes and then a gentle vacuum applied for a further 5 minutes to ensure even sample loading. The membrane was allowed to air dry overnight before carrying out hybridisation. UV irradiation of the membrane was not necessary.

2.2.8.1. Dot Blot Hybridisation using Allele Specific Oligonucleotides Probes

The method used is that of Farr et al (1988a). The filters were prehybridised for 30 minutes at 55°C in the following solution :

3M tetramethyl ammonium chloride (TMAC)

50MM Tris.HCl pH7.5

2MM EDTA

0.3% SDS

100µg ml^{-1} sheared denatured salmon sperm DNA

5x Denhardt's solution (appendix 5)

The DNA probes used were 19bp oligonucleotide probe "cocktails" which were specific for the various base substitutions possible at positions 1 and 2 of codons 12 and 13 of the K-ras gene. The sequences of these allele-specific oligonucleotides is given in appendix 9.1. Approximately 250ng of the probe "cocktail" was end-labelled using T4 polynucleotide kinase. The reaction mix comprised the following:

1µl 10x T4 kinase buffer (appendix 6)

1µl γ ^{32}P ATP

1µl T4 polynucleotide kinase

250ng probe "cocktail" (appendix 9.1)

ddH_2O to 10µl

The reaction mix was incubated at 37°C for 1 hour and then purified by spinning through a G-25 sephadex (Pharmacia) column in TE buffer. The labelled probe was then added directly to the prehybridisation mix and hybridised for 1 hour at 55°C. Washing was carried out as follows:

2 x10 minutes room temperature 2xSSPE, 1% SDS

1 x 3 minutes room temperature TMAC solution *

1 x 30 minutes 65°C TMAC solution

* As prehybridisation mix minus salmon sperm DNA

Autoradiography was carried out overnight at -70°C with intensifying screens.

2.2.9. Determination of K-*ras* base substitutions using coupled amplification and direct sequencing of genomic DNA samples

This method utilises a technique developed by Ruano et al. (1990) which exploits the ability of Taq polymerase to incorporate dideoxynucleotides as part of the normal pcr procedure and thus produces chain termination events as in normal Sanger dideoxy sequencing. The pcr procedure was carried out as follows:

15 cycles of the pcr program given in 2.2.7.1. were carried out using reduced concentrations of primers and nucleotides in a total volume of 50µl:-

500ng-1µg genomic DNA

1 x PCR buffer (Cetus or Promega)

10µM each deoxynucleotide

5 pMol each primer (K1 and K2)

2U Taq polymerase (Cetus or Promega)

ddH_2O to 50 µl

For each individual sample, 4 such reactions were set up and following the first 15 cycles, one dideoxynucleotide was added to each tube at the concentrations given below:

ddATP	640µM
ddTTP	640µM
ddCTP	400µM
ddGTP	100µM

One of the primers (K2) was end-labelled to a specific activity of 2 µCi $pMol^{-1}$ and 5 pMol was added to each reaction tube. Another 15 cycles was then carried out and 10% of the product was run on a 6% acrylamide, 7M urea sequencing gel (for gel preparation see appendix 10). The gel was then exposed overnight at -70°C. The sequence obtained in this way was compared with the normal sequence and any base changes noted.

3. RESULTS

3. Results

3.1. Allele loss studies using hypervariable DNA probes.

Initial studies of allele loss were carried out on a sample of 26 sporadic colorectal carcinomas; 3 APC carcinomas; 48 adenomas from 21 APC patients; one APC desmoid tumour and two sporadic adenomas using a panel of 5 highly polymorphic, locus-specific minisatellite probes (Wong et al., 1987).All the sporadics used in this study were adenocarcinomas except sample 244 which was undifferentiated. DNA was extracted from the flash frozen tumour tissue and also either from normal mucosa or blood in all cases. In light of previous evidence for loss of heterozygosity at chromosome 5 loci in at least 20% of colorectal carcinomas (Solomon et al., 1987) and linkage of APC to the region 5q21-22 (Bodmer et al., 1987) the probe λMS8 (D5S43; 5q35-qter) was chosen for this study (appendix 11.1). Two minisatellite probes specific for each arm of chromosome 1 were also chosen - λMS1 (D1S7; 1p33-p35) and λ MS32 (D1S8; 1q42-q43) along with two chromosome 7 probes - λMS31 (D7S21; 7p22-pter) and pλg3 (D7S22; 7q36-qter) (appendix 11.4).The advantage of minisatellite probes is that they detect extremely variable loci with heterozygosities ranging from 90-99%. The basis for investigation of chromosome 1 is that , as with most malignancies, frequent structural alterations of this chromosome are found in colorectal cancer (Reichmann et al., 1984). Also, prior to this study, isoenzyme analysis of 2/6 informative colorectal cancers showed evidence of loss of expression at the α-fucosidase locus (1p34)whilst retaining expression of phosphoglucomutase I (1p22) suggesting a partial deletion of chromosome 1p (S.H. Rider, M.B. Davis and J.D.A. Delhanty - unpublished data). Investigation of chromosome 7 is based on the observation of trisomy of this chromosome in colorectal carcinogenesis (Reichmann et al., 1985).

3.1.1. Allele loss in carcinomas

The results obtained using the above-mentioned minisatellite probes in 26 sporadic and 3 familial colorectal carcinomas are presented in table 3.1. A total of 23 carcinoma

patients were informative for the probe λMS1, all of which retained heterozygosity in the tumour tissue.

25 patients were constitutionally heterozygous for the second chromosome 1 probe, λMS32; a clear case of allele loss was seen in one of the tumours (APC 72 - corresponding to the colorectal cancer cell line JW2) whilst two sporadic carcinomas (samples 151 and 184) exhibited different sized bands when compared with their normal DNA counterpart

Of the 22 carcinoma patients informative for the chromosome 5 probe λMS8, 2 exhibited clear loss of one allele in the tumour tissue (195 and 315) whilst a further three showed a marked reduction in the intensity of one allele (185, 219, HB). All five of these were sporadic carcinomas (figure 3.1).

Using the chromosome 7p probe λMS31, 19 patients proved informative, with two showing changes in the tumour tissue. Patient 260 exhibited reduced intensity together with the appearance of 2 new bands and patient 311 also showed extra bands in the tumour DNA . The appearance of additional bands or altered band sizes seen with λMS1, λMS31 and λMS32 may reflect the high rate of somatic mutation seen in this type of material with these probes (Jeffreys et al., 1989).

21 carcinoma samples were informative for the pλg3 probe, of which two sporadic cases showed a definite reduction in intensity of one allele when compared with the normal DNA (185 and HB). Chromosomes prepared from a short-term culture from patient HB revealed four copies of chromosome 7 in diploid cells (figure 3.3) possibly indicating duplication of one parental chromosome at the expense of the other.

Reduced intensity of one of a pair of allelic fragments rather than complete loss possibly reflects the presence of normal stromal tissue in the neoplasm; the co-existence of more than one cellular clone or the duplication of one allele at the expense of the other.

Table 3.1. Allele changes at minisatellite loci in sporadic and APC colorectal carcinomas.

Patient no.	Site[d]	Dukes stage	Probe and chromosomal location λMS1 1p33-p35	λMS32 1q42-q43	λMS8 5q35-qter	λMS31 7p22-pter	pλg3 7q36-qter
sporadics							
CRC3	R	B	1.2	-	-	1.2	1.2
CC4	C		1.2	1.2	1.2	-	1.2
CC21	C		1.2	1.2	1.2	1.2	1.2
CC22	R	B	1.2	1.2	1.2	-	1.2
142	R	C1	1.2	1.2	1.2	1.2	1.2
149	R	C1	1.2	1.2	1.2	1.2	1.2
150	R	C1	1.2[a]	1.2	-	-	1.2
151	R	C1	1.2	1.2[a]	1.2	1.2	-
177	R	C1	1.2	1.2	1.2	1.2	1.2
183	R	C2	1.2	1.2[a]	1.2	-	1.2
184	R	C1	1.2	1.2	-	1.2	1.2
185	R	C1	1.2	1.2	**1.(2)**	1.2	**(1).2**
192	R	B	1.2	1.2	-	1.2	1.2
193	R	C1	1.2	1.2	1.2	1.2	1.2
195	R	C1	1.2	1.2	**2**	1.2	-
200	R	B	-	1.2	1.2	1.2	1.2
202	DC	B	1.2	-	1.2	1.2	1.2
214	R	B	1.2	1.2	-	1.2	1.2
219	R	C1		1.2	**(1).2**		
225	R	B		1.2	1.2		
244	R	C1		1.2	1.2		
254	R	B			1.2		
260	R	A	1.2	1.2	-	**(1).2**[b]	1.2
311	AC	B	1.2	1.2	1.2	1.2[b]	1.2
315	SC	C2	1.2	1.2	**2**		
HB			1.2	1.2	**(1).2**	-	**1.(2)**
APC							
72[c]	R	C		**1**	-	1.2	1.2
112	SC	B	1.2	1.2	1.2	1.2	-
119		C	1.2		1.2	1.2	1.2

Homozygosity in the constitutional DNA is indicated as a dash. Where the normal tissue was informative the tumour genotype is indicated in the table. Heterozygosity is indicated by 1.2. The continued presence of the larger allelic restriction fragment is indicated by '1' whereas '2' indicates the continued presence of the smaller allele. Reduction of intensity is indicated by ( ). Absence of an entry indicates not tested or no result obtained.

[a] altered band size in tumour

[b] additional band(s) in tumour

[c] equivalent to colorectal cancer line JW2

[d] R = rectal; C = colon; AC= ascending colon; DC= descending colon; SC = sigmoid colon.

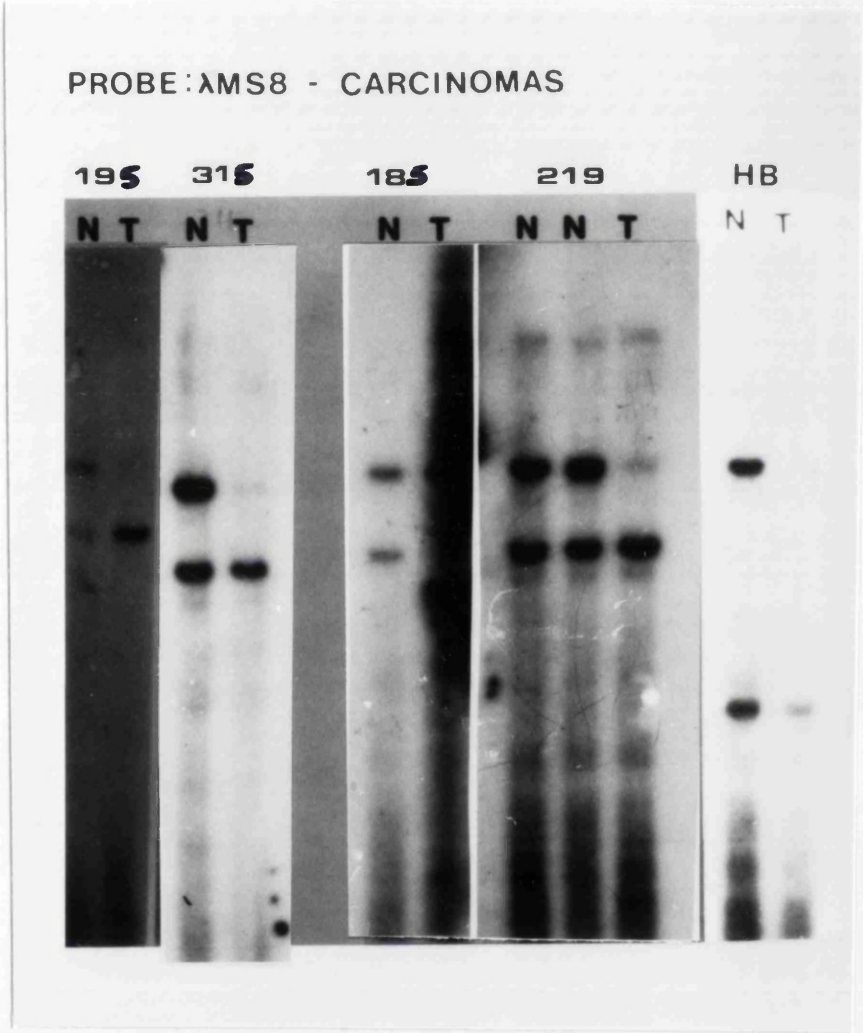


Figure 3.1 Allele loss on chromosome 5 in sporadic colorectal carcinomas - autoradiographs of Southern hybridisation of HinfI digested DNA with the minisatellite probe λMS8 (5q35-qter). T=tumour DNA; N=normal DNA counterpart.

3.1.2. Allele loss in adenomas

The results of this study are presented in table 3.2 along with the numbers of adenomas which were examined from each patient and any size data which was available.

Thirteen adenoma patients (11 APC) were constitutionally informative for λ MS1, none of which showed any alterations in the tumour tissue. Of the patients (1 APC) informative for λMS32, only 1/5 adenomas from patient 70 showed an altered band size. This specimen was a 2cm diameter villous polyp . Again, as with the carcinoma samples in table 3.1, this may reflect a high rate of somatic mutation at this locus. The relevance of such a high mutation rate with respect to carcinogenesis is at present unknown.

Among the 19 (17 APC) patients informative for λMS8, three showed evidence of allele loss in the adenoma DNA. A clear reduction in intensity was seen in two of the three adenomas examined from one APC patient (57). DNA from a single adenoma from a second APC patient (93) showed complete loss of the smaller allele, whilst DNA from the sporadic adenoma of patient 52 showed a similar loss (figure 3.2).

No changes were detected in the adenoma DNA of the 1 patients informative for the chromosome 7 probe λMS31 or in that of the 15 patients informative for pλg3.

The desmoid tumour (a benign neoplasm of mesenchymal origin) from patient 95 who was informative at one locus of each tested chromosome showed no change from the constitutional type.

A summary of the results obtained with the minisatellite probes is given in table 3.3.

Table 3.2 Allele changes at minisatellite loci in familial and sporadic colorectal adenomas.

Patient no.	Adenoma number	Size[a] (mm)	λMS1	λMS32	λMS8	λMS31	pλg3
APC							
30	3	6mm max.	1.2	1.2	-	1.2	1.2
33	1		-	1.2	1.2	-	1.2
35	3		1.2	1.2	-	1.2	1.2
36	3		1.2	1.2	-	1.2	-
37	3		-	1.2	1.2	1.2	1.2
38	2		1.2	1.2	1.2	1.2	1.2
39	2		1.2	1.2	1.2		
41	2	6mm max.			1.2	1.2	
42	1	7mm			1.2		
43	3		1.2	1.2	1.2	1.2	1.2
44	2		-	1.2	1.2	1.2	-
45	2		-	1.2	1.2		1.2
46	2		1.2	1.2	1.2		-
47	1				1.2		
48	3		-		1.2		1.2
49	1		1.2	-	-		1.2
50	2	15mm, 10mm	1.2	1.2	1.2	1.2	1.2
57	3	6mm max.	1.2	1.2	(1).2[c]	1.2	1.2
70	5	largest 7mm	1.2	1.2[b]	1.2	1.2	1.2
84	3		1.2	1.2	1.2	1.2	1.2
93	1				1		1.2
sporadic							
51	2	10mm	1.2	1.2	1.2	1.2	1.2
52	1				1		
desmoid							
95	-		1.2	1.2	1.2		1.2

Homozygosity in the constitutional DNA is indicated by a dash; where the normal tissue was informative the tumour genotype is shown in the table. Heterozygosity is indicated by 1.2. The continued presence of the larger allelic restriction fragment is indicated by '1' and '2' indicates the continued presence of the smaller allele. Reduction of intensity is indicated by ( ). Absence of an entry indicates not tested or no result.

[a]Adenomas < 5mm diameter unless otherwise stated.

[b]altered band size in DNA from largest polyp

[c]reduced intensity of larger allele in DNA from two separate adenomas

Table 3.3 Summary of results obtained with minisatellite probes

		Allelic Changes			
Probe	No. het. patients	Allele loss	Decreased allele intensity	Altered allele size	% showing changes
Carcinomas					
λMS1	23	-	-	1	4.3
λMS32	25	1	-	2	12.0
λMS8	22	2	3	-	22.7
λMS31	19	-	1	2	15.7
pλg3	21	-	2	-	9.5
Adenomas					
λMS1	13	-	-	-	-
λMS32	16	-	-	1	6.3
λMS8	19	2	2[a]	-	9.7
λMS31	13	-	-	-	-
pλg3	15	-	-	-	-

[a] Two adenomas from one patient

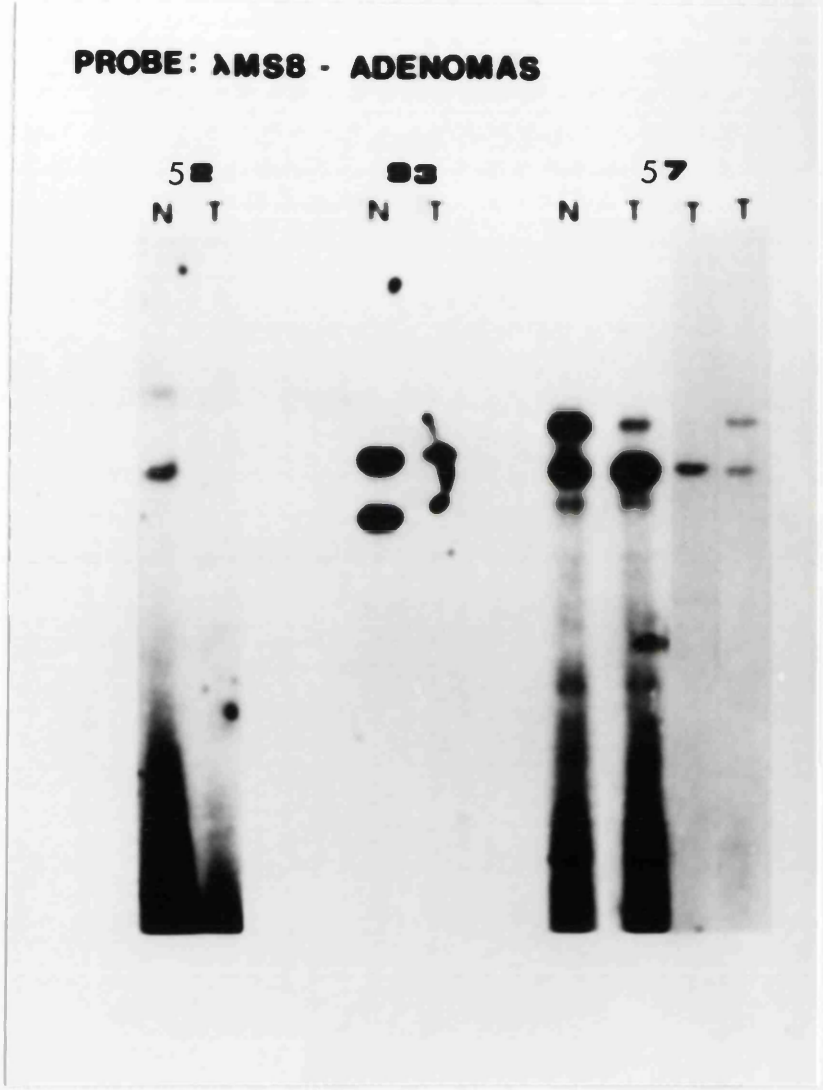


Figure 3.2. Chromosome 5 allele loss in colorectal adenomas using the minisatellite probe λMS8 (5q35-qter) hybridised to HinfI-digested genomic DNA samples. T=tumour (i.e. adenoma) DNA; N=normal DNA counterpart. Note in patient 57, allele loss in 2/3 adenomas and retention of heterozygosity in the third.

3.2. Further allele loss studies

In light of the results obtained in section 3.1. above with respect to loss of heterozygosity (LOH) using the chromosome 5 hypervariable minisatellite probes, it was decided to undertake further detailed studies on a series of APC carcinomas and on several large series of APC adenomas. Three desmoid tumours derived from APC patients were also included in the study together with a series of hamartomas derived from a patient with juvenile polyposis. Details of all samples used are given in tables 3.4 to 3.8. LOH was examined in these samples using polymorphic DNA probes not only on chromosome 5 but also on chromosomes 17 and 18, regions of which have been implicated in a large percentage of colorectal cancers and sporadic adenomas (Vogelstein et al., 1988). In all cases DNA extracted from corresponding normal tissue was available for comparison.

Investigation of allele loss was also carried out on a sporadic carcinoma HB which was also included in the previous study (table 3.1)for which detailed karyotypic analyses (figures 3.3 and 3.4) were available in the hope of correlating the cytogenetic and molecular genetic data. The pseudodiploid karyotype produced as a result of a short-term culture of this tumour is illustrated in figure 3.3. A tetraploid karyotype seen in some cells of the tumour thought to be produced by a "doubling-up" of a diploid cell is shown in figure 3.4. Various abnormalities are evident including a 4:5 translocation; four copies of chromosome 7 and various chromosome 17 rearrangements. Figure 3.5 is an interpretation of the possible breakpoints involved with respect to the formation of the translocation chromosome (J.D.A.Delhanty, pers. comm.). The breakpoint on chromosome 5 appears to be at or near 5q21, the proposed site of the polyposis gene, resulting in the possible deletion of this region. For this reason, detailed allele loss studies were deemed necessary to establish the extent of the deletion involved.

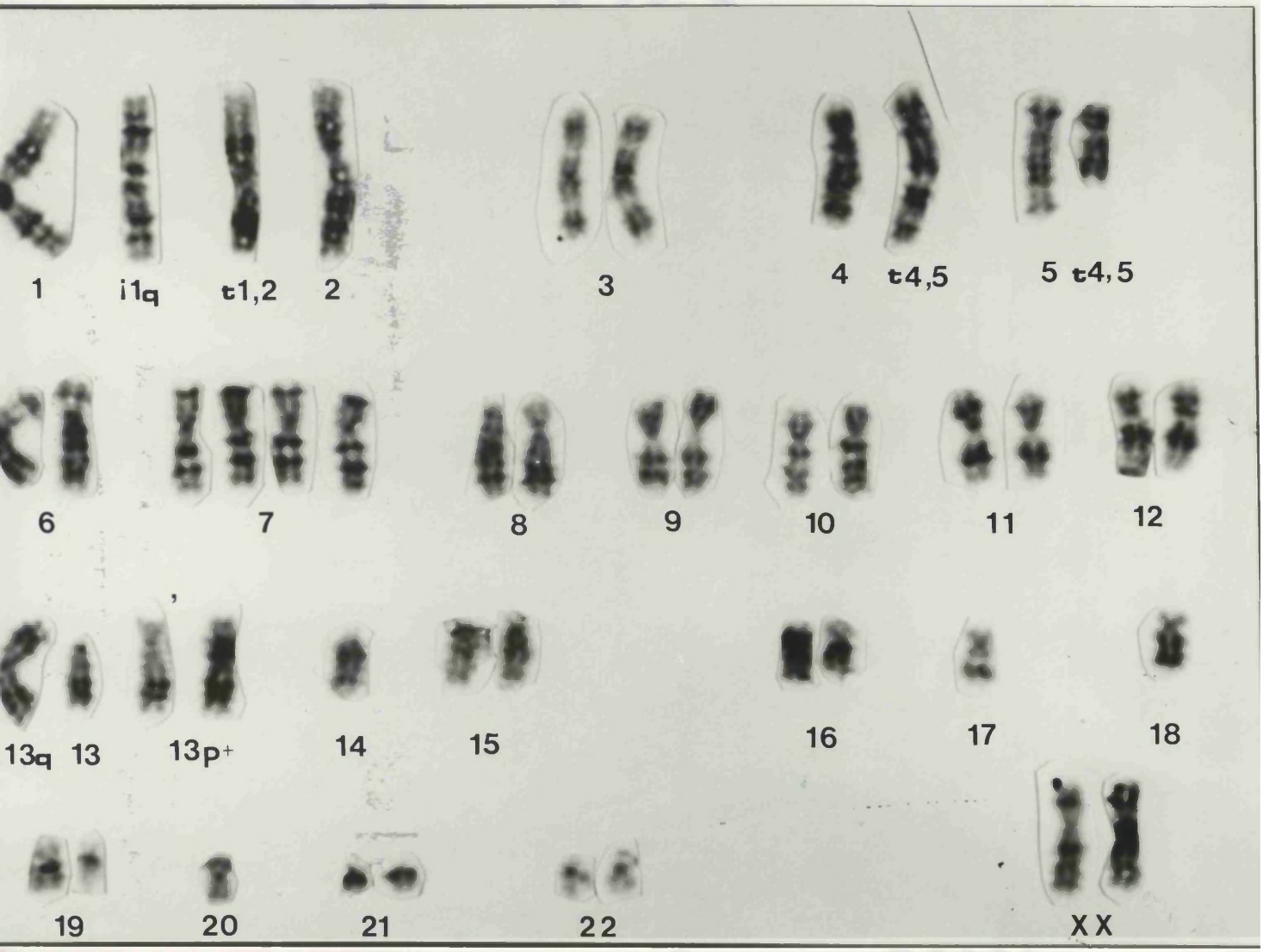


Figure 3.3 Pseudodiploid karyotype of metaphase spread obtained from short-term culture of sporadic carcinoma HB. Among the abnormalities to note are the t (4, 5) translocations and the over-representation of chromosome 7.

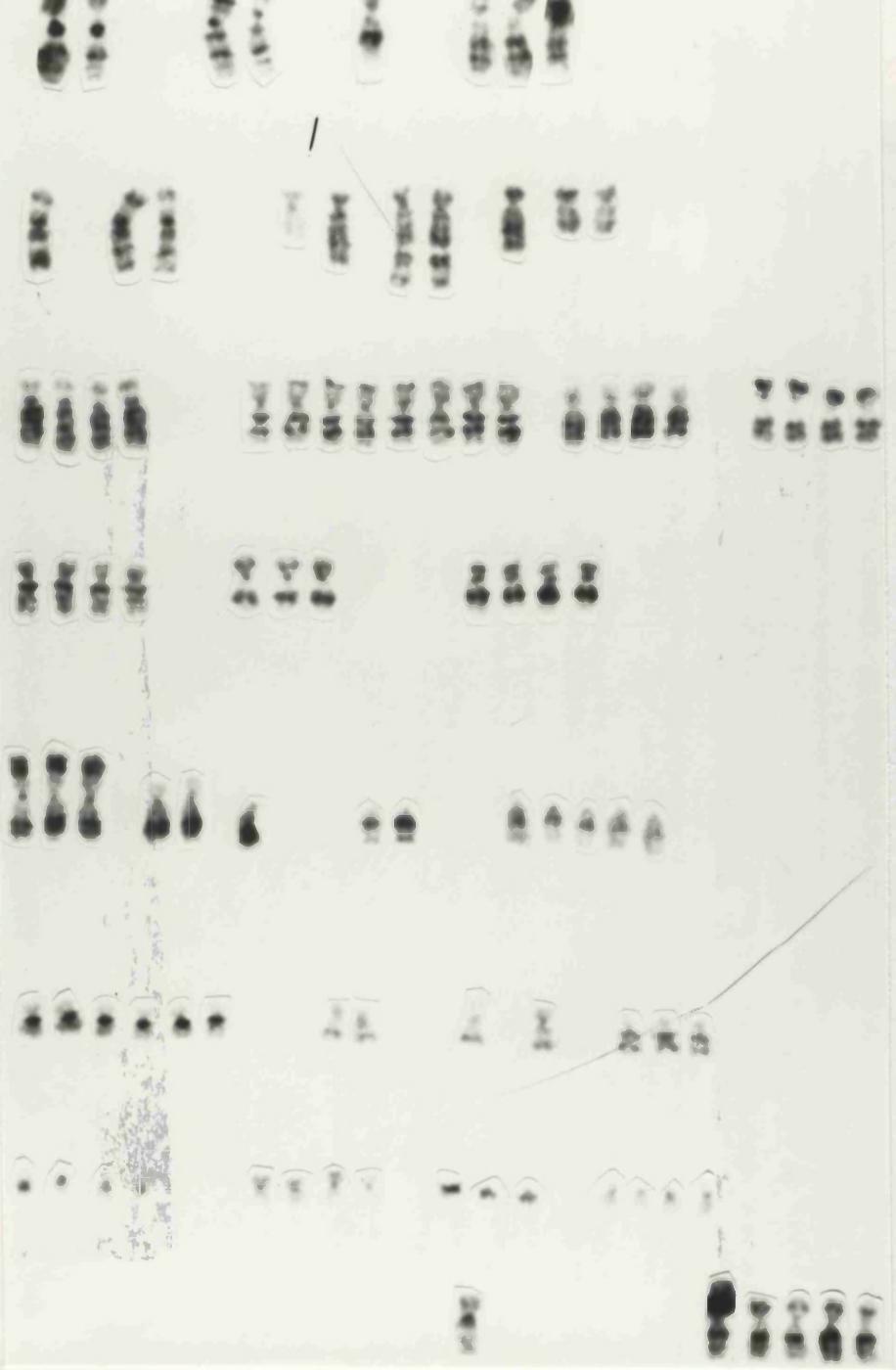


Figure 3.4. Tetraploid karyotype obtained from carcinoma HB initially formed by a "doubling up" of a diploid cell and subsequently by other changes. Diploid and tetraploid cells were essentially present in a 1:1 ratio at the time of presentation of the tumour.

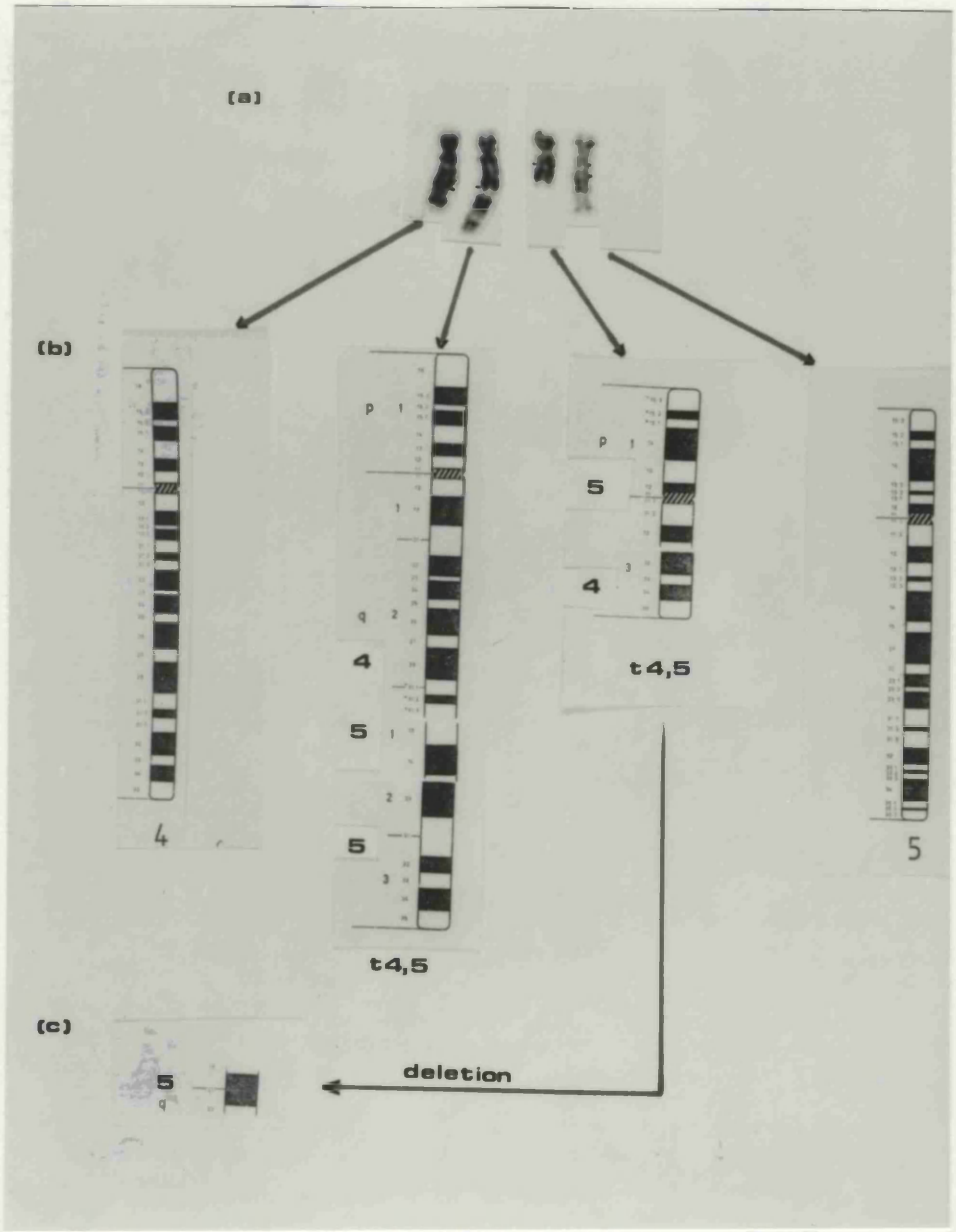


Figure 3.5. Patient HB tumour karyotype - Interpretation of the breakpoints leading to the formation of the t(4,5) chromosomes shown in figure 3.3 and (a) above; (b) diagrammatic representation of the chromosomes shown in (a) with possible breakpoints at 4q31 and 5q13, 15 and 22; (c) probable deleted region of 5q resulting from formation of translocation chromosome.

Table 3.4. Patient/sample description - APC carcinoma samples

Patient no.	Description/ Site	Details	Use of material
72	JW2 colon carcinoma cell line	Also shown in table 3.1	Hypervariable minisatellite probe analysis; allele loss studies: chromosomes 5,17,18; K-*ras* activation studies
112	Sigmoid, Dukes' B	Also shown in table 3.1	As above
119	Dukes' C	Also shown in table 3.1	As above
130	Desc. Colon		Allele loss studies; chromosomes 5,17,18; K-*ras* activation studies
131	Rectal, Dukes' B		As above
134	Liver metastasis		As above
141	Caecal, Dukes' B		As above
145	Rectal, Dukes' B	Sample 5 in table 3.7	As above
147	Dukes' C	Sample 1 in table 3.6	As above
150	Rectal, Dukes' B		As above
158	Primary jejunal, moderately differentiated carcinoma	Very fatty tissue - little DNA obtained	As above
161	Sigmoid, Dukes' A		As above

Table 3.5. Description of adenomas from APC patients 136 and 139

Patient no.	No. of adenomas investigated	Size range of adenomas (mm)	Histological type	Degree of dysplasia	Use of material	Additional information
136	10	6-15	tubular	mild	Allele loss studies on chromosomes 5,17,18; K-*ras* activation studies	Patient aged 21 years at colectomy 7/7/89 98 polyps > 2mm; 15 > 10mm + hundreds of smaller ones. Specimens taken from an area 300-400mm from ileal junction in descending order.
139	12	10-30	tubulo-villous	mild-moderate	As above	Patient age 24 years at date of colectomy 3/10/89

Table 3.6. Description of adenomas from APC patients 146 and 147

Patient no.	Adenoma no.	Size (mm)	Histological type	Degree of dysplasia	Use of material	Additional information
146	1	50	tubular	mild	Allele loss studies on chromosome s 5,17,18; K-*ras* mutation analysis	Possible new mutation patient
147	1 (carcinoma)	30	invasive Dukes' C adenocarcinoma	severe	As above	
	5	20		mild	As above	Adenomas throughout large intestine but concentrated in distal half
	7	10		mild		
	11	5		mild		

Table 3.7. Description of samples from APC patient 145

Sample no.	Position and tumour type	Size(mm)	Histological type	Degree of dysplasia	Tumour tissue as % cellular population	Use of material
1	transverse adenoma	7	tubular	mild	50	Allele losses on
2	descending adenoma	6	tubular	mild	50	chromosomes 5,17,18;
3	transverse adenoma	6	tubular	mild	50	K-ras mutation
4	ascending adenoma	<5	-	-	-	analyses
5	carcinoma		Dukes' B		-	
6	descending adenoma	10	tubular	mild	60	
7	ascending adenoma	<5	-	-	-	
8	transverse adenoma	7.5	tubular	mild	50	
9	transverse adenoma	5	tubular	mild	55	
10	descending adenoma	9	tubular	mild	60	
11	descending adenoma	15	tubular	moderate	60	

Patient 145 aged 23 years at date of colectomy 24/1/90. Represents possible new mutation i.e. no prior family history..

Total no. adenomas ~ 2500. 12 = 1-2cm; 2 = 5cm (one of which had carcinoma arising).

Table 3.8. Description of duodenal adenoma series

Patient no.	Size (mm)	Histological type	Degree of dysplasia	Use of material
D1	5	Tubulovillous	moderate	Allele loss with
D2	5	tubular	mild	hypervariable probes;
D3	10	tubular	mild	K-ras mutation analyses
D4	25	tubulovillous	moderate	
D5	1	tubular	mild	
D6	1	tubular	mild	
D7	5	tubulovillous	mild	
D8	2	tubular	mild	
D9	5	tubular	mild	
D10	2	tubular	mild	
D11	-	normal mucosa*	-	

* Histological examination revealed this "adenoma" to comprise normal epithelial cells only.

3.2.1. Investigation of Allele Loss on Chromosome 5.

Details of the probes used and associated references are given in appendix 11.1. A total of 10 probes was used to cover the length of the chromosome. Seven of these (C11p11, D5S71; ECB27, D5S98; π227,D5S37; YN5.48, D5S81; MC5.61, D5S84; EF544 and L562) are clustered in the region 5q21-22 and have all been reported as being linked, in varying degrees, to the APC gene. The other three probes used map to 5p (MS621, D5S110); 5q35-qter (MS8, D5S43) and 5q33-35 (v-fms, CSFIR). As far as is known, EF544 and L562 have not as yet been assigned HGM locus symbols but as previously shown (figure 1.6) both exhibit extremely tight linkage to the APC locus. More recently a polymorphic cDNA clone corresponding to the .MCC gene was made available to us and was added to the panel of chromosome 5 probes.

3.2.1.1. Chromosome 5 Allele Loss in Carcinomas.

Table 3.9. shows details of the results obtained using the various chromosome 5 probes on thirteen normal and tumour DNA pairs. Of the 13 patients (12 APC and 1 sporadic) used in this study, 12 were informative for one or more of the chromosome 5 markers, 7 of which (58.3%) exhibited loss of one or more of these markers. Figure 3.6. illustrates some of the losses observed. Such LOH was expressed as a reduction in intensity rather than complete loss of one allele probably reflecting the population of normal stromal cells which contaminate the tumour. In the case of carcinoma 134 (a liver metastasis of an APC colon carcinoma), family linkage data indicates that in the case of π227 and YN5.48, it is the normal allele which was retained suggesting that in this tumour loss of chromosome 5 sequences occurred as a secondary event probably associated with the metastatic process.

The distribution of allele loss along chromosome 5 in these carcinomas is illustrated graphically in figure 3.7. It can be seen that the loss is concentrated around the region of the APC gene and is therefore highest with the markers most tightly linked to the disease locus. The highest percentage loss (50%) was seen with the marker YN5.48 (D5S81) although this includes the data obtained from the metastasis, tumour 134. No loss was seen using the hypervariable minisatellite probe MS621 (D5S110) on the short

arm of chromosome 5 indicating that whole chromosome loss does not play a major role in the aetiology of these tumours.

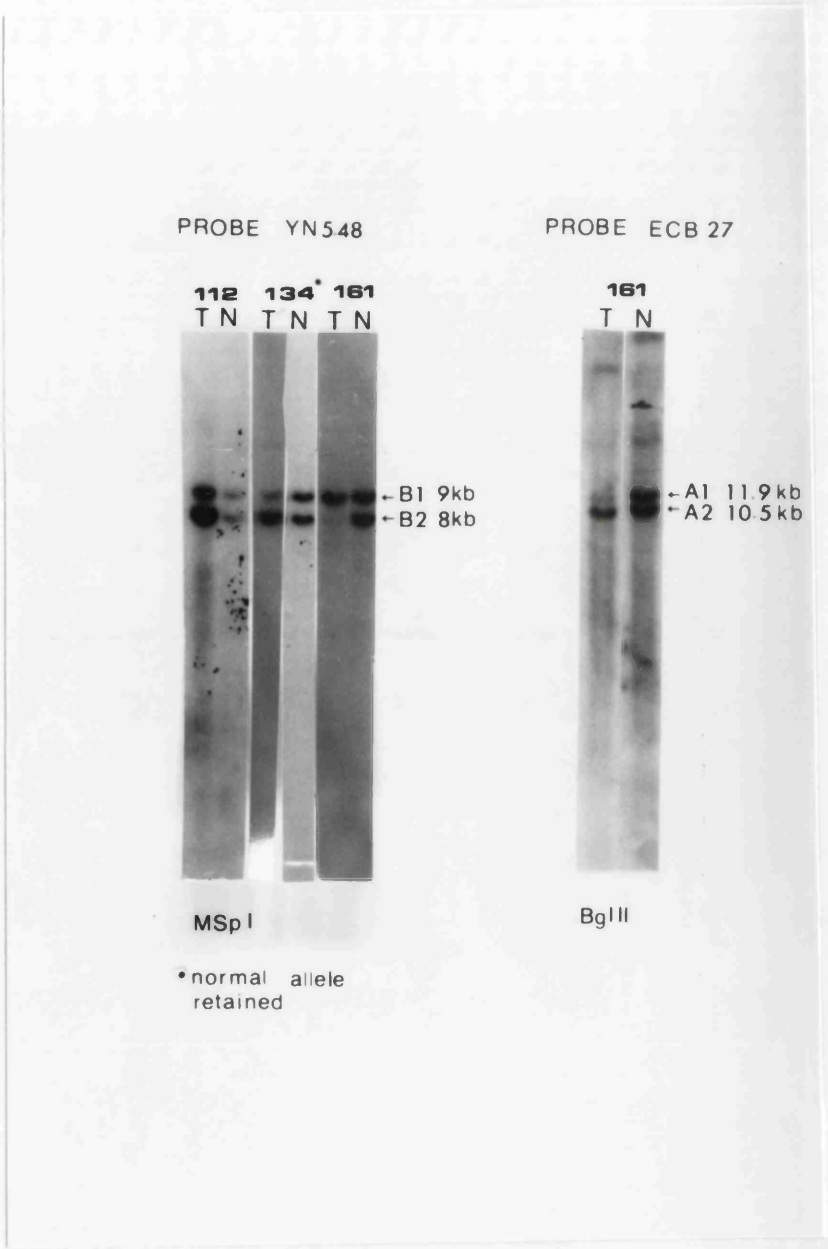


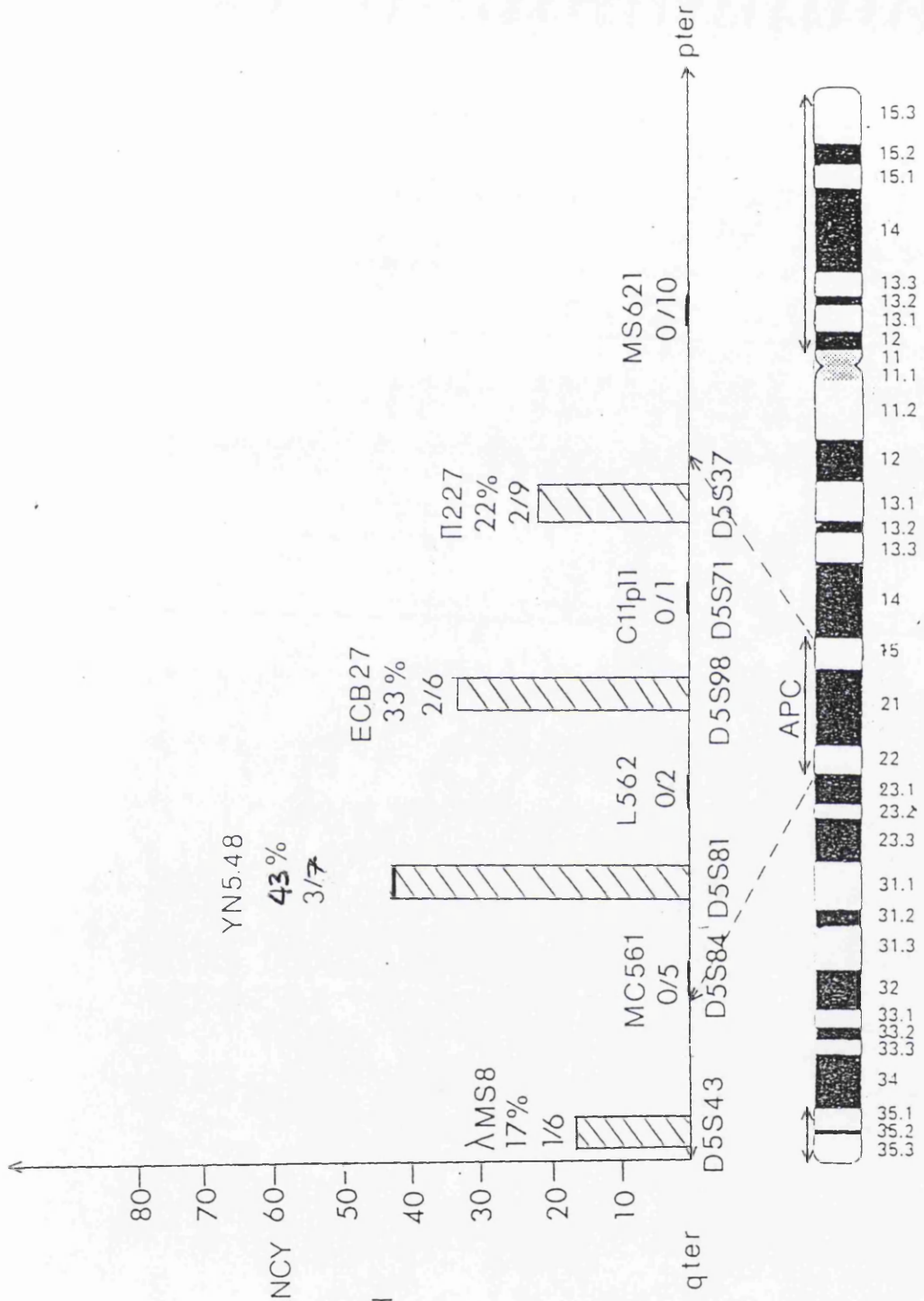
Figure 3.6. Chromosome 5 allele loss in familial carcinomas using the APC gene flanking markers YN5.48 and ECB27. Data obtained from APC 161 illustrates the involvement of the APC region in this tumour. T=tumour; N=normal DNA. * Family linkage data suggests that the normal allele is retained in this tumour (liver metastasis).

Table 3.9. Chromosome 5 allele loss in colorectal carcinomas

APC PATIENT NO.	PROBES										
	MS621	π227	C11p11	ECB27	L562	EF544	YN5.48	MC5.61	v-fms	λMS8	MCC
72	1.2	1.2	-	-	-	-	-	1.2	-	-	-
112	-	1.2	-	1.2	-	-	**(1)2**	-	-	1.2	-
119	1.2	1.2	1.2	-	-	-	1.2	1.2	-	1.2	-
130	1.2	1.2	-	-	-	-	1.2	-	-	-	-
131	1.2	1.2	-	1.2	1.2	-	1.2	1.2	-	-	-
134[a]	-	**1(2)**	-	1.2	-	-	**(1)2**	1.2	-	-	-
141	1.2	1.2	-	1.2	1.2	-	-	1.2	-	1.2	-
145	1.2	1.2	-	-	-	-	-	-	-	1.2	-
147	1.2	-	-	-	-	-	-	-	-	1.2	-
150	1.2	-	-	-	-	-	1.2	-	-	-	-
158	-		-		-	-	-	-	-		
161	1.2		-	**(1)2**	-	-	**1(2)**	-	-	-	-
sporadic											
HB	1.2	**1(2)**	-	**1(2)**	-	-	-	-	-	**(1)2**	-

Homozygosity in the constitutional DNA is indicated by a dash. Heterozygosity in the normal tissue and retention of both alleles in the tumour is indicated by 1.2.even though some probes recognise multiallelic systems. The continued presence of the larger allelic restriction fragment is indicated by '1' whereas '2' indicates continued presence of the smaller allele. Reduction in heterozygosity is indicated by ( ). Absence of an entry indicates not tested or no result obtained.

[a]Normal allele apparently retained, according to family data. Maybe secondary event in metastasis.


FREQUENCY OF LOH (%)
80
70
60
50
40
30
20
10
qter
pter
D5S43
D5S84
D5S81
D5S98
D5S71
D5S37
APC
λMS8
17%
1/6
MC561
0/5
YN5.48
43%
3/7
L562
0/2
ECB27
33%
2/6
C11p11
0/1
Π227
22%
2/9
MS621
0/10
15.3
15.2
15.1
14
13.3
13.2
13.1
12
11
11.1
11.2
12
13.1
13.2
13.3
14
15
21
22
23.1
23.2
23.3
31.1
31.2
31.3
32
33.1
33.2
33.3
34
35.1
35.2
35.3

3.2.1.2. Chromosome 5 Allele Loss in Adenomas.

Table 3.10 illustrates the results obtained using chromosome 5 markers on a total of 37 colorectal adenomas from 6 patients (tables 3.5-3.7); 10 hamartomas from a patient with juvenile polyposis and 4 APC desmoid tumours. A benign cyst from an APC patient (PS) with a karyotypically detectable chromosome 5q constitutional deletion (Cross et al., 1991) was also investigated.

No LOH was detected in 10/11 duodenal adenomas informative for the marker λMS8. Unfortunately, the tissue biopsy received in each of these cases was extremely small (<2mm) and consequently little DNA was obtained. Allele loss studies on these tumours were therefore limited. In general, very little DNA was extracted from the desmoid tumours as most of the tumour mass is comprised of collagen with very few cells present. Therefore, these samples were also only subjected to limited analysis.

From table 3.10, it can be seen that no losses were observed in the 22 adenomas looked at from patients 136 and 139. In the case of patient 136, 3/11 of the chromosome 5 markers used were informative, two of which, C11p11 and π227 are linked to the APC locus. Both these markers are proximal to the APC region. Unfortunately none of the distal markers were polymorphic in this patient so it is difficult to say with any certainty that LOH has not occurred but in light of the results with C11p11 and π227 it seems unlikely. . Patient 139 proved to be highly uninformative with most of the markers used, being heterozygous for λMS8 (5q35-qter) only. Both alleles were retained in all the adenomas from this patient. Very little can be concluded about possible events occurring in the area around the APC gene in this patient since none of the APC-linked markers were informative but other possible markers are under investigation .

In the case of patient 145, LOH was detected in 4/10 adenomas using the minisatellite probe λMS8 (tables 3.10 and 3.12) which maps to the telomere of 5q. Interestingly, the carcinoma obtained from this patient showed no such loss (table 3.9). Heterozygosity was retained in all samples with both π227 (5q15-21) and MS621 (5p) indicating that loss of the whole chromosome is not the mechanism by which these allele losses occur (figure 3.8a). This patient was constitutionally homozygous for the

remaining markers used so it is impossible to predict how far up the chromosome the deletion extends.

Loss of heterozygosity was detected in the 5cm adenoma from patient 146 using the markers ECB27, YN5.48 and EF544, and therefore incorporates the APC locus (figure 3.8b). In contrast heterozygosity was retained with π227. The other three distal markers, MC5.61, *v-fms* and λMS8 were uninformative in this patient which makes it difficult to predict how far the deletion extends telomeric to the disease locus.

No losses were seen in the samples investigated from patient 147 but, as with patient 139, only MS621 and λMS8 were informative. A carcinoma from this patient similarly exhibited no loss (table 3.9).

Three adenomas from patient 57 were investigated in the initial study using the minisatellite probes (table 3.2) where LOH was detected in 2/3 adenomas using the λMS8 probe. One of the adenomas showing loss of an allele was therefore subjected to further analysis and exhibited loss of the ECB27 region also proving that the deletion originally detected using λMS8 incorporates the APC locus (figure 3.8c).

In the case of the series of hamartomas taken from a patient with juvenile polyposis no allele loss was observed with either MS621 or λMS8. All seven other markers except π227 were uninformative in this patient. Results obtained with π227 were interesting. In 6/10 of the polyps examined, no differences were detected when compared with the normal DNA sample. Of the 4 remaining polyps, all showed distinct extra fragments in addition to the allelic ones expected (figure 3.9). In some cases (lanes 3&4)these extra fragments hybridised to the probe more strongly than did the allelic ones. These results are suggestive of possible rearrangements in the region of the π227 marker which may or may not involve the APC locus.

Results obtained from studies of desmoid tumours are presented in table 3.11. As previously mentioned, because of limited DNA resources, only restricted analysis was possible. In the case of desmoid 95, only studies using the initial minisatellite probes and C11p11 were possible (table 3.2). The only informative chromosome 5 marker was therefore λMS8 both alleles of which were retained in the tumour DNA. Patients

Table 3.10. Chromosome 5 allele loss in adenomas

	PROBES										
Patient no.	**MS621**	**π227**	**C11p11**	**ECB27**	**L562**	**EF544**	**YN5.48**	**MC561**	***v-fms***	**λMS8**	**MCC**
Adenomas											
136 (10)	1.2	1.2	1.2	-	-	-	-	-	-	-	
139 (12)	-	-	-	-	-	-	-	-	-	1.2	
145[a](10)	1.2	1.2	-	-	-	-	-	-	-	**(1).2**	-
146 (1)	1.2	1.2	-	**1**	-	**2**	**1**	-	-	-	-
147 (3)	1.2	-	-	-	-	-	-	-	-	1.2	-
57 (3)			-	**(1).2**	-	-	-	-	-	**(1).2**	-
Hamartomas											
JB (10)	1.2	1.2[b]	-	-	-	-	-	-	-	1.2	

Homozygosity in the constitutional DNA is indicated by a dash; where the normal tissue was informative the adenoma genotype is shown in the table. Heterozygosity is indicated by **1.2.** The continued presence of the larger allelic restriction fragment is indicated by '1' whilst '2' denotes the continued presence of the smaller allele. Reduction of intensity is indicated by ( ). Absence of an entry indicates not tested or no result obtained.

[a] Loss of heterozygosity detected in some adenoma samples and results are expanded in table 3.12.

[b] Indicates presence of extra bands in some polyp samples in addition to allelic fragments.

Number in brackets after sample no. indicates the number of adenomas/hamartomas looked at from each patient.

Table 3.11. Investigation of chromosome 5 allele status in APC -derived desmoid tumours and an epidermal cyst

	PROBES									
Patient no.	**MS621**	**π227**	**C11p11**	**ECB27**	**L562**	**EF544**	**YN5.48**	**MC5.61**	**v-fms**	**λMS8**
desmoids										
89	1.2	1.2	-							1.2
95			-							1.2
100	1.2	1.2	-							1.2
JM		1.2	1.2							
cyst										
PS[a]		1.2	-	-	-	-	-			1.2

Nomenclature in above table as used previously.

[a] Patient is hemizygous with the probes most closely linked to the APC locus (EF544; YN5.48) due to a constitutional deletion of the region surrounding the gene. Investigation of allele loss was therefore carried out to detect possible loss of the remaining allele. However no such loss was observed, in this case indicated by a dash.

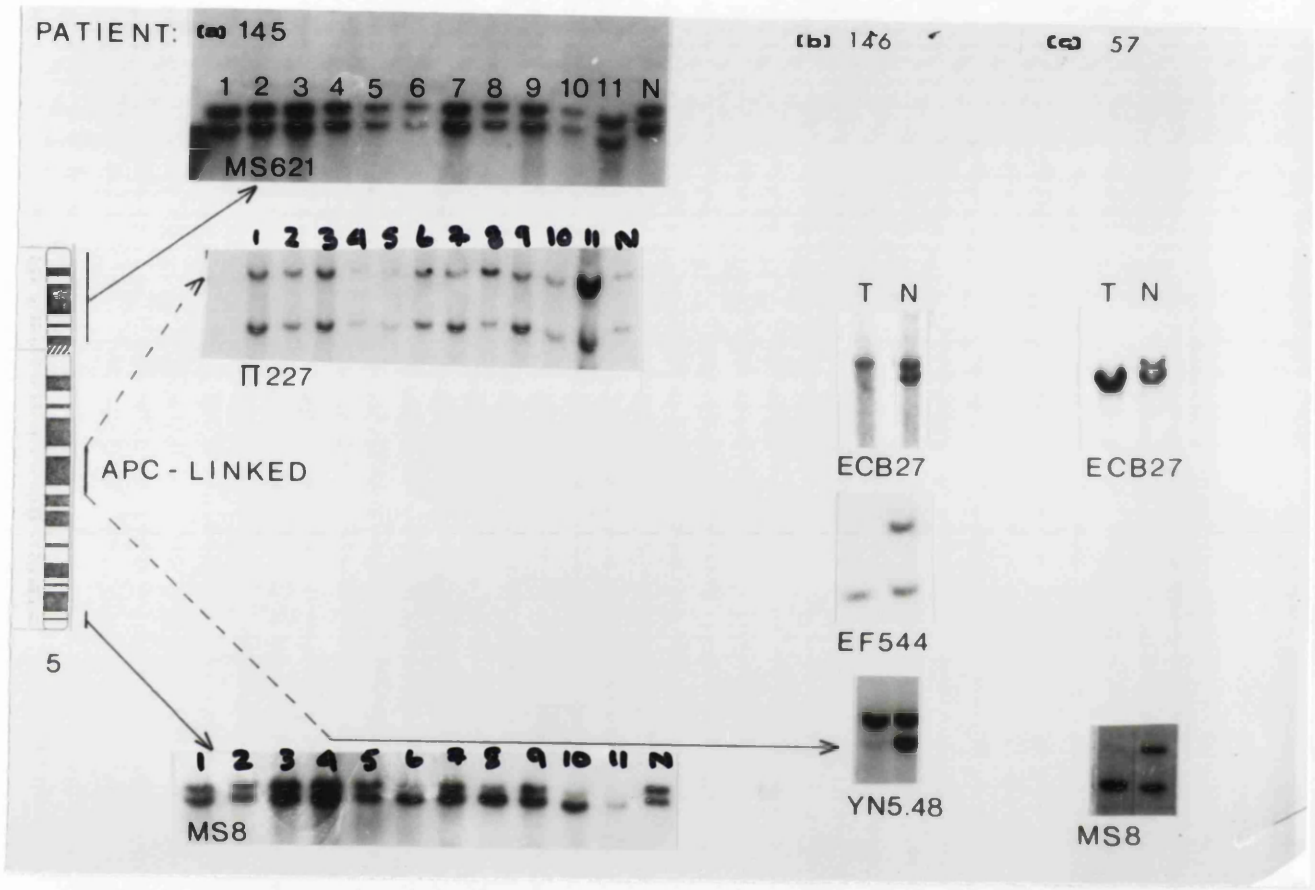


Figure 3.8. Chromosome 5 allele loss in adenomas of three APC patients

(a) Patient 145 - LOH in 4/10 adenomas with λMS8 and retention with π227 and MS621 **(b)** Patient 146 - LOH in 50mm adenoma with ECB27, EF544 and YN5.48 **(c)** Patient 57 LOH in adenoma with λMS8 and ECB27 .

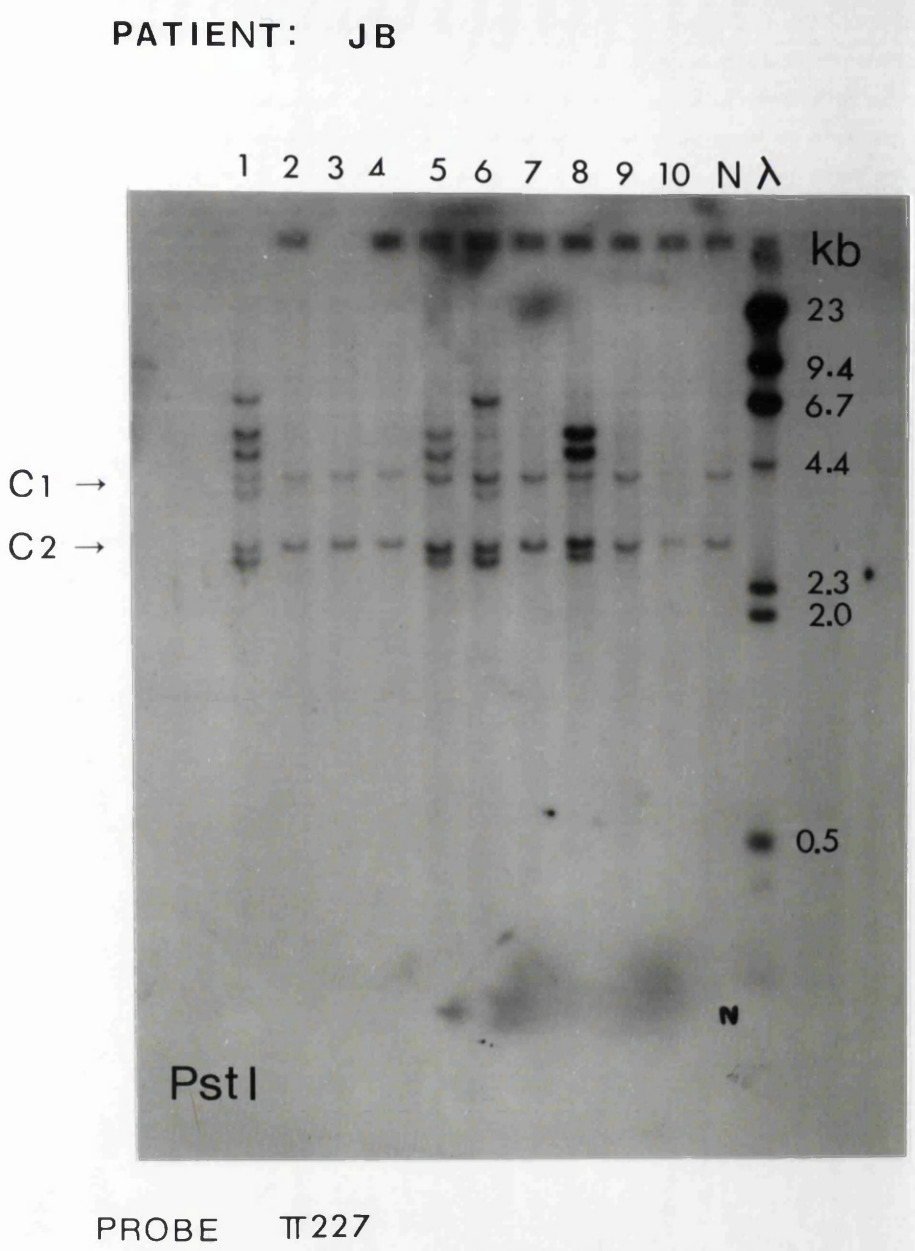


Figure 3.9. Appearance of novel bands in DNA extracted from a series of hamartomas (1 - 10) digested with PstI and probed with the APC-linked marker, π227. The expected polymorphism is indicated by the arrows. DNA extracted from normal colonic mucosa is indicated by 'N'. Sizes of the λ/HindIII molecular weight marker are included for comparison.

89 and 100 were found to retain heterozygosity with the markers MS621, π227 and λMS8. Patient JM proved informative with both π227 and C11p11 and the desmoid retained both alleles in each case. Little can be concluded from these results except to say that a larger sample number is necessary since allele loss has been demonstrated in desmoid tumours but only rarely (Okamoto et al., 1990). It is, however, possible to say that if losses have occurred in these tumours they are probably small, interstitial deletions. Whole chromosome loss can certainly be ruled out as a major contributory factor.

Another extracolonic manifestation of APC or Gardners syndrome is the occasional appearance of epithelial tumours or epidermal cysts presumably also governed by the APC gene since they are another example of cellular hyperproliferation. Such a cyst was obtained from an APC patient with a constitutional heterozygous chromosome.5q deletion - (del(5)(q22-q23.1) (Cross et al., 1991; Griffin et al., 1991). This cyst was also included in the allele loss study. The constitutional deletion in this patient is known to include the markers YN5.48, EF544 and MCC and the cyst was investigated in order to determine if the remaining allele was deleted. As shown in table 3.11, heterozygosity was retained using π227 and λMS8, whilst the remaining constitutional allele was retained with YN5.48 and MCC.

3.2.2. Investigation of Allele Loss on Chromosome 17.

For the purposes of this study, four chromosome 17 markers were used, all of which map to the region 17p13, which is the region showing the highest percentage loss of heterozygosity in colorectal cancers. The probes used were pYNZ22 (D17S5); p144-D6 (D17S34); pMS228 (D17S1) all of which are hypervariable VNTR probes and the polymorphic p53 gene probe pProsp53 (D17S). Details of these markers can be found in appendix 11.2.

Table 3.12. Patient 145 - details of allele loss using the hypervariable minisatellite probe λMS8 (5q35-qter)

Sample no.	Position	Size (mm)	λMS8 Alleotype
Adenomas			
1	transverse colon	7	1.2
2	descending colon	6	1.2
3	transverse colon	6	1.2
4	ascending colon	<5	1.2
6	descending colon	10	(1).2
7	ascending colon	<5	1.2
8	transverse colon	7.5	(1).2
9	transverse colon	5	1.2
10	descending colon	9	(1).2
11	descending colon	15	(1).2
Carcinoma			
5	-		1.2

Retention of constitutional heterozygosity indicated by 1.2; significant reduction in intensity of larger allele indicated by (1).2.

3.2.2.1. Chromosome 17p Allele Loss in Carcinomas.

The results obtained from the 11 APC and 1 sporadic colorectal carcinoma/ normal DNA pairs studied are presented in table 3.13. (APC carcinoma 158 was not investigated with these probes due to insufficient DNA). Again, apart from APC 72 (colorectal cancer line JW2), LOH was seen as a reduction in intensity of one allele probably reflecting the presence of normal stromal cells in the tumour. Unfortunately, in this study the p53 gene probe proved uninformative in all patients tested. Using the other 17p13 markers , 8/12 (66%) of samples showed a definite reduction in intensity with one or more of the markers used. 6/11 informative samples showed allele loss using the MS228 marker and these results are illustrated in figure 3.10. (samples 72, 112, 119, 131, 141, 145).Sample 72 also exhibited loss with the 144-D6 marker whilst the carcinomas from patients 134 and HB exhibited loss with both YNZ22 and 144-D6 but not with MS228 in the case of sample 134 (HB not tested with MS228).

Table 3.13. Chromosome 17p allele loss in colorectal carcinomas

		PROBES		
Patient no.	**YNZ22**	**144.D6**	**MS228**	**pProsp53**
APC				
72	-	**1**	**2**	-
112	1.2	-	**1.(2)**	-
119	1.2	1.2	**1.(2)**	-
130	1.2	1.2	1.2	-
131	-	1.2	**1.(2)**	-
134*	**1.(2)**	**1.(2)**	1.2	-
141			**1.(2)**	-
145	-	1.2	**1.(2)**	-
147	1.2	-	1.2	-
150	1.2	-	1.2	-
161	1.2	.-	1.2	-
sporadic				
HB	**1.(2)**	**1.(2)**		-

Constitutional homozygosity is indicated by a dash. Where the normal tissue was informative the normal genotype is given in the table. Heterozygosity is indicated by **1.2.** The continued presence of the larger polymorphic restriction fragment is indicated by '1' whereas '2' indicates continued presence of the smaller allele. Reduction of intensity is indicated by ( ). Absence of an entry indicated not tested or no result. * Liver metastasis.

3.2.2.2. Chromosome 17 allele loss in adenomas

The adenomas investigated with the chromosome 17p markers are as for the chromosome 5 probes. Again for comparative purposes, the 10 hamartomas from the juvenile polyposis patient JB were used. The results are presented in table 3.14. From the results it can be seen that none of the 37 adenomas investigated showed evidence of allele loss on chromosome 17. The adenomas of patient 145 all retained heterozygosity with all three markers for which this patient was informative even though the carcinoma

from this patient exhibited LOH with MS228 (table 3.13). Adenomas from patients 136, 139 and 147 also showed no evidence of loss. There appears to be a marked difference between the frequency of chromosome allele loss in adenomas and carcinomas pointing to a late role in the tumorigenic mechanism.

Table 3.14. Investigation of chromosome 17p allele loss in APC adenomas and desmoids.

		PROBES		
Patient no.	**YNZ22**	**144-D6**	**MS228**	**pProsp53**
adenomas				
136 (10)	1.2	1.2	1.2	-
139 (12)	1.2	1.2	1.2	-
145 (10)	-	1.2	1.2	-
146 (1)	1.2	1.2	1.2	-
147 (3)	1.2	-	1.2	-
57 (1)	1.2	1.2	1.2	-
desmoids				
89			1.2	
100			1.2	
cyst				
PS	-	1.2		
hamartomas				
JB (10)	1.2	1.2	1.2	1.2

Constitutional homozygosity is indicated by a dash. Where the normal tissue was informative the genotype indicated in the table is that shown by all the benign lesions examined from that patient. LOH is expressed as in the previous tables, reduction in intensity being indicated by ( ). Number in brackets after sample no. = no. of adenomas/hamartomas looked at from each patient.

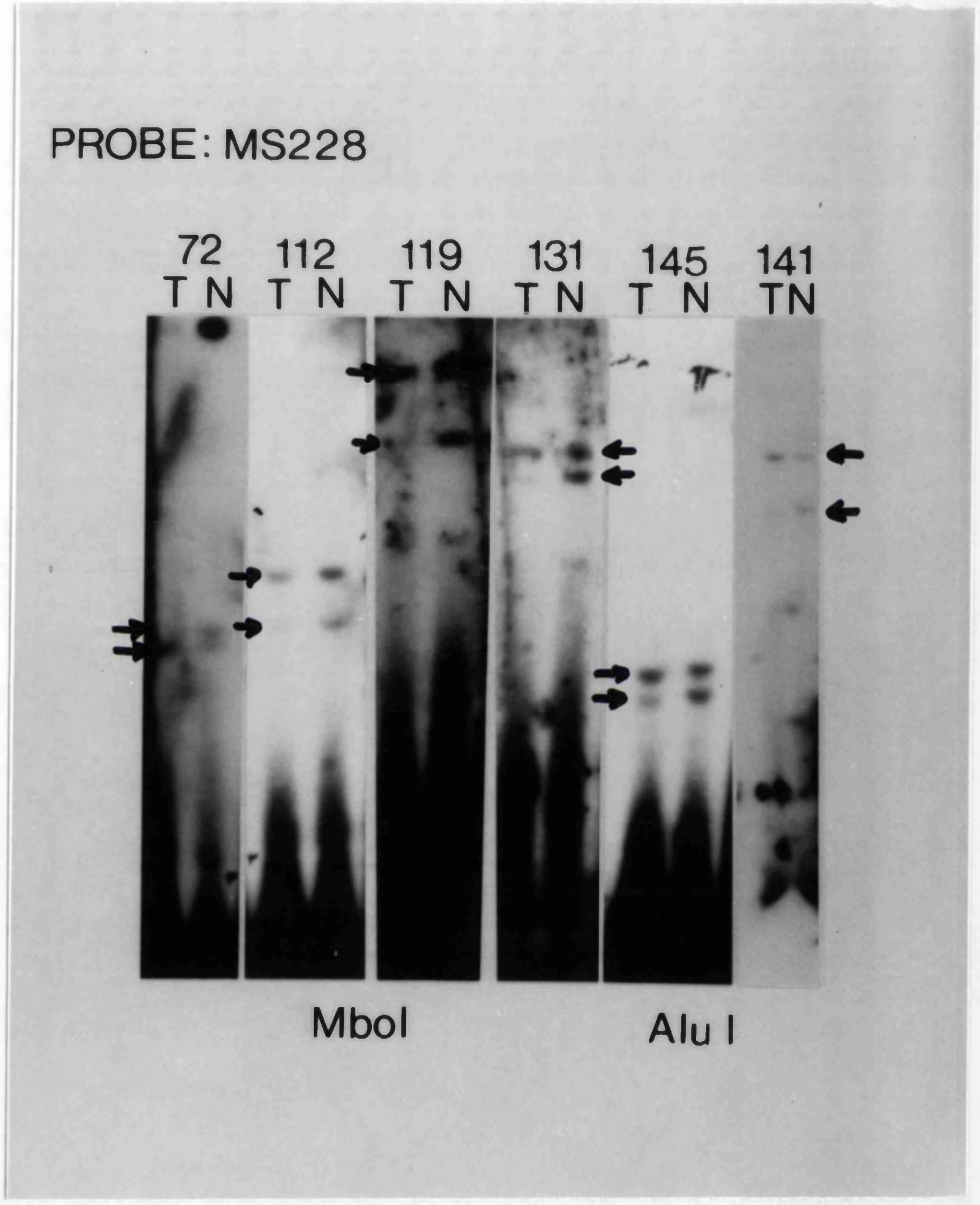


Figure 3.10. Chromosome 17p allele loss in APC colorectal carcinomas using the locus-specific minisatellite probe pMS228 (17p13). N=normal; T=tumour DNA.

3.2.3. Investigation of allele loss on chromosome 18.

Allele loss on chromosome 18 has been widely implicated in colorectal carcinogenesis and the recent isolation and characterisation of the DCC (deleted in colorectal carcinomas) gene at 18q21 has substantiated the previously circumstantial evidence for the existence of a tumour suppressor sequence at this locus.

A total of six polymorphic markers mapping to the long arm of chromosome 18 were used in this study (appendix 11.3). Three of these markers, Sam 1.1, p15-65 and Josh 4.4 specifically hybridise to different regions of the DCC gene and were invaluable in establishing the region of most common loss.

3.2.3.1. Chromosome 18 allele loss in colorectal carcinomas.

The results obtained from the study of 12 APC and 1 sporadic colorectal carcinomas are presented in table 3.15. Ten out of the 12 carcinomas investigated (77%) which were informative for one or more of the markers looked at, exhibited either LOH or a rearrangement of one allele with at least one of the probes used. Again this LOH was, in most cases, manifested as a reduction in intensity due to the presence of contaminating normal tissue. The highest level of loss (80%) was seen by using the DCC probe Sam 1.1 which is specific for a coding region of the gene. The distribution of allele loss within the DCC gene is illustrated graphically in figure 3.12 and a hypothetical orientation of the three DCC markers is suggested based upon the pattern of deletion observed. Of the nine carcinomas showing evidence of allele loss , four (72, 130, 145 and HB) demonstrated loss with more than one marker. In the other four cases, one showed loss only using Sam 1.1 (131) and the other three with MS440 (112); OLVIIE10 (119) and OS-4 (147). In the case of 112 and 147 the lack of loss seen in the region of the DCC gene maybe a consequence of the fact that both samples were constitutively homozygous for 2/3 of the polymorphic DCC markers (Sam 1.1 and Josh 4.4) whilst retaining heterozygosity with p15-65. In contrast sample 119 was informative for all the DCC markers and retained both alleles with all three. The marker OLVIIE10 however is very close to, and was used to isolate, the DCC gene so that the region involved in this tumour may still lie within the DCC sequence but is not detected

by the three DCC probes. Alternatively, it could be that a deletion in an adjoining sequence is affecting the control of the DCC gene.or this marker may lie within the DCC region (Fearon et al., 1990). Some of the losses detected using the chromosome 18 markers are shown in figure 3.11.

Two of the samples investigated showed evidence of gene rearrangements using the Josh 4.4 marker (samples 158 and HB - figure 3.13). Normal DNA from carcinoma 158 was homozygous for the 15kb allele whilst the tumour DNA sample was homozygous but for a novel fragment possibly indicating that one copy of the 15kb allele had been lost whilst the remaining one had undergone a rearrangement leading to the inactivation of the DCC gene. In contrast, the normal DNA of sample HB was heterozygous for the 15kb and the 10 and 5kb alleles whilst the tumour DNA was homozygous for a novel fragment, which happens to be of the same size as that seen with 158.

3.2.3.2. Chromosome 18 allele loss in adenomas.

The results of this study are presented in table 3.16. Again as with the chromosome 17 investigation,none of the adenomas investigated showed evidence of loss with any of the chromosome 18 probes. No losses were seen in the 10 adenomas from patient 136, although only the Josh 4.4 probe was informative in this patient. Neither were there any losses seen in 12 adenomas from patient 139 or in the 10 adenomas from patient 145 although the carcinoma from this patient did show LOH (table 3.15). Loss of heterozygosity in the carcinoma of patient 145 and retention of heterozygosity in the adenomas using the DCC marker Josh4.4 is illustrated in figure 3.14. The three adenomas from patient 147 all retained heterozygosity with the four markers for which this patient was informative, whereas the carcinoma from this patient exhibited LOH with the marker OS-4 (table 3.15).

In addition, no allele loss was seen in 2/11 duodenal adenomas tested or in 2/2 desmoids (89 and 100) informative for MS440.

Table 3.15. Chromosome 18 allele loss in colorectal carcinomas.

	PROBES					
Patient no.	**OLVIIE10**	**Sam 1.1**	**p15-65**	**Josh 4.4**	**OS-4**	**MS440**
APC						
72	**2**	**2**	**2**	-	-	
112	1.2	-	1.2	-	1.2	**(1).2**
119	**(1).2**	1.2	1.2	1.2	1.2	
130		**(1).2**	1.2	1.2	**(1).2**	
131	1.2	**(1).2**	1.2	1.2	-	1.2
134	-	-	-	-	1.2	1.2
141	1.2	-	**(1).2**	-	-	
145	-	-	-	**(1).2**	1.2	
147	1.2	-	1.2	-	**(1).2**	1.2
150	-	-	-	1.2	-	
158		-	1.2	**R**[a]	-	
161	-	-	-	1.2	1.2	
sporadic						
HB	-	-	**(1).2**	**R**[b]	-	

Nomenclature in table as used previously. '1' and '2' are used to represent the larger and smaller restriction fragments respectively even though some probes recognise more than two alleles.

R = rearrangement
[a] Sample constitutionally homozygous, tumour homozygous for different sized fragment.
[b] Normal DNA sample heterozygous, tumour homozygous for novel fragment.

Table 3.16 Chromosome 18 allele status in APC adenomas

	PROBES					
Patient no.	**OLVIIE10**	**Sam 1.1**	**p15-65**	**Josh 4.4**	**OS-4**	**MS440**
adenomas						
136 (10)	-	-	-	1.2	-	-
139 (12)	1.2	1.2	1.2	1.2	-	1.2
145 (10)	-	1.2	-	1.2	1.2	-
146 (1)	1.2	1.2	1.2	1.2	-	1.2
147 (3)	1.2	-	1.2	-	1.2	1.2
57 (1)	-	1.2	-	1.2	-	1.2
desmoids						
89		-		1.2		
100		1.2		1.2		
cyst						
PS		-	-	1.2		

Nomenclature in table as used previously. Number in brackets after sample no. = no. of adenomas looked at from each patient. '1' and '2' are used to represent the larger and smaller allelic restriction fragments respectively even though some probes recognise more than two alleles.

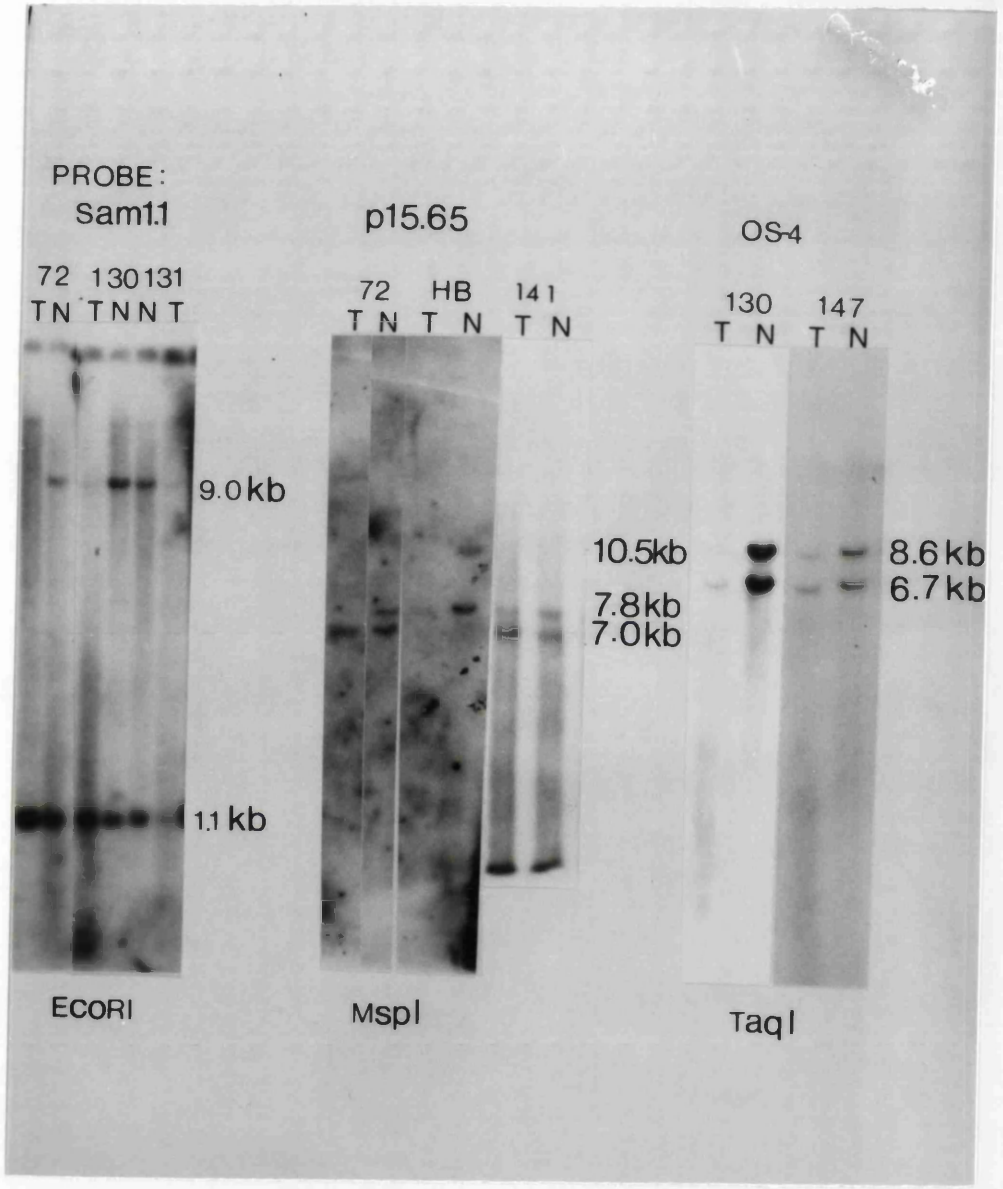


Figure 3.11. Chromosome 18q allele loss in colorectal carcinomas

N=normal; T=tumour DNA samples.

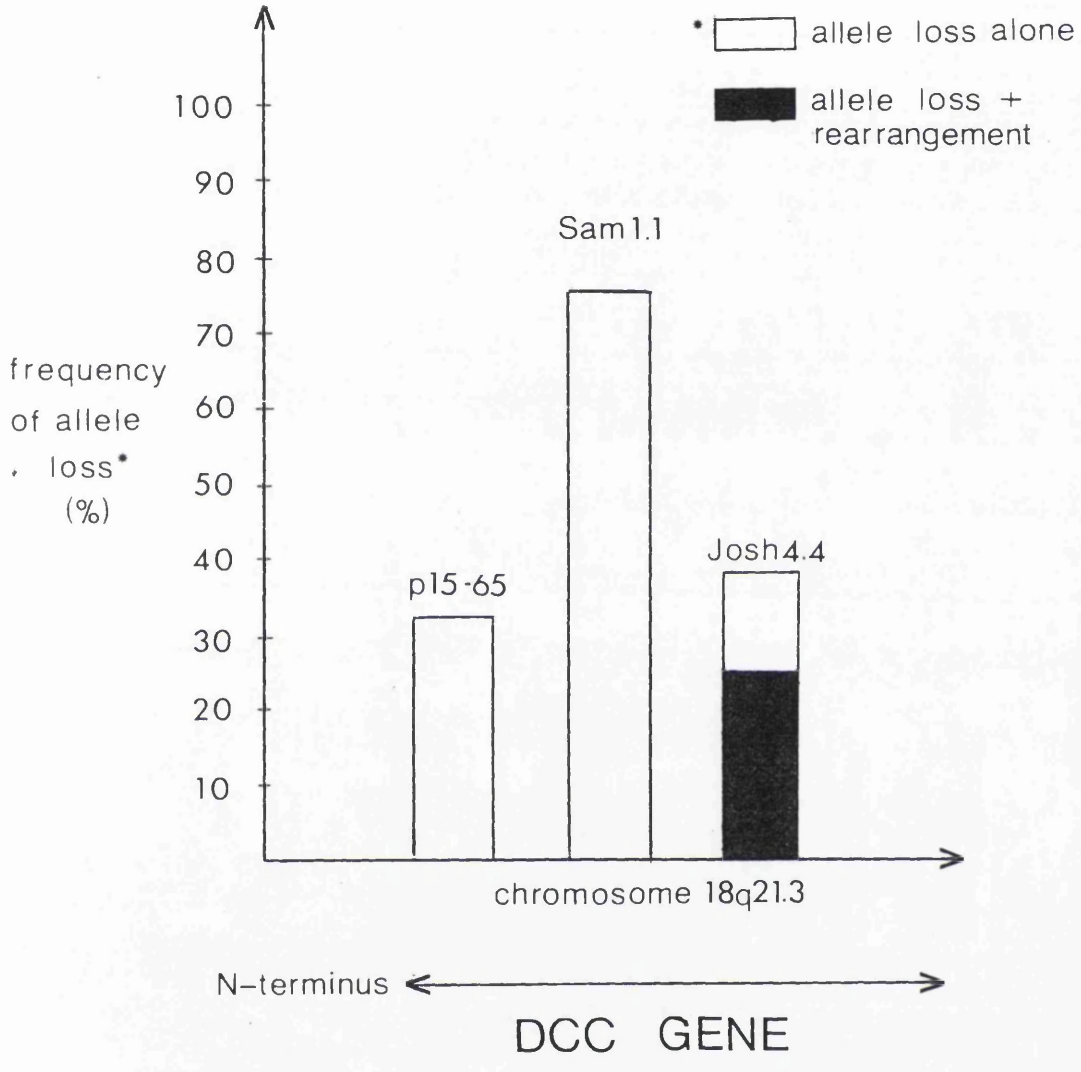


Figure 3.12. Distribution of allelic losses within the DCC locus at 18q21.3 as recognised by the three polymorphic cDNA clones p15-65 (N-terminus); Sam1.1 and Josh4.4 and possible orientation of these markers within the DCC locus as determined by deletion patterns.

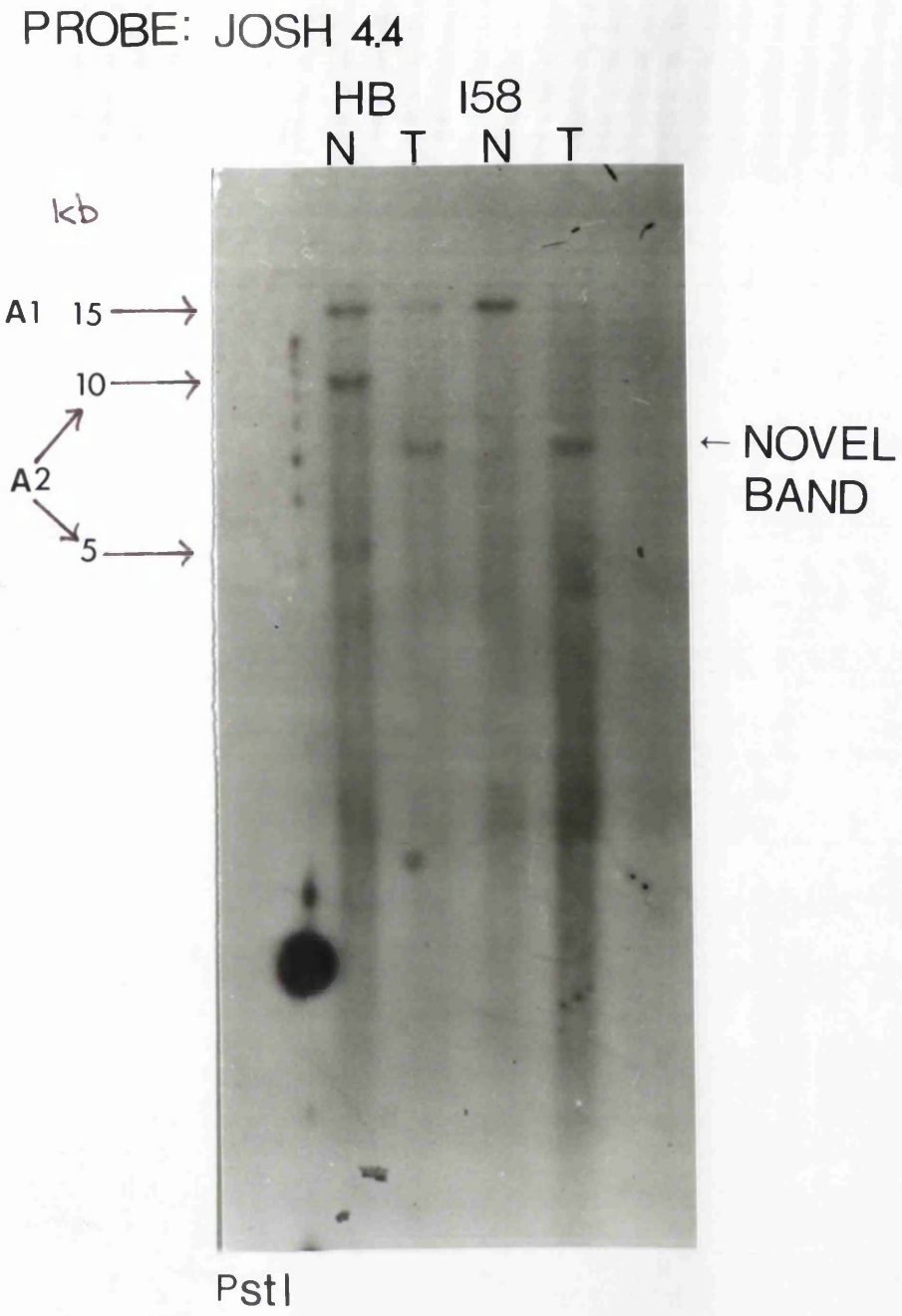


Figure 3.13. Rearrangements within the coding sequence of the DCC gene (18q21.3) as illustrated by PstI digestion of HB and 158 DNA and hybridisation to the cDNA probe Josh4.4. N=normal; T=tumour.

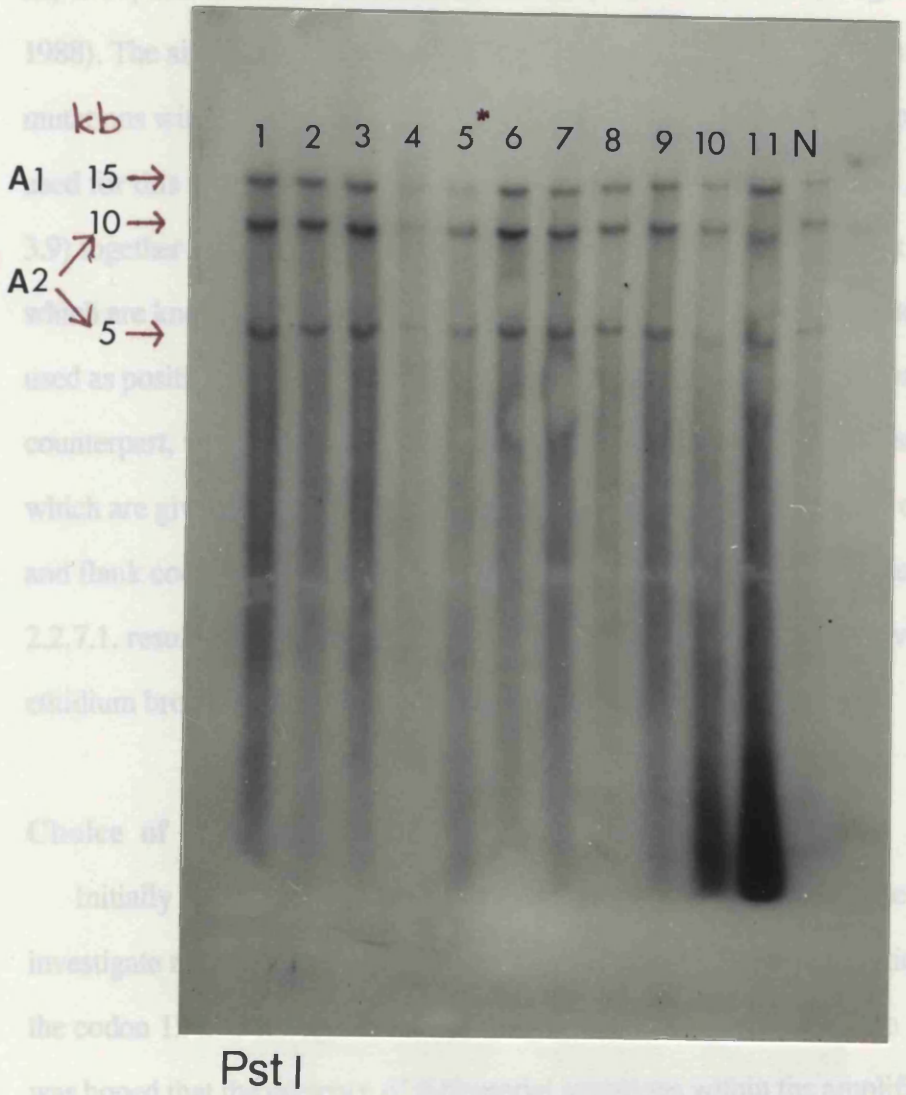


Figure 3.14. Involvement of the DCC gene region in APC 145 carcinoma* (lane 5) as illustrated by the reduction in intensity of the 15kb allele (A1) when compared with the adenoma DNA samples (lanes 1-4 and 6-11) on hybridisation to the cDNA probe Josh4.4.

3.3. Investigation of K-ras mutations.

Point mutations at codon 12 (and occasionally codon 13) of the K-ras gene are known to occur in around 40% of colorectal carcinomas (Bos et al., 1987; Forrester et al., 1987) and to a lesser extent in adenomas (Farr et al., 1988b; Vogelstein et al., 1988). The aim of this study was to attempt to correlate the occurrence of such mutations with the stage of tumour development and its degree of dysplasia. Samples used for this study included all those previously described (tables 3.1, 3.2 and 3.4 - 3.9) together with a panel of nine colorectal cancer cell lines (appendix 12), three of which are known to contain a mutation in codon 12 of the K-ras gene and hence were used as positive controls (KMS-4; SW480; JW2). Each tumour DNA sample, along with its normal DNA counterpart, was initially amplified using the primers K1 and K2 the sequences of which are given in appendix 8. These primers are specific for exon 1 of the K-ras gene and flank codons 12 and 13 (figure 3.15a). Using the program specified in section 2.2.7.1. results in the production of a 111-bp fragment which can be viewed on an ethidium bromide-containing minigel (figure 3.15b).

3.3.1. Choice of system for detection of mutations.

Initially a computer search was carried out using the SITe database program to investigate the distribution of useful restriction endonuclease recognition sites around the codon 12 and 13 region. The results of the search are presented in figure 3.16. It was hoped that the presence of differential mutations within the amplified fragments derived from different samples would lead to the generation or loss of restriction sites thereby facilitating a simple diagnostic restriction enzyme digest. Unfortunately, in most cases such restriction sites were not present and the cases where such an approach would be possible either represented rarely seen mutations, as described by other workers, or otherwise required the use of obscure restriction enzymes in which case the cost was prohibitive.

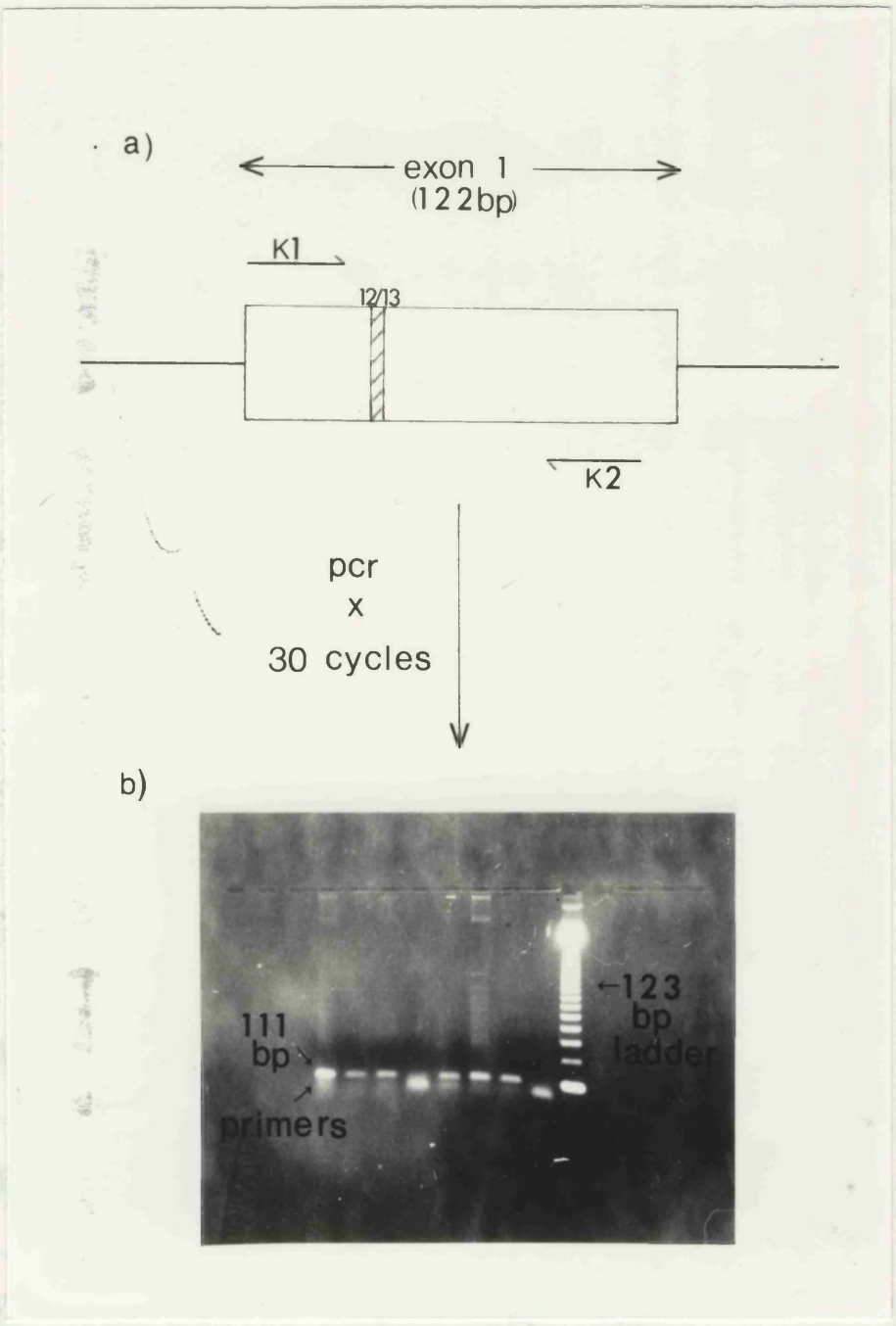


Figure 3.15. **(a)** Relative positions of amplification primers K1 and K2 with respect to exon 1 of the K-ras gene and **(b)** 111 base pair fragment produced as a result of 30 rounds of amplification of 500ng - 1µg of genomic DNA.

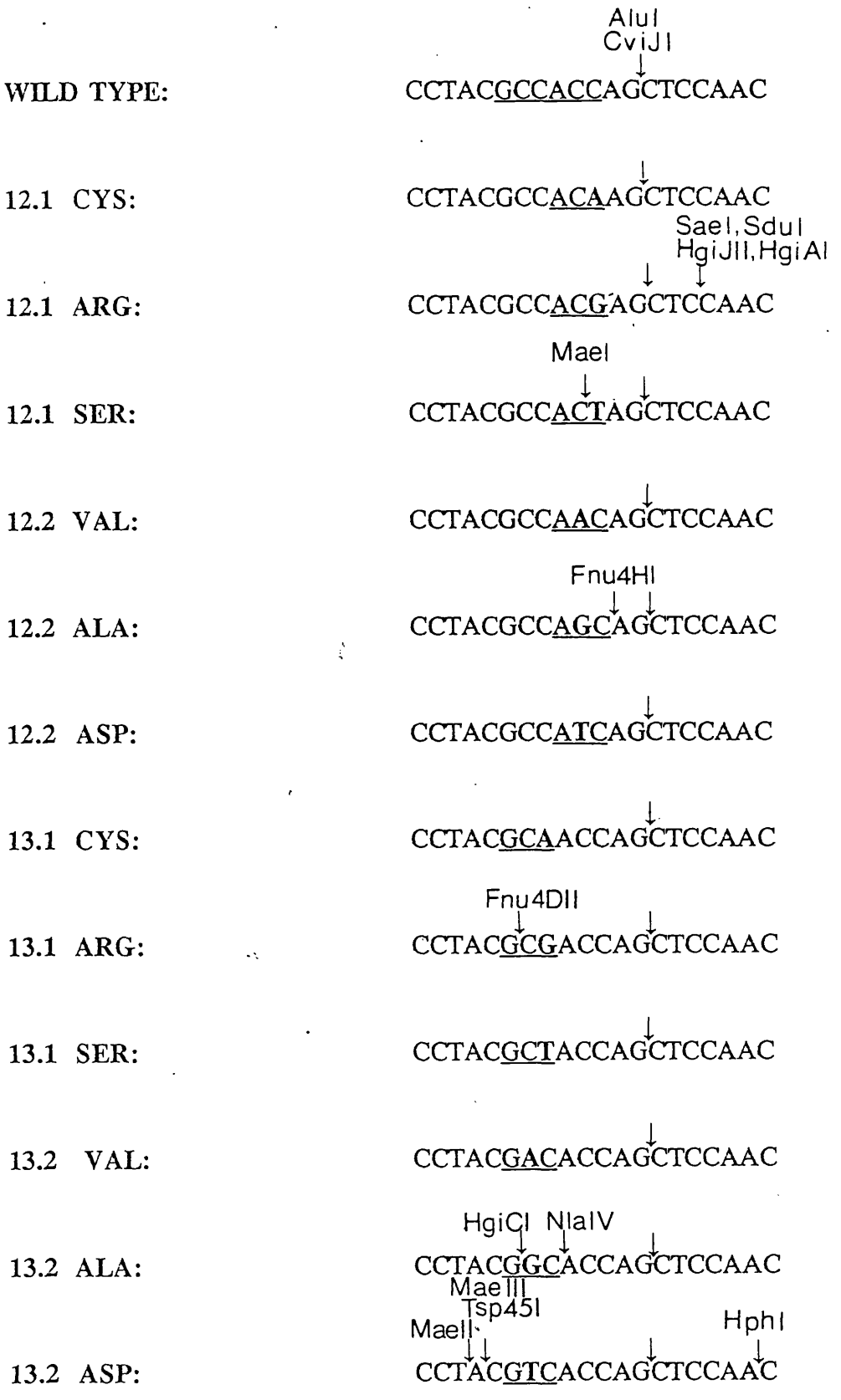


Figure 3.16. Antisense sequences illustrating the RFLP search of the region surrounding codons 12 and 13 of the K-ras gene to investigate possible use of restriction digests as a diagnostic method for detection of point mutations.

The approach adopted, therefore, was that of dot-blot DNA hybridisation of the pcr products to a series of radiolabelled allele-specific oligonucleotide (ASO) probe 'cocktails', the details of which are given in section 2.2.8. and appendix 9.1. Briefly, one-tenth of each pcr product was loaded in duplicate onto a Hybond N+ hybridisation membrane and this membrane was then hybridised in turn to the four antisense probe 'cocktails' representing all possible mutations at codon 12 positions 1 and 2 and codon 13 positions 1 and 2 respectively. The filters were washed under such stringent conditions that only perfectly matched oligonucleotides will hybridise to the immobilised target DNA and on autoradiography, amplified products possessing a mutation at the this position will be detected. Following autoradiography, the membrane was stripped and hybridised to the next mutant 'cocktail'. Finally the filters were hybridised to the labelled primer K2 to assess the loading of the DNA.

Such an approach as described above will only result in the detection of amplified products containing a mutation and will reveal at which base position the mutation occurs. In order to establish the exact base substitution involved all samples proving positive in the ASO assay were verified by direct sequencing of the pcr products. This protocol is described in section 2.2.9.

3.3.2. Investigation of K-ras mutations in a panel of colon cancer cell lines

The results obtained in this study are illustrated in figure 3.17 In the case of these samples, detection of mutations by the ASO technique (figure 3.17a) was followed by a second detection procedure in order to confirm the reliability of the dot-blot assay. This second approach utilised the modified pcr procedure (section 2.2.7.1. and appendix 9.2), which if the test sample does not contain a codon 12 mutation, results in the introduction of an artificial MspI restriction site (C^CCG) into the 100bp amplified product. (figure 3.17b). MspI digestion will therefore result in the cleavage of the pcr product to give two smaller fragments of 79 and 21 base pairs. However, if there is a codon 12 mutation in the sample being amplified, such digestion will not occur resulting in only the intact 100bp product being detectable on electrophoresis. A sample containing one mutated and one wild-type codon 12 results in the presence of the

digested 79bp fragment as well as the undigested 100 bp fragment. In practice the 21bp fragment is too small to visualise by conventional agarose gel electrophoresis (figure 3.17b). Unfortunately, a constituent of the pcr reaction apparently caused inhibition of the MspI digestion and so, even in the samples which did not contain a mutation, a residual 100bp band can be seen representing undigested wild-type DNA. Because of this problem, this method was not deemed suitable to use on the other tumour samples due to the possible production of spurious results. Verification and determination of codon 12 and 13 mutations in these colorectal cancer cell lines by direct sequencing of the pcr product is shown in figure 3.18. As shown in figure 3.17. 4/9 (44%) of the colorectal cancer cell lines contained codon 12 mutations. KMS-4 contained a base substitution at position 1 codon 12, whilst JW2, SW480 and LIM 1899 were mutated at the second base position. No result was obtained using the modified pcr approach for either the KMS-4 or CaCO2 lines. The problem encountered with this method is highlighted in tracks 1,3,5,6 and 8 (figure 3.17b) In these cases a slight residual 100bp band can be detected which would normally signify the presence of a mutation but here merely represents undigested normal product. This can be verified by comparison of lanes 2 and 3 containing JW2 tumour and normal DNA respectively. A residual 100bp band in lane 3 (normal DNA) is indicative of incomplete digestion of the wild-type pcr product as is the case in lanes 1 (COLO320); 5 (HCA-7); 6 (HT29) and 8 (LIM1215). From these results it can be predicted that both JW2 and LIM 1899 are heterozygous for a codon 12 mutation since the 100bp band in these samples is much stronger than in lane 3. SW480 appears to be homozygous for a codon 12 mutation as no 79bp digestion product is visible. These results were verified by direct sequencing of the products of amplification and the exact nature of the mutations seen are presented in table 3.17.

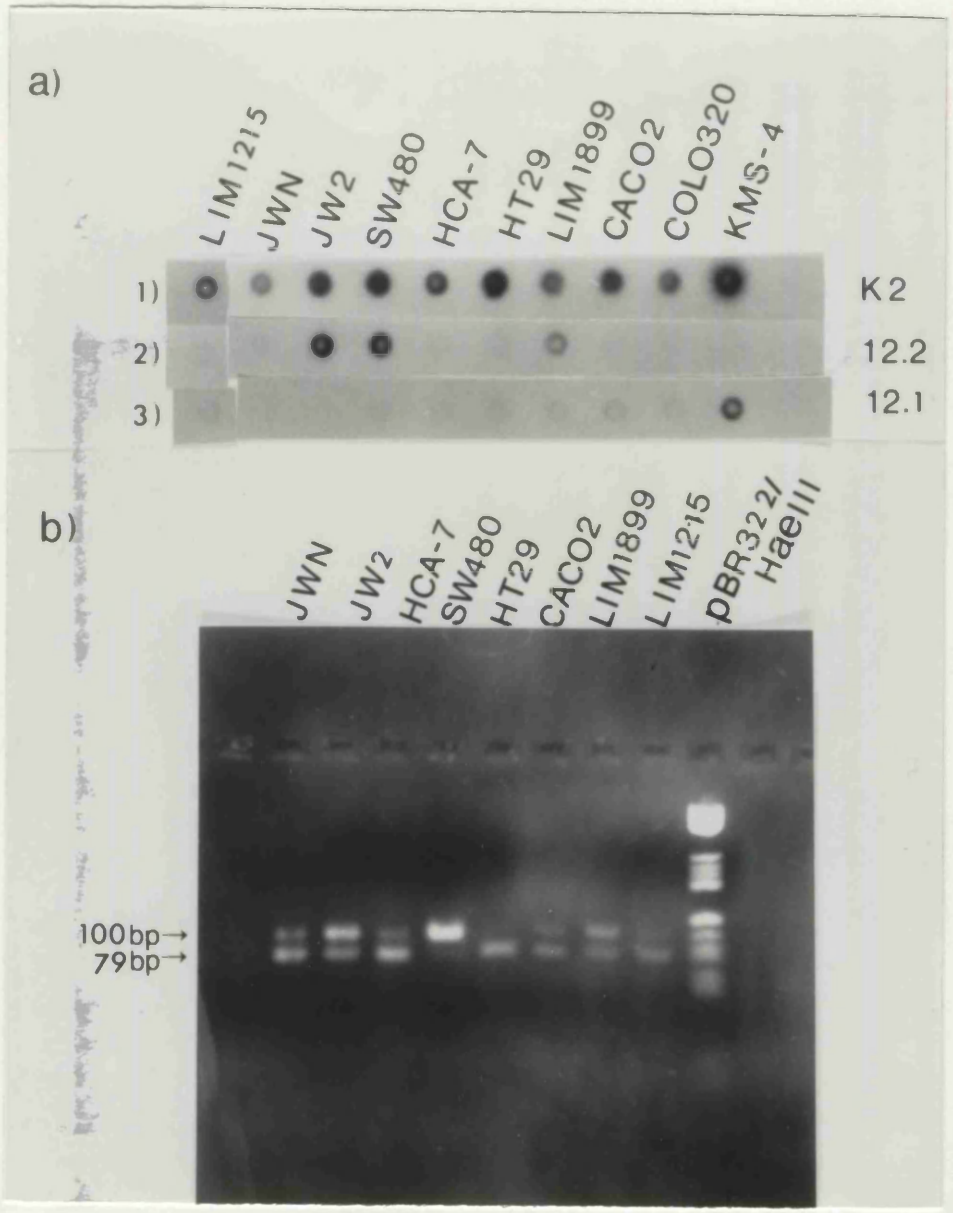


Figure 3.17 (a). Allele specific oligonucleotide hybridisation of colorectal cancer cell line DNA to :- (1) K2 amplification primer to assess the loading of the sample (2) mutant oligonucleotide probe cocktail 12.2 (3) mutant oligonucleotide probe cocktail 12.1 **(b)** Modified pcr using primer K1a and restriction endonuclease cleavage of the amplification product with MspI. JWN = normal DNA counterpart of cell line JW2.

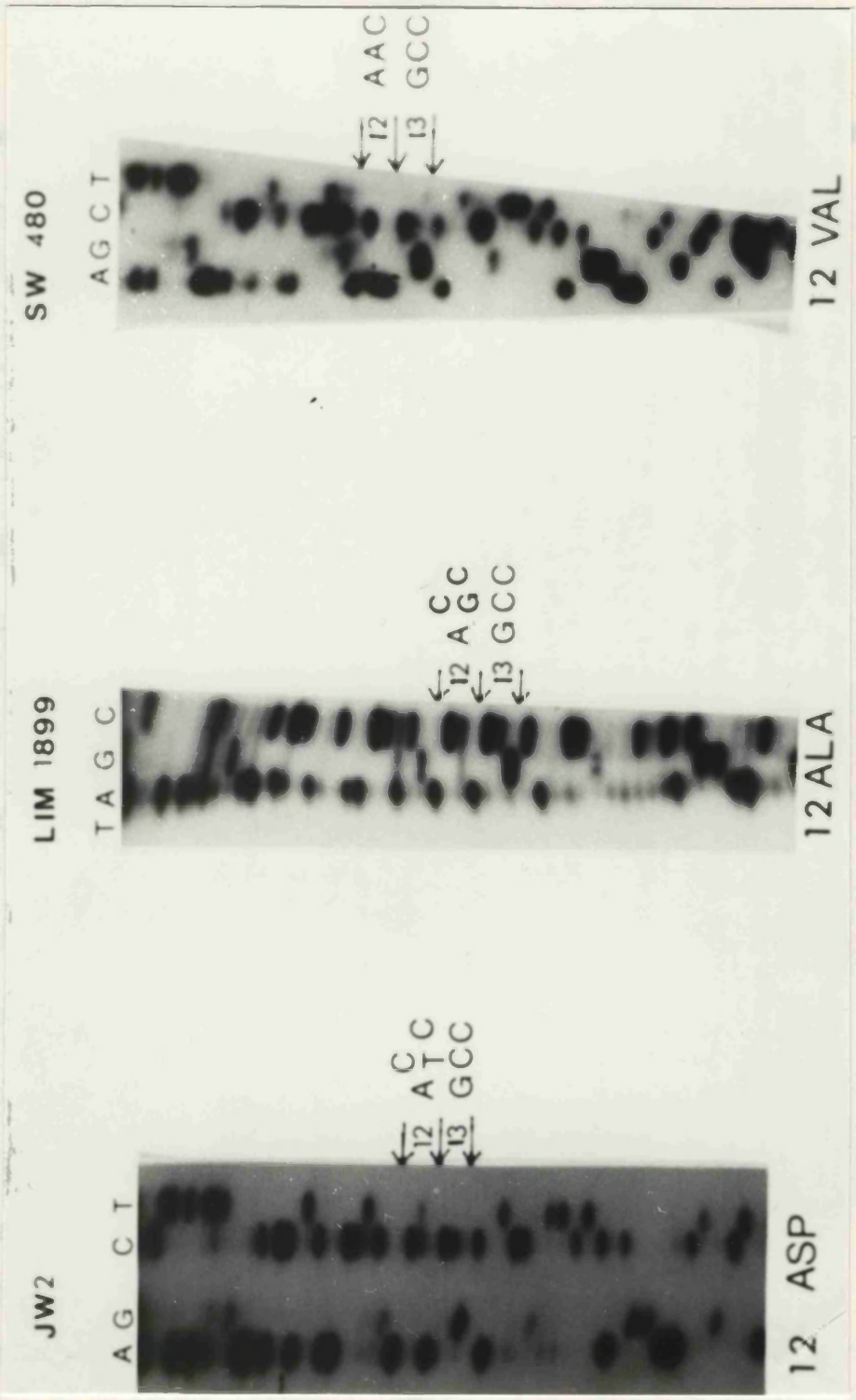


Figure 3.18. Sequence analysis of K-ras mutations in colorectal cancer cell lines (sequences shown are antisense).

Table 3.17. K-ras mutations in colorectal cancer cell lines.

Cell line	Description	Mutation	Codon	Base subst.	A.A. subst.	Hom/Het
SW480	Sporadic, Dukes B	+	12.2	G --> T	Gly --> Val	Hom
JW2	APC - Dukes' C	+	12.2	G --> A	Gly --> Asp	Het
KMS-4	APC - lymph node metastasis	+	12.1	G --> T	Gly --> Cys	Het
LIM1899	Sporadic, Dukes C	+	12.2	G --> C	Gly --> Ala	Het
LIM1215	HNPCC	-				
HCA-7	Sporadic Dukes B	-				
HT29	Sporadic	-				
COLO320	Sporadic C2	-				
CaCO2	Sporadic	-				

3.3.3. Investigation of K-ras mutations in sporadic and familial colorectal carcinomas

In this study 16 sporadic colorectal and 12 APC carcinomas were available for investigation. One of the APC carcinomas (72) is equivalent to the colorectal cancer cell line JW2. The results are presented in figure 3.19..8/16 sporadic cancers proved positive in the dot-blot assay (50%) compared with 5/12 of the APC carcinomas (41.7%). Where possible the results were verified by sequencing and the results are shown in table 3.18 and in figures 3.20 and 3.21. The majority of these mutations

occurred at position 2 of codon 12 and most of these represented glycine to aspartic acid transitions. Sample 147A was, on sequencing found to contain two mutations, one

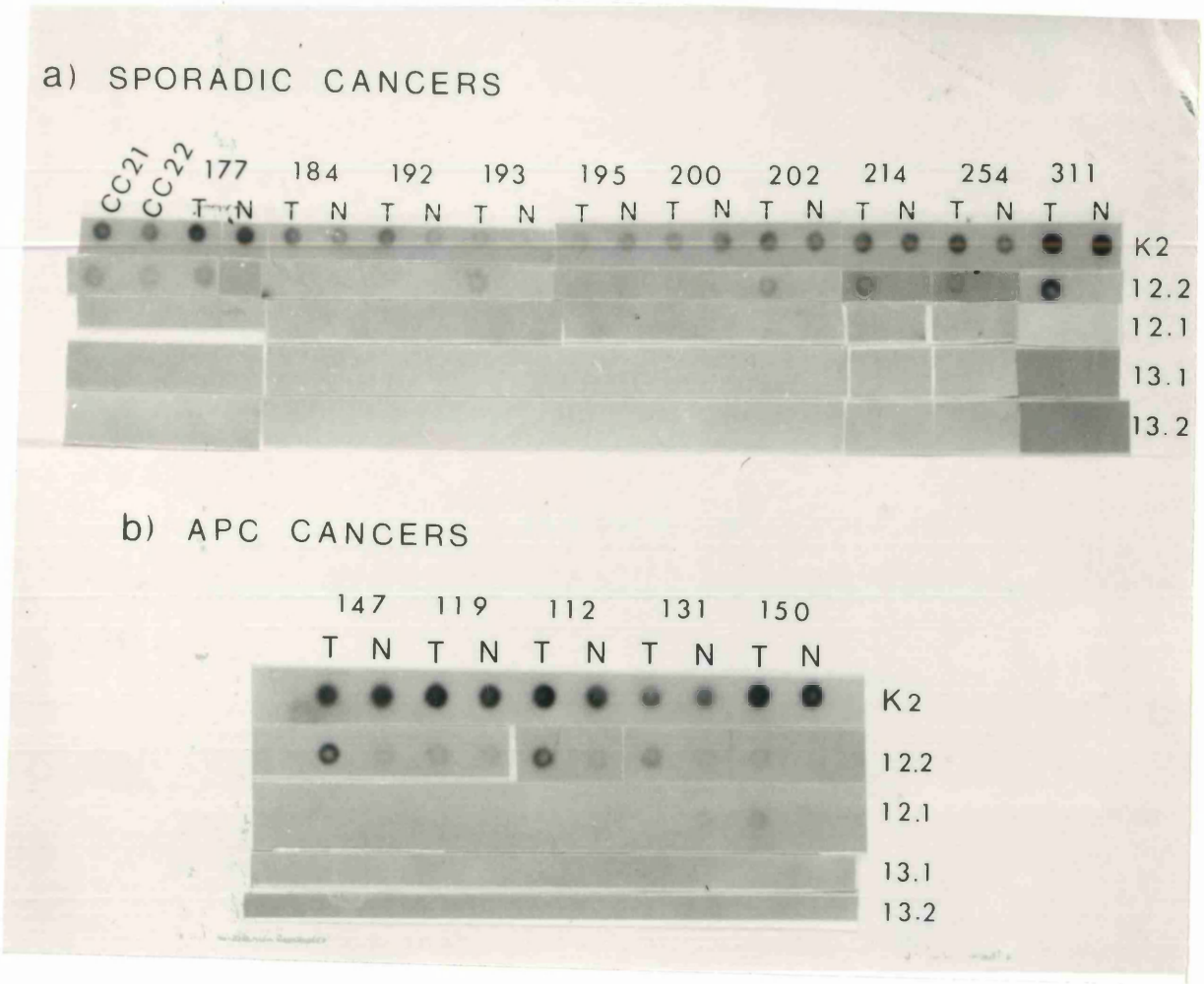


Figure 3.19. Allele specific oligonucleotide analysis of K-ras exon 1 amplification product in **(a)** sporadic and **(b)** familial carcinomas hybridised with end-labelled K2 amplification primer and mutant cocktails 12.2; 12.1; 13.1 and 13.2.

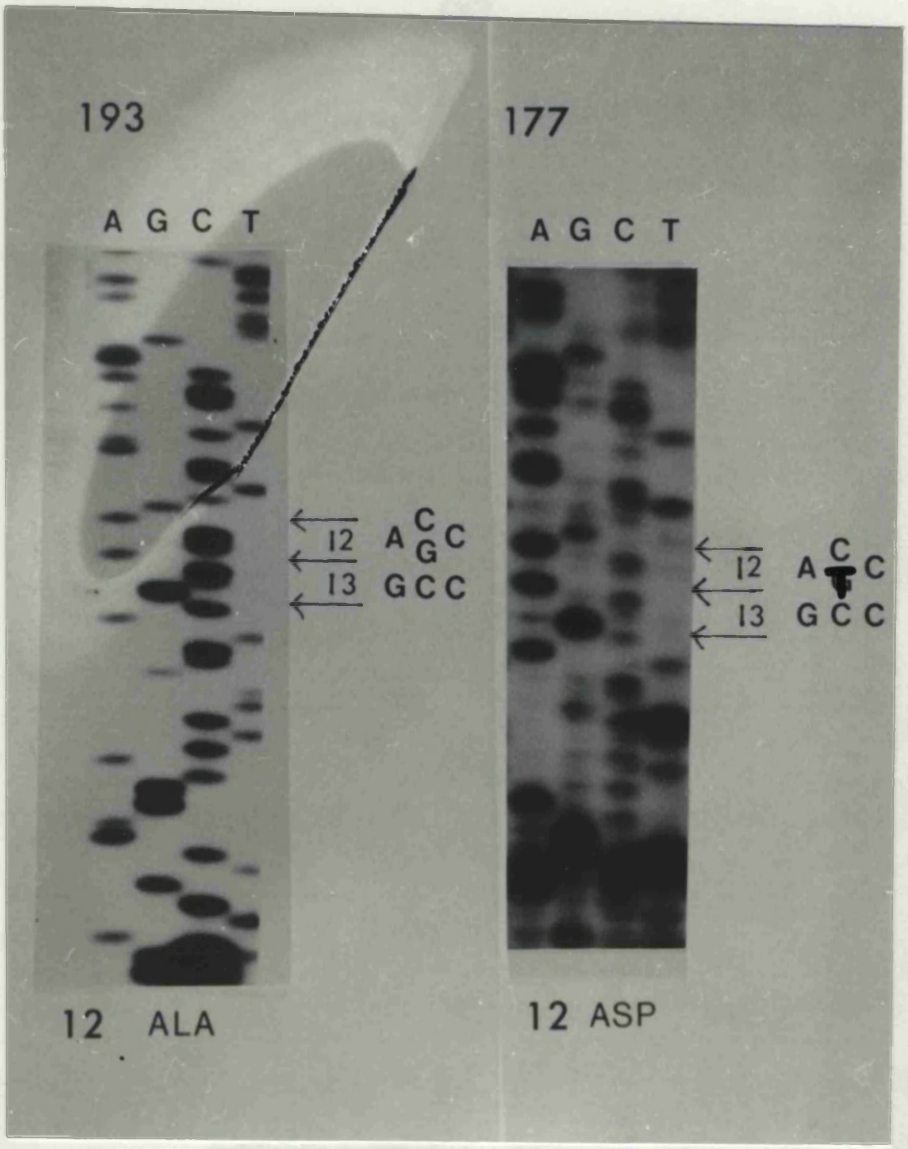


Figure 3.20. Sequence analysis of K-ras exon 1 amplification product in two sporadic colorectal carcinomas illustrating two different point mutations.

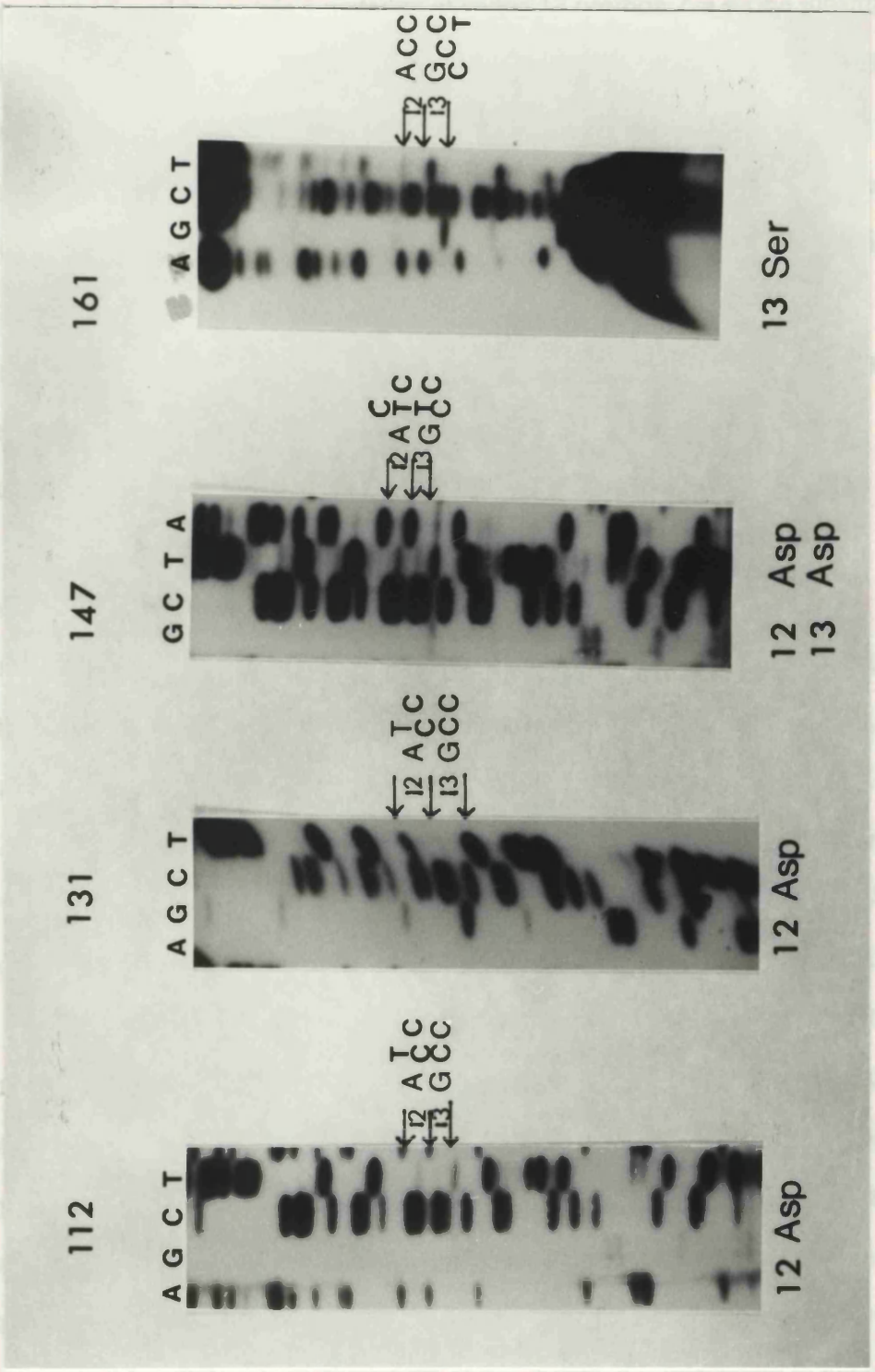


Figure 3.21. Sequence analysis of K-ras exon 1 amplification product in APC carcinomas

each at codons 12 and 13 respectively, both of which were glycine to aspartic acid base changes. Sample 161 was not screened in the original ASO analysis but was sequenced and found to contain a mutation at codon 13 position 2, a serine substitution. Only the colorectal cancer cell line SW480 was homozygous for its mutation which is in agreement with the published karyotypic data that it only has one copy of chromosome 12 and that the remaining K-ras gene is mutated (reference). The other positive cancer cell lines (JW2, LIM1899 and KMS-4) were heterozygous for their respective mutations. The remaining carcinoma samples sequenced all appeared heterozygous for their respective mutations. In most cases the mutated band appeared weaker than that of the normal allele probably reflecting the population of normal stromal cells which infiltrate the tumour.

3.3.4. Investigation of K-*ras* mutations in familial adenomas.

In this study, 82 APC colorectal adenomas (tables 3.2, 3.5-3.7) were investigated together with 11 duodenal APC adenomas (table 3.8). For comparative purposes, 11 hamartomas from a juvenile polyposis patient were also used. A total of 5/82 (6%) of the APC colorectal adenomas proved positive for point mutations of the K-ras gene and were verified by direct sequencing. None of the duodenal adenomas or the juvenile hamartomas contained mutations. The results are presented in figure 3.22. and table 3.19. From table 3.19., it can be seen that in the case of the adenomas, as with the carcinomas, the majority (all in this case) of mutations occur at position 2 of codon 12. 3/4 of the pcr products sequenced revealed the mutation to consist of a G -> A base change resulting in a glycine to aspartic acid substitution. From the sequencing data (figure 3.23.), it can be seen from the faintness of the band that this mutation has perhaps occurred in a small clone of cells and may have conferred a selective growth advantage on this cellular clone. In support of this theory, it is interesting to note that in the case of patient 139 only 1/12 adenomas investigated showed a mutation and that this was the only adenoma which was able to be cultured. In the case of patient 145, 1/10 adenomas was positive in the K-ras assay. This was a 9mm mildly dysplastic adenoma.whereas a 15mm moderately dysplastic adenoma and a carcinoma from this

patient were both normal. The other large series of 10 adenomas (patient 136) were all negative for K-ras mutations as were 4 adenomas from patient 147. In contrast to the situation with patient 145, a carcinoma from this patient (147A) was found to contain two mutations, both of which were aspartic acid substitutions at codons 12 and 13 respectively. Superficially, there appears to be no immediate correlation between the frequency of K-ras mutation and the histopathological status of the tumour.

Table 3.18. K-ras mutations in colorectal carcinomas

Sample	Codon	Base subst.	A.A. subst.	Hom/Het
sporadics				
177	12.2	G --> A	Gly --> Asp	Het
193	12.2	G --> C	Gly --> Ala	Het
202	12.2	G --> A	Gly --> Asp	Het
214	12.2			
254	12.2			
311	12.2	NOT	SEQUENCED	
CC21	12.2			
CC22	12.2			
APC				
112	12.2	G --> A	Gly --> Asp	Het
147	12.2	G --> A	Gly --> Asp	Het
	13.2	G --> A	Gly --> Asp	Het
131	12.2	G --> A	Gly --> Asp	Het
161	13.1	G --> A	Gly --> Ser	Het

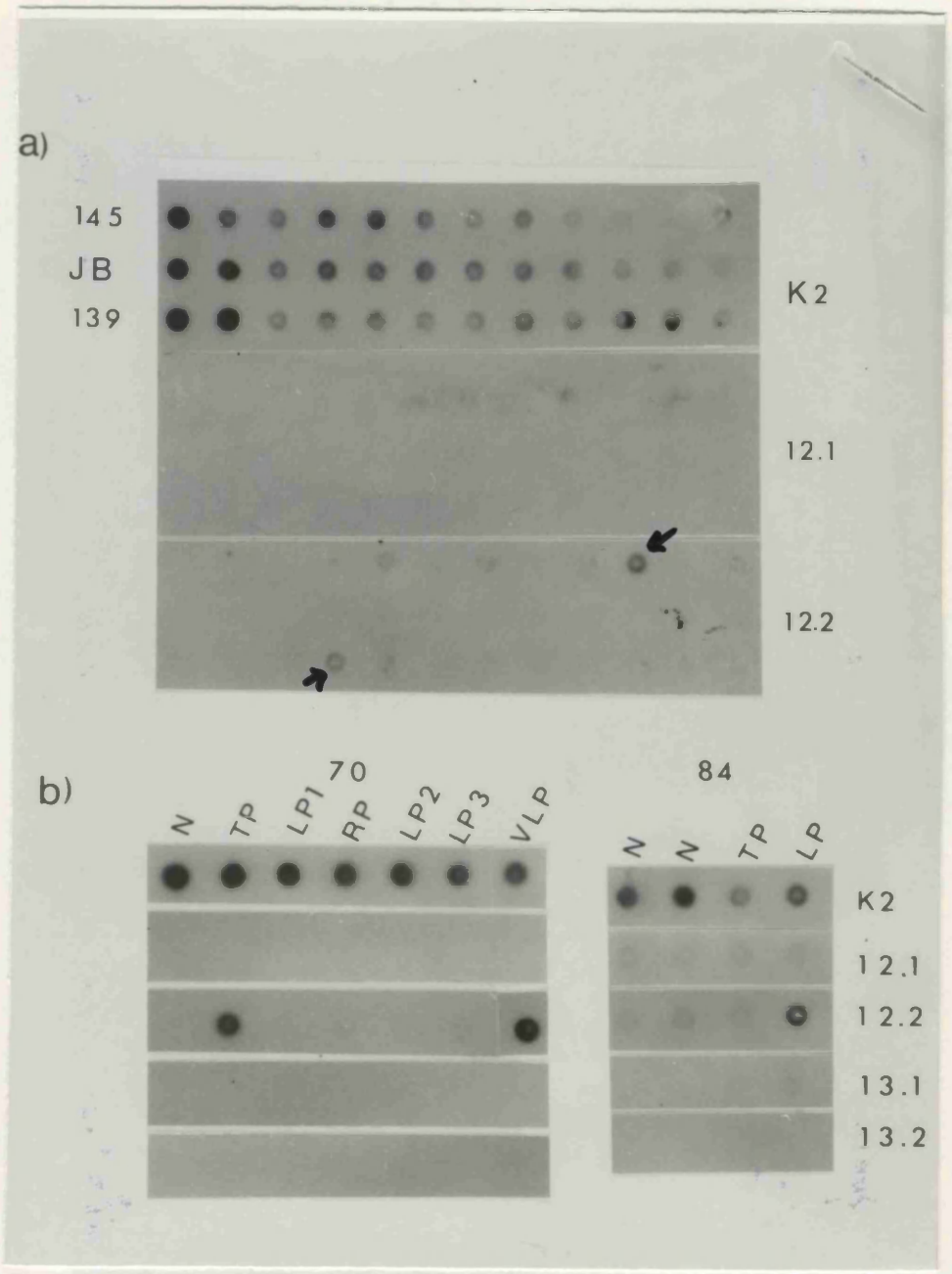


Figure 3.22. K-ras exon 1 ASO analysis of APC adenomas and juvenile hamartomas **(a)** samples from patient 145 (L to R samples 1-11 and normal DNA); JB (hamartoma samples 1-11 + normal); 139 (adenoma samples 1 to 12) **(b)** adenoma and normal DNA samples from APC patients 70 and 84. TP=transverse polyp; RP=right polyp; LP=left polyp; VLP=villous left polyp; N=normal.

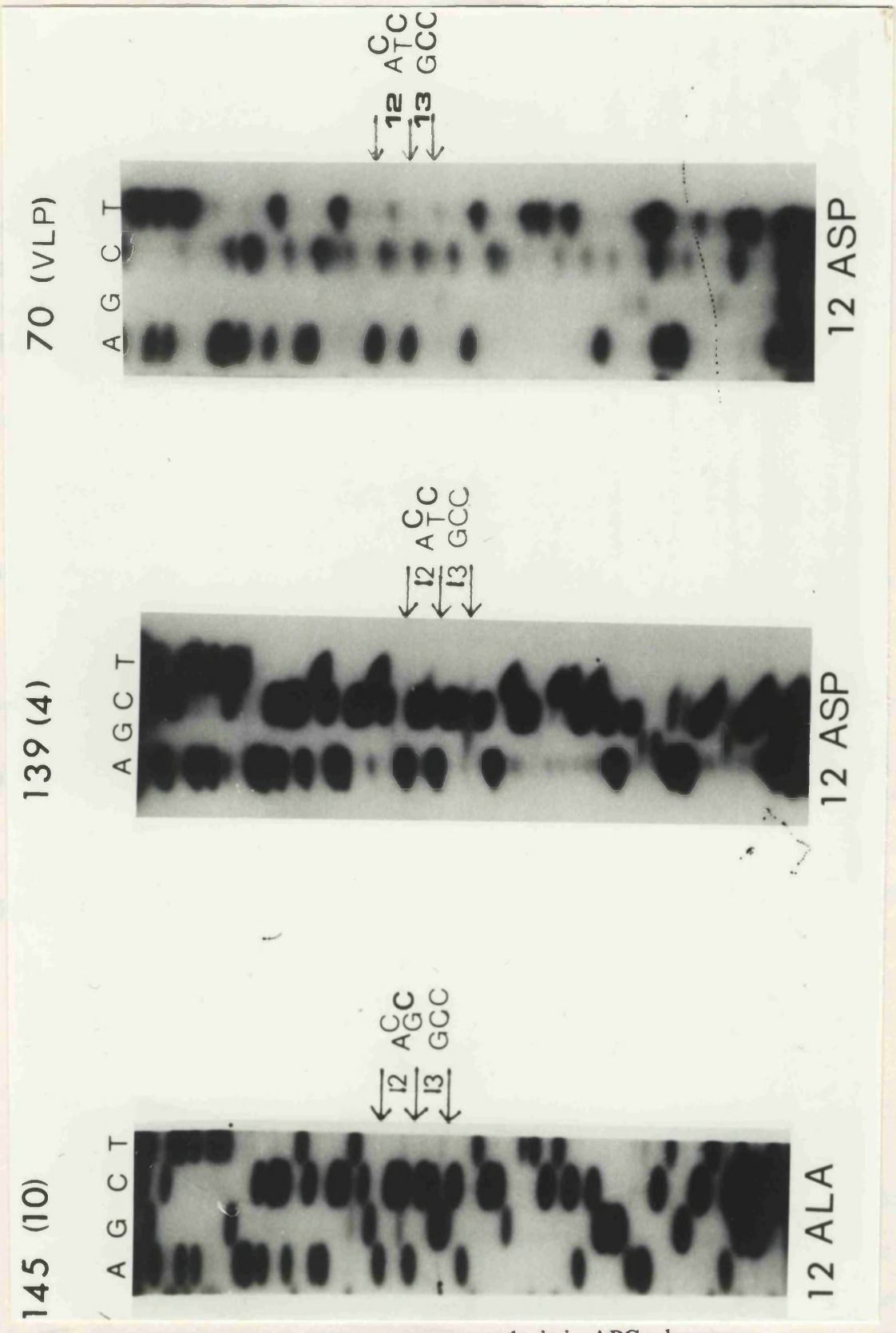


Figure 3.23. K-ras exon 1 - sequence analysis in APC adenomas

Table 3.19. Analysis of K-ras mutations in APC adenomas

Patient (no.of adenomas investigated)	Adenoma sample	Size (mm)	Type	Degree of dysplasia	Codon	Base subst.	A.A. subst
70 (6)	Villous left polyp	7	sessile, villous	mild -> mod. + severe	12.2	G -> A	Gly -> Asp
70 (6)	Transverse polyp	?	?	unknown	12.2	G -> A	Gly -> Asp
84 (3)	left polyp	5	tubular	mild	12.2	Not	sequenced
139 (12)	4 (table 3.5)	10	tubular	mild -> mod.	12.2	G -> C	Gly -> Ala
145 (10)	10 (table 3.7)	9	tubular	mild	12.2	G -> A	Gly -> Asp

3.4 Prevalence of New Mutations in APC

It is often stated that the frequency of new mutations in APC is very high (Rustin et al., 1990). However, the possibility of non-paternity being a reason for there being no prior family history has apparently not been investigated.Three of the APC patients already described (145, 146 and 57) have no prior family history of APC and are therefore presumed to represent "new mutations" i.e. germline mutations which have arisen spontaneously. Blood was obtained from the parents of patients 145 and 146 together with blood samples from three other proposed "new mutations" and their parents making five "pedigrees" in all for which normal lymphocyte or normal mucosal DNA from both parents and patient was available. Unfortunately no blood samples were obtained from the parents of patient 57 for use in this study. Prior to investigation of the nature of the inherited mutations, it was necessary to establish the paternity in each case in order to be certain that these cases represented true new mutations and were not merely cases of non-paternity.

3.4.1. Confirmation of Paternity.

Paternity was established by restriction enzyme digest of the genomic DNA samples with HinfI, AluI or MboI and subsequent Southern hybridisation to a series of highly polymorphic minisatellite DNA probes (Appendix 11.1). Three probes were generally used for each pedigree and allelic inheritance in all three cases was taken as good evidence for correct paternity and therefore as verification of true "new mutation" status. The pedigrees and results are shown in figures 3.24 to 3.27. As is evident in figures 3.24 and 3.25 complete agreement with respect to expected inheritance of alleles is seen in families 535 (patient 145) and 540 (patient 146) using the three minisatellite probes MS1; MS8 and pλg3. The paternal DNA obtained in family 409 was very dilute and consequently the paternal alleles are extremely faint in the case of the MS1 probe and cannot be seen at all in the MS8 autoradiograph. There is good agreement, however, between inheritance of the alleles seen with both MS1 and pλg3 and it seems likely therefore that patient 409 II 1 also represents a true "new mutation". In the case

of family 551 only the results with two probes (MS1 and MS621) are illustrated but correct paternity was also verified using the probe MS440. In the case of family 417, only the result obtained with the MS1 probe is shown but the probes MS440 and MS621 were also used and correct paternal inheritance was verified in all cases. As further verification of true new mutation status all parents of proposita (except those of patient 146 - family 540) were examined by sigmoidoscopy and no evidence of polyposis was found. In addition, family 535 were also examined for the presence of CHRPEs. Both parents were negative whilst the patient 145 (540 III 2) was found to have 3 in one eye and 1 in another. This in itself is not significant but together with the other data suggests that patient 145 has undergone a new mutation at the APC locus.

In the case of patients 145, 146 and 57, all of which showed evidence for LOH at chromosome 5 loci in some of their adenoma DNA, all three presented at an early age with severe manifestations of the disease. Patient 145 presented at age 23 with ~2500 adenomas and two cancers; patient 146 had several large adenomas and a cancer with lymphatic metastases at age 29 indicating a very poor prognosis; patient 57 underwent a colectomy at 15 years of age and patient 417 II 1 presented at 23 years with a Duke's B carcinoma. It is possible that these "new mutation" cases exhibit a more virulent form of APC and are indicative of allelic heterogeneity at the APC locus.

It seems from the results obtained therefore that all five patients assessed seem to represent true "new mutations". However, the possibility of non-paternity always remains and should be investigated in all putative cases before any estimation of the mutation frequency can be made. In contrast to the observation of a high mutation rate the number of patients presenting with the disease remains more or less constant. It is possible to explain this apparent paradox if the severity of the disease and the early age of onset precludes reproduction in these patients

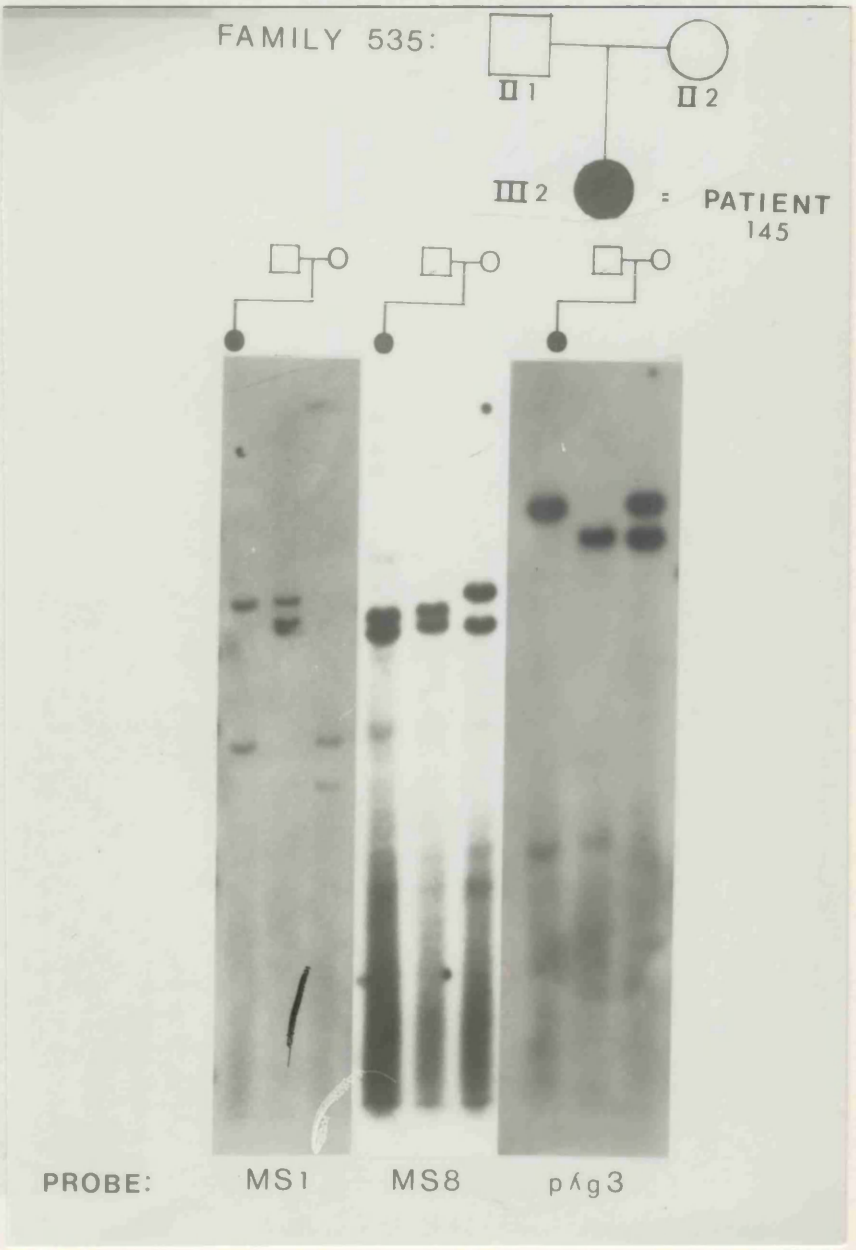


Figure 3.24. Establishment of paternity in "new" mutation patient 145 (535 III 2) using a panel of highly polymorphic minisatellite probes.

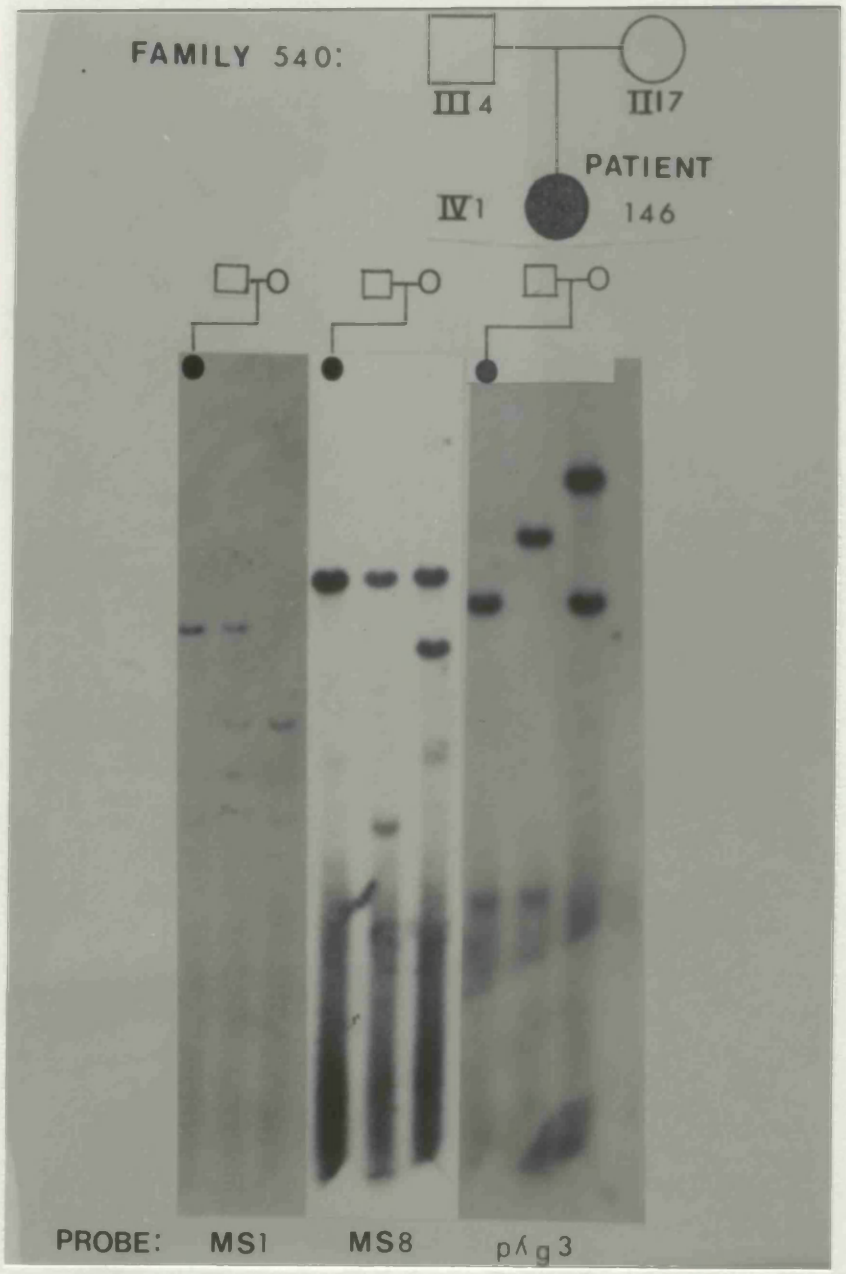


Figure 3.25. Establishment of correct paternity in "new" mutation patient 146 (540 IV 1) using a panel of highly polymorphic minisatellite probes.

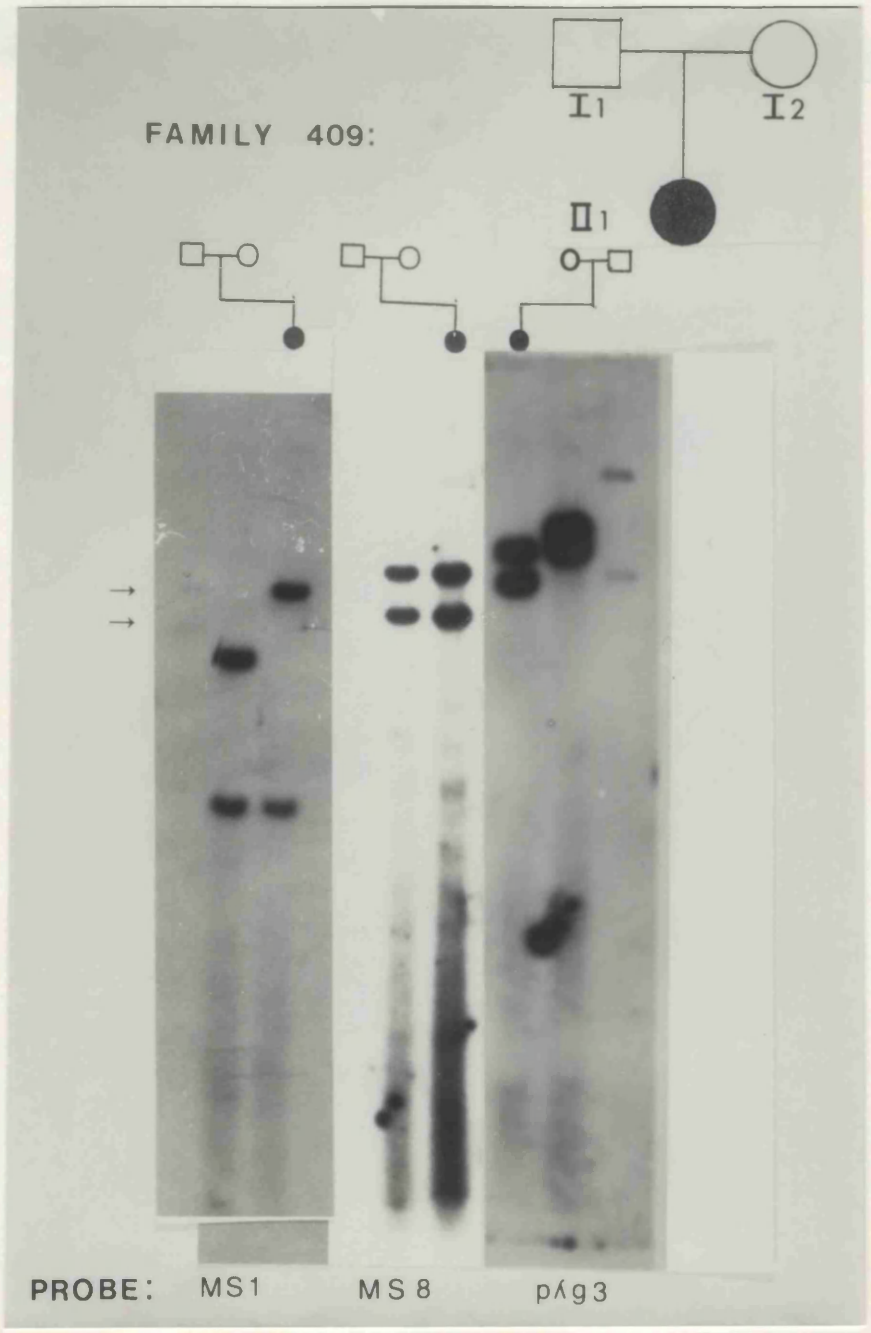


Figure 3.26. Establishment of correct paternity in "new" mutation patient 409 II 1 using a panel of highly polymorphic minisatellite probes.

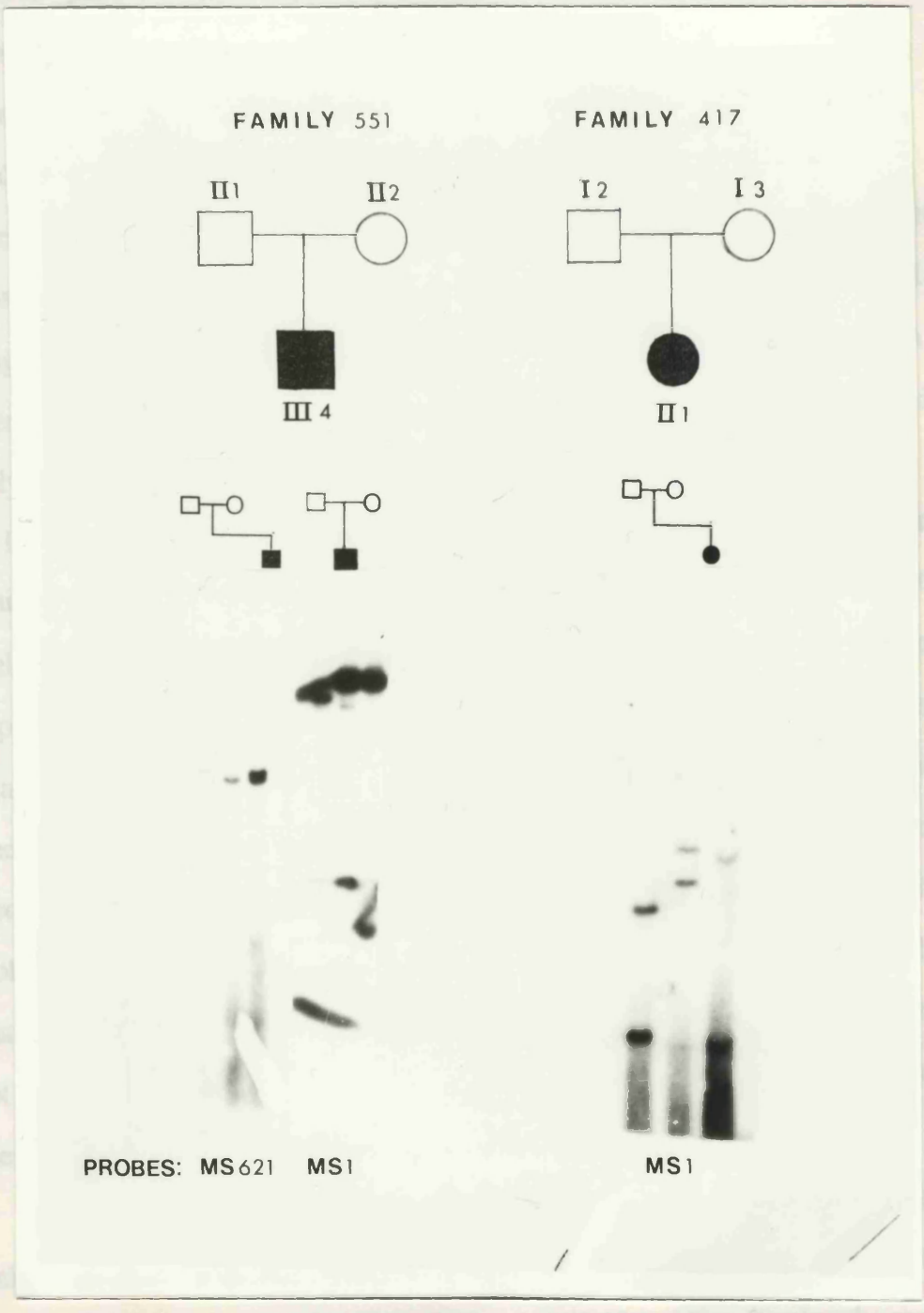


Figure 3.27. Establishment of correct paternity in "new" mutation patients 551 III 1 and 417 II 1 using highly polymorphic minisatellite probes.

3.4.2. Parental Derivation Of New Mutations

In two of the pedigrees previously shown in figures 3.24 and 3.25 (535 - patient 145; 540 - patient 146) it was possible to trace the parental derivation of the newly mutated allele by utilising the fact that both patients exhibited allele loss with chromosome 5 markers. Using the classic tumour suppressor gene paradigm of Knudson (1971), inheritance of a mutant allele is followed by the loss of the corresponding normal allele leading to a complete absence of genetic information at that locus. Examination of normal DNA from each of the parents of patients 145 and 146 using the probes with which allele loss was seen, will therefore show from which parent the chromosome carrying the mutation was inherited. The results obtained in this study are shown in figures 3.28 and 3.29.

In the case of patient 145, figure 3.28 shows allele loss in 4/10 adenomas using the minisatellite probe λMS8 which maps to 5q35-qter (the sample numbers in the first 11 tracks relate to those in table 3.7). Tracks 12 and 13 contain DNA from father and mother respectively. It can be seen that in the 4 cases in which allele loss is evident (lanes 6, 8, 10 and 11) the larger allele is lost in all cases and this is the allele which must have been inherited from the father. It therefore follows that the mutation must have arisen on the chromosome 5 inherited from the mother. Obviously, the lack of informativity of the probes closer to the APC locus means that involvement of the APC gene cannot be guaranteed but the fact that the same allele is lost in all four cases suggests that this is not a random event and the likelihood of APC gene involvement in these deletions seems high.

Figure 3.29 illustrates the results obtained in family 540 using three probes closely linked and flanking the APC locus. In this case, allele loss in the 5cm adenoma of patient 146 was seen with ECB27; EF544 and YN5.48 and therefore involvement of the APC gene in this deletion is certain. Tracks 1 and 2 of each panel contains normal and tumour DNA of patient 146 respectively whilst tracks 3 and 4 contain lymphocyte DNA from the mother and father respectively. All three probes proved informative with respect to determination of the parental derivation of the newly mutated allele since for all three probes the parents had a different genotype. In the case of ECB27, the smaller

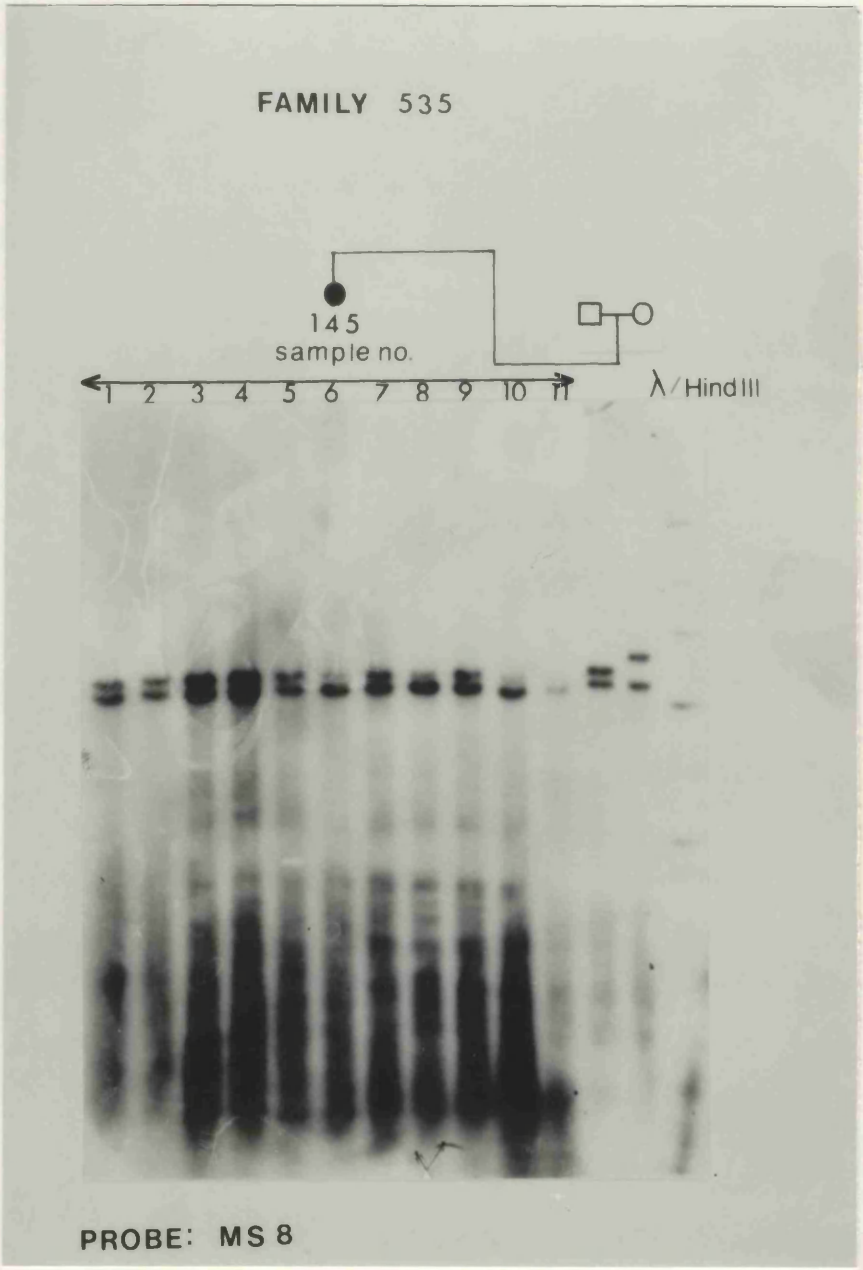


Figure 3.28. Parental derivation of the newly mutated allele in patient 145 determined by hybridisation with λMS8.

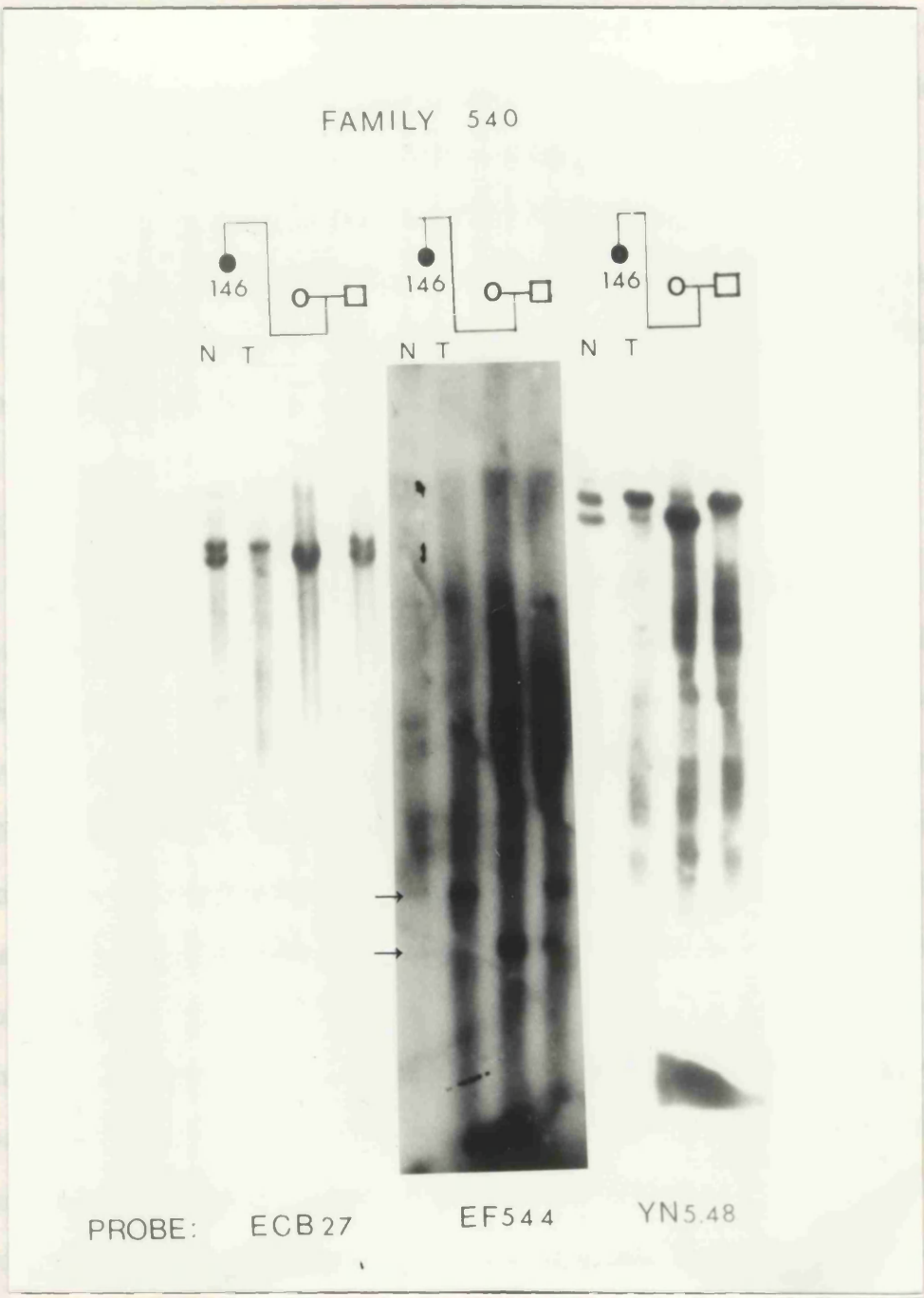


Figure 3.29. Parental derivation of the newly mutated allele in patient 146 as determined by hybridisation with ECB27; EF544 and YN5.48.

allele (10.5 kb) was lost in the tumour and this allele must have been inherited from the mother indicating that the chromosome 5 on which the mutation arose was derived from the father and in the case of the ECB27 locus carried the 11.9 kb allele). In the case of the probe EF544, the normal bands in track 1 are very faint (for verification of the heterozygous state in the normal DNA of patient 146 see figure 3.8) but the adenoma clearly shows loss of the smaller (2.1 kb) allele. Again this is the allele inherited from the mother indicating paternal derivation of the chromosome 5 on which the mutation arose. A similar result was obtained using the probe YN5.48, in which the maternal allele was again lost in the tumour.

3.4.3. MCC Gene Status in New Mutation Pedigrees

The cDNA clone MCC40 which exhibits an EcoRI polymorphism was used to investigate the status of the MCC putative tumour suppressor gene (Kinzler et al., 1991) in the new mutation patients described above. Unfortunately in most cases investigated this probe was uninformative as seen in families 540 and 551 (figure 3.30). Patients 145 (family 535) and patient 57 were also homozygous using this probe. However material from family 417 gave some interesting results. The middle panel of figure 3.30 contains EcoRI-digested DNA from the parents (lanes 1 and 2) and from the patient (lanes 3-5). Lane 5 contains DNA prepared from a fresh blood sample whereas lanes 3 and 4 contain DNA prepared several years previously from normal mucosa (N^+) and an adenoma (T^*) respectively and stored at -70°C. As can be seen from the autoradiograph, the father is homozygous for the larger 4.3 kb allele whereas the mother is heterozygous for the 4.3 and 4.1 kb alleles. The fresh normal DNA from the patient is also homozygous for the larger allele, as is the DNA labelled T. The other "normal" DNA sample however displays an extra band in addition to the expected 4.3 kb allele. This extra band is smaller in size (approximately 3 kb) and shows strong hybridisation to the MCC40 probe. There are two possible explanations for the appearance of this aberrant band:

1) The DNA originally extracted from normal colonic mucosa has become degraded during its period of storage OR

2) The DNA samples labelled N^+ and T^* have been mixed up during their period of storage and the aberrant band represents a rearrangement in the adenoma DNA.

It is obvious that the extra band does not represent a constitutional rearrangement because the fresh normal DNA shows the patient to be constitutionally homozygous. This same Southern filter was then hybridised to another probe recognising an EcoRI polymorphism (Sam1.1) in order to check that the correct sized alleles were present which would therefore rule out the possibility of the DNA being degraded. However, although the expected 1kb fragment was detected, the other allele (A1=9kb) was not and instead there was an extra band of around 5kb suggesting that this DNA sample was in fact degraded and that the novel band seen on hybridisation to MCC40 was an artefact and not a rearrangement at the MCC locus.Therefore, of the three pedigrees presented in Figure 3.30 following hybridisation to the MCC40 cDNA probe none showed any evidence of gross constitutional rearrangements at the putative tumour suppressor gene locus.

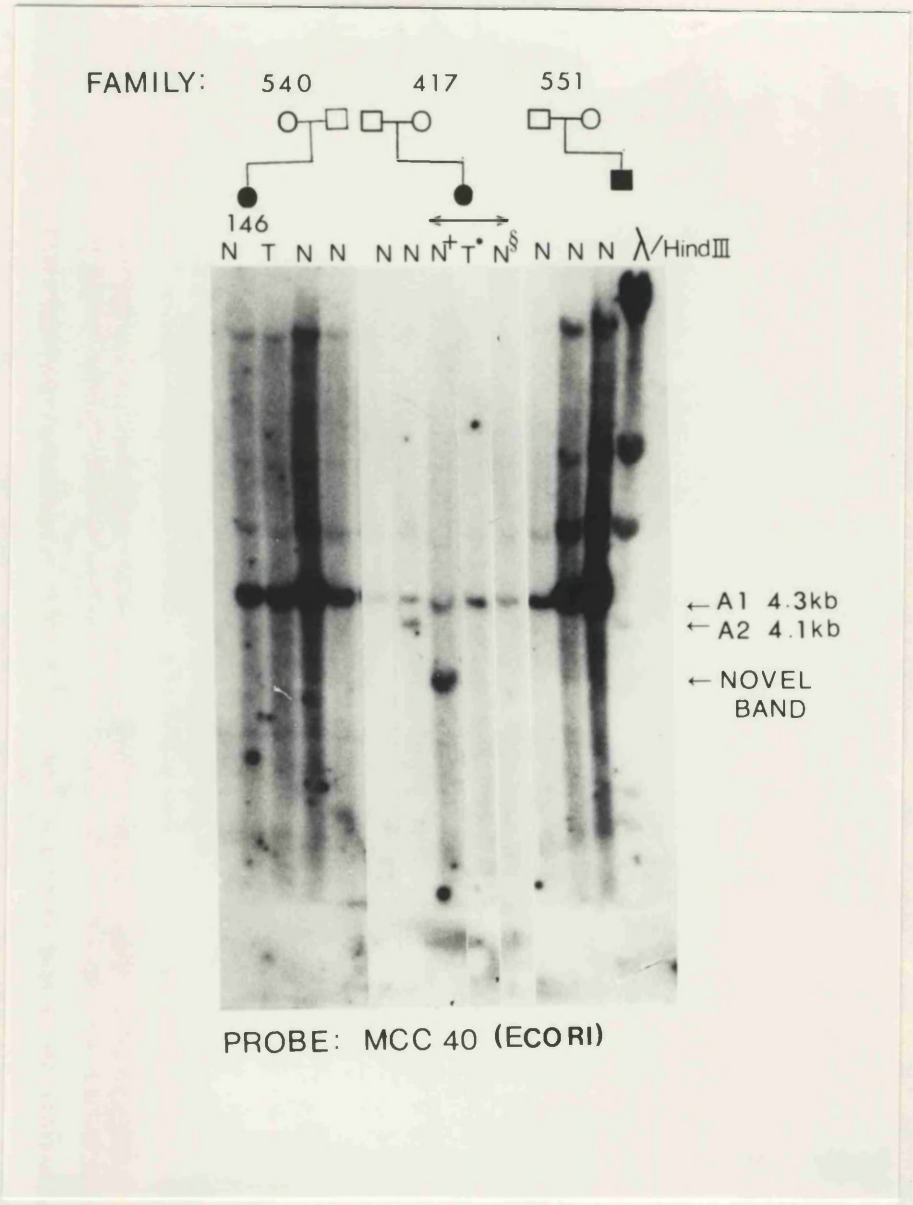


Figure 3.30. MCC gene status in three new mutation pedigrees as determined by hybridisation with the cDNA clone MCC40.

4 DISCUSSION

4. Discussion

The first suggestion of the presence of sequences within the human genome with the ability to suppress tumorigenicity was suggested by the hybrid work of Harris et al. (1969) and Stanbridge et al. (1976). It now seems that loss or inactivation of such tumour suppressor sequences plays a vital role in the development of many cancers sometimes in association with other changes.Fearon and Vogelstein (1990) suggest the involvement of 4-5 independent genetic events in colorectal carcinogenesis including allelic changes on chromosomes 5, 17 and 18, point mutations of the K-ras protooncogene and hypomethylation.

This study was therefore initiated with the aim of investigating the precise molecular genetic events which form an integral part of colorectal carcinogenesis. This approach involved employing the techniques of restriction endonuclease digestion and Southern hybridisation on a range of tumour and normal DNA samples to polymorphic DNA probes specific for the regions of the human genome previously implicated in the progression of the disease. These polymorphic probes included those in the vicinity of the APC gene on chromosome 5q and of the p53 and DCC genes on chromosomes 17p and 18q respectively. Loss of constitutional heterozygosity at these loci in the tumour samples of informative patients is thought to implicate tumour suppressor sequences in the aetiology of the tumour (Green, 1988; Knudson, 1989) and use of allele loss observations has been used to isolate the chromosome 18 gene deleted in colorectal cancer (Fearon et al., 1990). In the context of this study, it was hoped that such loss of heterozygosity could be correlated with the stage of tumour development and possibly with the occurrence of K-ras point mutations which occur in up to 50% of colorectal cancers (Bos et al., 1987; Forrester et al., 1987; Vogelstein et al., 1988) and to a lesser extent in adenomas (Farr et al., 1988b; Vogelstein et al., 1988) Throughout the course of this study great attention has been paid to the histopathology of the tumours involved: in the case of adenomas the size , degree of dysplasia, and whether the tumour was of the tubular or villous type was deemed important and was recorded wherever possible. Similarly, in the case of the carcinomas, the Dukes' staging and

tumour type was again noted. Unfortunately, such information was not available for all the tumour samples.

4.1 Allele loss at minisatellite loci.

This study utilised a panel of 5 highly polymorphic, locus-specific minisatellite probes mapping to 1p, 1q, 5q, 7p and 7q (Wong et al., 1987) to detect allelic changes in DNA extracted from a range of colorectal neoplasms when compared to normal (lymphocyte or normal mucosal) DNA from the same patients. The rationale for using these probes was that with their heterozygosities ranging from 90-99% (Wong et al., 1987) , it was hoped that the majority of patients would prove informative. In this type of study, lack of constitutional heterozygosity is the greatest limitation on detection of allele loss.

4.1.1 Allele loss in carcinomas

29 carcinomas (26 sporadic and 3 familial -table 3.1) were investigated, the majority of which were of rectal origin and well-developed (Dukes' B or C). In accordance with the findings of others (Solomon et al., 1987; Okamoto et al., 1988) using the marker λMS8 (D5S43), chromosome 5 allele loss was detected in 23% of informative carcinomas (table 3.3). Most usually this was seen as a reduction in intensity of one of the alleles when compared with the allele intensities of the normal corresponding DNA (figure 3.1). The faint residual alleles is probably due to infiltration of the tumour by normal stromal cells. Of the 5 tumours in which LOH was found, 4 were Dukes' stage C and all were sporadic adenocarcinomas. Of the three familial carcinomas, two were informative with the λMS8 probe but neither showed evidence of LOH. This is probably reflective of the small number of familial cancers looked at rather than to any inherent difference in the aetiology of the sporadic and familial cancers as chromosome 5 allele loss has been shown to occur at roughly the same frequency in both tumour types (Vogel stein et al., 1988; Sasaki et al., 1989). The highest proportion of allele loss on chromosome 5 has been shown to occur in the

vicinity of the APC gene (Ashton-Rickardt et al., 1989), and it seems likely that more of the carcinomas examined would show allele loss with markers closer to the APC gene (Sasaki et al., 1989). The carcinoma from patient HB gave an interesting result with the λMS8 probe. Fortunately karyotypic data is available for this tumour (J. Delhanty, pers. comm.) and figure 3.3 shows a typical pseudodiploid karyotype obtained from a metaphase spread. Several karyotypic aberrations are apparent, most notably two translocation chromosomes formed by breakage of chromosomes 4 and 5. The probable result of such an event is illustrated graphically in figure 3.5 indicating that a deletion of a small region possibly surrounding the *APC* gene has occurred. However no large scale deletion incorporating the telomere of either chromosome 5 can be observed in the diploid karyotype. However, the tetraploid karyotype (figure 3.4) also obtained from this tumour reveals an extra chromosome 5 aberration involving a visible deletion of the 5q31-qter region and it is likely that this is the dele tion detected by the observed loss of heterozygosity using the telomeric probe λMS8.It seems, therefore, that this deletion represents a secondary event in this tumour and does not equate with events at the *APC* locus involved in the generation of the deletion shown in figure 3.5. It has been suggested that reduction of telomeric sequences occurs in some tumours and that such telomeric loss makes the chromosome less stable and subject to further deletions (Hastie et al., 1990).

Using the minisatellite probes specific for chromosome 1 (λMS1 and λMS32), a couple of interesting results were obtained. The reason for interest in this chromosome was that, in common with other malignancies, structural alterations of this chromosome are frequent (Reichmann et al., 1984) and evidence obtained from studies of an adenoma-derived cell line have suggested a role for chromosome 1 in both tumour progression and in *in vitro* immortalisation (Paraskeva et al., 1988; Paraskeva et al., 1989).Allelic loss at 1p22 and 1p36 has also been implicated as a common late event in melanomas (Dracopoli et al., 1989) and the 1p36 region has also been implicated in ductal carcinoma of the breast (Genuardi et al., 1989). Prior to this study there was evidence for loss of expression at the α-fucosidase locus (1p34) in 2/6 colorectal carcinomas but retention of expression was observed at the phosphoglucomutase locus

(1p22) suggesting a deletion of part of the short arm (S.H. Rider, M.B. Davis and J.D.A. Delhanty - unpublished observations using isoenzyme analysis). However, use of the chromosome 1p33-35 marker failed to detect such a deletion in 23 informative carcinomas. Using the λMS1 probe only 1/23 carcinomas showed any deviant banding pattern when compared to its normal DNA counterpart and this was detected as an alteration in fragment size. Similarly 2/25 informative carcinomas showed alterations in band sizes using the chromosome 1q marker λMS32. These observations may be explained on the basis of the high rate of somatic mutation seen at these loci in this type of material (Jeffreys et al., 1988). An unequivocal case of allele loss was seen in the case of the familial carcinomas cell line JW2 using the chromosome 1q probe. However, detailed karyotypic data analysis of this tumour cell line (Paraskeva et al., 1984) has indicated that this tumour possesses one normal copy of chromosome 1 and one chromosome iso1q with an apparent deletion of chromosome 1p. Thus there appears to be disagreement between the karyotypic and molecular data. Unfortunately no result was obtained for this tumour with the 1p λMS1 probe. Possibly there is a small deletion of 1q either on the normal chromosome or on both copies of the isochromosome. The present study finds little support for the results of Leister et al. (1990) in which 39% of colorectal carcinomas showed allele loss at 1p35 most of which were detected using the λMS1 probe although a high percentage of these losses were concentrated in metastatic tumours again suggesting that this chromosomal region is involved in tumour progression rather than as a primary event.

The importance of trisomy of chromosome 7 in colorectal cancer is well-documented (Reichmann et al., 1985), and it is thought that increased chromosome copy number is important in the general aetiology of solid tumours (Van Der Berghe, 1987).Frequent structural abnormalities have also been observed in non-malignant tumours (Teyssier and Ferre, 1989). It may be that the various protooncogenes mapping to this chromosome play a part in the tumorigenic process and a significant increase in the expression of EGF receptor has been shown in well-established colorectal carcinomas (Steele et al., 1990). Use of the polymorphic markers pλg3 and λMS31 detected allele loss in 3/24 carcinomas informative for one probe or the other.

One of these carcinomas (HB) is known to contain multiple copies of chromosome 7 (figure 3.3) and this DNA result indicates duplication of one parental allele at the expense of the other. It may be that a similar situation applies in the case of the other tumours for which chromosome 7 allele loss was demonstrated (185 and 260) but no karyotypic data is available in these cases. One of these tumours (260) along with sample 311 demonstrated the presence of novel bands on hybridisation with λMS31 which can possibly again be explained by the high rate of somatic mutation seen st such loci (Jeffreys et al., 1988) and is probably a consequence of the genetic instability of the tumour cells rather than a process which is directly related to malignant transformation. Previous involvement of chromosome 7 has been demonstrated in ~10% of colorectal tumours (Vogelstein et al., 1989).

These results demonstrate the importance, wherever possible, of obtaining both molecular and karyotypic data. For example, in the cases of samples HB or 72 (JW2), either the karyotypic or DNA data alone would offer completely contrasting interpretations of the events involved in the genesis of these tumours. However, taken side by side, they offer a clear view of the changes involved in the evolution of these tumour samples.

4.1.2. Allele loss in adenomas

To date there has been little evidence for the occurrence of chromosome 5 allele loss in familial adenomas although such loss is a relatively frequent event in sporadic adenomas (Vogelstein et al., 1988). Using the probe λMS8 which maps to 5q35-qter, allele loss has been demonstrated in 3/34 familial (APC) adenomas and in 1/2 sporadic adenomas. Again, this was either seen as a reduction in intensity of one allele with respect to the other or as complete loss of one fragment (figure 3.2). Interestingly, two out of three of the APC adenomas showing loss were derived from one patient (57 - table 3.2) whilst a further adenoma from this patient exhibited retention of both alleles. This patient possessed extremely well-developed adenomas at age 15 and is thought to represent a spontaneous mutation since there is no family history of the disease. Chromosomes obtained from a 48h culture of an adenoma from this patient showed

random loss or gain of chromosomes in 11/26 cells analysed.The largest of these adenomas used for the DNA study was 6mm and whilst several other adenomas examined were much larger (e.g. patient 50 - table 3.2) it seems that there is no immediate correlation between adenoma size and deletion of chromosome 5 sequences. Complete allele loss was seen in the adenoma examined from patient 93 and in a 10mm sporadic adenoma from patient 51 (figure 3.2). Although sample 51 was initially thought to represent a sporadic sample because it was a solitary adenoma in a patient of advanced years and there was a lack of family history, evidence of CHRPEs has now been found so it may be a case of low expression at the APC locus leading to a late onset of the disease as a brother also shows signs of the presence of adenomas. The patient's colon is currently being examined for the presence of microadenomas but no result has been received. It is possible that this patient represents a pedigree in which the expression of the APC gene is very low similar to that described in a large Utah kindred by Leppert et al. (1989) in which one or two members appeared to have classical polyposis and the other affected members had either microadenomas or one or two polyps. It is possible, therefore, that this study indicates chromosome 5 allele loss in 4/35 familial adenomas.

No significant changes were seen in any of the adenomas with the chromosome 1 or 7 probes. One adenoma (patient 70) showed an altered band size using the λMS32 probe but again this is probably a consequence of the high mutation rate at these loci and not directly related to the malignant process. These results suggest significant involvement of chromosome 5 with respect to benign adenomas and that although chromosome 5 allele loss is not a frequent event in terms of numbers of adenomas it may well be important in those adenomas in which it occurs, possibly bestowing on them a higher malignant potential. The observation that chromosome 1 and 7 allele loss occurs in carcinomas but not in adenomas suggests that these are later events possibly related to tumour progression and such a role has been suggested for chromosome 1 (Paraskeva et al., 1988). It is clear from pathological reports of APC cases that not all adenomas present possess the inherent ability to become carcinomas e.g. if one takes the average number of adenomas in a typical APC patient to be ~1000, the fact that the

highest recorded number of cancers in such a patient is 7 (Bussey, 1975) indicates the overall rate of malignant conversion is low. Law et al. (1988) found no chromosome 5 allele loss in 40 informative APC adenomas. The diiference between the results presented here and those previously published may simply be due to a difference in sampling or may represent significant differences between patients.

Obviously, loss of the chromosome 5 marker λMS8, does not automatically implicate the APC locus in the observed deletion and the use of markers flanking the APC gene are necessary to show that the samples in which chromosome 5q35-qter allele loss has been demonstrated are also deleted further up the chromosome. Involvement of the APC gene in the deletion observed in one of the adenomas from patient 57 is discussed in the following section.

4.2. Further Allele Loss Studies.

4.2.1. Chromosome 5.

4.2.1.1. Chromosome 5 allele loss in carcinomas.

Investigation of chromosome 5 allele status was carried out using 11 polymorphic DNA probes (appendix 11.1) several of which have been shown to exhibit tight linkage to the APC gene (Dunlop et al., 1990). The polymorphic cDNA probe MCC40 specific for the recently isolated MCC gene (a candidate APC gene) was also used. This probe exhibits an EcoRI polymorphism recognising two alleles of 4.3 and 4.1kb but unfortunately proved uninformative in the panel of 13 carcinomas used.12/13 carcinoma samples were informative for one or more of the markers used and 4/12 exhibited reduction of intensity with at least one marker (112, 134, 161 HB λ - table 3.9). It is evident that this loss is clustered near the marker YN5.48 (figure 3.7) which is consistent with results obtained by other workers (Ashton-Rickardt et al., 1989; Sasaki et al., 1989). Sample 134 is a liver metastasis obtained from an APC patient who originally presented with a colorectal carcinoma. DNA from this tumour exhibited loss with both YN5.48 and π227 probes. Knudson's hypothesis predicts that in the familial case, an allele lost at a tumour suppressor gene locus during tumorigenesis will be the

normal allele and that the retained allele will be mutated (Knudson, 1971). However, family linkage data obtained from patient 134, indicates that the allele lost in both cases is the mutated one, the normal allele being retained (M.B. Cachon-Gonzalez - pers. comm.) suggesting that this is not a primary event in this tumour and maybe related to the metastatic process, probably a result of the increased chromosomal instability of the tumour cells.

Because of the clustering of chromosome 5q allele loss around the APC locus and the lack of evidence for such loss on chromosome 5p (as shown by retention of the marker MS621), it seems that whole chromosome loss is not a significant means by which LOH is attained. Similarly only 1/6 carcinomas showed loss with the 5q telomeric marker λMS8 suggesting that interstitial deletion is probably the most common mechanism as previously suggested (Ashton-Rickardt et al., 1989). This latter observation also indicates that the extent of chromosome 5q allele loss in carcinomas in section 4.1.1. above is an underestimate of the numbers of theses samples which would have shown loss had other informative markers closer to the APC locus been available.

It is unfortunate that as with MCC40, the marker EF544 was also uninformative in this panel of tumours as it is the marker showing the tightest linkage to the APC locus (Dunlop et al., 1990). Similarly, another closely linked probe L562 was only informative in two cases and heterozygosity was retained in both.

In the case of sporadic carcinoma HB, loss was detected with the markers π227, ECB27 and λMS8. As previously discussed a detailed (tumour) karyotype was available and is illustrated in figures 3.3-3.5. It seems that in course of formation of the two translocation chromosomes a small segment of chromosome 5q (15-22?) was deleted. This is reflected in the loss of alleles detected with ECB27 and π227 suggesting that these markers lie within the deletion and the close linkage demonstrated between these markers and the APC locus provides good evidence for the presence of the APC gene within the deletion also. However, the loss shown with λMS8 probably represents a scondary event which occurred during the formation of the tetraploid cell and is not related to the 5q15-22 deletion (as previously discussed). The involvement of

the APC locus in the allelic events in these tumours seems unequivocal but the lack of informativity of some of the most closely linked markers remains the single most limiting factor in diagnosing allele loss. The extent of allele loss detected may also be masked by the proportion of normal cells infiltrating the tumour and having the effect of "diluting out" the tumour cell population, It has been suggested that LOH maybe masked if the proportion of normal cells present exceeds 30% (Vogelstein et al., 1988) and this has led to methods of microdissecting out homogeneous islands of malignancy (Law et al., 1988) to avert this possibility. There is no doubt that the ability to do this would have made the task of assessing allele loss easier but the expertise involved was not available prior to the onset of the study. The ability to produce epithelial cell lines from the tumours used would have been another approach by which a homogeneous cell population could have been obtained (as illustrated by the allele loss results obtained in tumour 72 - cell line JW2) but such cultures are not always easy to obtain.

4.2.1.2. Chromosome 5 allele loss in adenomas

A total of 37 APC colorectal adenomas were investigated in this second stage allele loss study from a total of 6 patients. Three adenomas from patient 57 were originally investigated with respect to allele loss using the λMS8 probe (table 3.2) but only one of the two showing loss at this locus was used in further studies.A series of 10 APC duodenal adenomas were also investigated using λMS8 only along with 10 hamartomas from a patient suffering from juvenile polyposis. 4 desmoid tumours and an epidermal cyst were also subjected to allele loss assessment.

Again the MCC gene probe was uninformative for all patients tested as were the v-fms oncogene and the APC-linked probe MC5.61. The probe YN5.48 which was the most informative in detecting allele loss in carcinomas was only informative in one of the adenoma patients and resulted in detection of allele loss in the 5cm adenoma examined from this patient. Similarly, allele loss was demonstrated in this tumour using the other APC-linked probes EF544 and ECB27 (figure 3.8b). Similar involvement of the APC locus was deduced from the loss of alleles detected in the adenoma of patient 57 using the probes λMS8 and ECB27 (figure 3.8c). None of the other markers which

map to the region between these two markers were informative in this patient and though it seems likely that the region between them is completely deleted in this tumour, the possibility remains that like the case of carcinoma HB, loss of the λMS8 marker could be a secondary event. However, even if this is the case, it seems likely that the APC gene is involved as genetically, ECB27 is closely linked to the disease locus (Dunlop et al., 1990). A similar explanation may exist for the case of patient 145, in which 4/10 adenomas exhibited allele loss with the λMS8 probe.This allele loss was represented by a reduction in intensity of the larger allele and the same allele was lost in all cases arguing against random loss. The residual band reflects the presence of normal cells in the tumour as described in table 3.7. Interestingly, the carcinoma from this patient did not show loss (lane 5, figure 3.8a) and retention of both alleles was apparent with both the other informative probes (π227 and MS621). However, it is possible that the APC locus is involved as MS621 showed no loss in the carcinomas and π227 shows sufficient recombination with the disease locus to put it around 16cM away (Cachon-Gonzalez et al., 1991). Clearly, establishment of the extent of the deletions in the adenomas of this patient requires a greater level of probe informativity particularly in the region flanking the APC locus. Recently, a C-A repeat microsatellite polymorphism of the type first described by Weber and May (1990), has been isolated in the L562 probe and current work is being focused on the utilisation of this polymorphism with a view to establishing the chromosome 5 allele status in the region immediately adjacent to the APC gene not only in patient 145 but in patients 136 and 139 both of whom were uninformative for the most closely linked APC markers. Patients 136 was seen to retain heterozygosity in all adenoma samples using the probes C11p11 and π227 but no informative marker is available on the telomeric side of the APC gene. In the case of patient 139, λMS8 was the only informative marker so very little can be concluded concerning events in the vicinity of the APC gene.

Investigation of the size, degree of dysplasia and histological type of the adenomas exhibiting allele loss was carried out. Tables 3.7 and 3.11 illustrate the results obtained in the case of patient 145. In the 4 adenomas showing loss, 3 originated in the descending colon and were all of the tubular type. There appears to be some correlation

between the size of the adenomas showing loss in that they were the four largest (6, 8, 10, 11) and ranged in size between 7.5 and 15mm. 3/4 of these exhibited mild dysplasia whereas the other (15mm - sample 11) was moderately dysplastic. It is interesting to note that the Dukes' B carcinoma (sample 5) from this patient showed no allele loss and again more informative markers would play a role here in establishing whether or not the carcinoma shows LOH closer to the APC locus as has been demonstrated in other studies (Miyaki et al., 1990). Loss of heterozygosity in this study using the λMS8 probe has been shown to extend to the APC locus in the case of patient 57.

The adenoma of patient 146 which showed complete allele loss with several markers was a 50mm mildly dysplastic tubular adenoma and this is supportive of the view that the frequency of chromosome 5 allele loss increases with increasing size of the tumour. However, no loss was seen in 3 adenomas from patient 147 which ranged in size from 5-20mm but only λMS8 and MS621 were informative. Very few details are available with respect to the three adenomas from patient 57, two of which showed loss at the λMS8 locus except that the largest of these was 6mm. It is possible that chromosome 5 allele loss enables a minority of adenomas to increase in size and perhaps become more villous with a higher malignant potential. This is supported by the recent study of Miyaki et al.(1990) where the proportion of observed allele loss on 5q increased from <2% in moderate adenomas to ~20% in severe adenomas.

The juvenile polyposis patient JB was constitutionally heterozygous for both the minisatellite probes, MS621 and λMS8 and no allele loss was detected. However, interesting results were obtained with the π227 probe for which this patient was also heterozygous (figure 3.9). 5/10 polyps possess the same pattern as the normal DNA sample (figure 3.9, lanes, 2,3,4,7,9). Apparent loss of the larger (4.3kb) allele was detected in one polyp (lane 10) although this may be due to differential loading of samples. In 4/10 polyps extra, strongly hybridising fragments were visible (lanes 1,5,6,8). Of these extra bands, one (~2.9kb) was common to all four hamartomas whilst a further two (~4.4-6kb) were common to three (lanes 1,5 and 8). No obvious explanation can be proposed to explain these results. A recent linkage study has

excluded linkage to the APC locus in juvenile polyposis (Peterson et al., 1990), so it is unlikely that the apparent allele loss in lane 10 signifies the involvement of a tumour suppressor gene in this disease.Similar observations have been noted in a large APC linkage study carried out in this laboratory (M.B. Cachon-Gonzalez, pers. comm.) in the constitutional DNA of APC patients which suggests that this probe maps to a recombination or mutation hotspot.Again there is the need for increased informativity with respect to other loci on this chromosome which may shed light on these observations. The extra bands observed do not appear to be the result of either (a) partial restriction endonuclease digestion or (b) degradation of the DNA samples as this filter has been used successfully with other probes (OS-4 and JOSH4.4 - chromosome 18) to give the expected fragment sizes.

Because of the shortage of DNA the series of 11 duodenal adenomas were only investigated using the minisatellite probe λMS8 and 10/11 of these were informative and showed no loss. One of these "tumours" was later found on histological examination to consist of normal mucosal cells only. Most of these adenomas were small <5mm and tubular exhibiting mild dysplasia (table 3.8). Four desmoid tumours were also subjected to a limited survey of chromosome 5 allele status (table 3.12) again due to the shortage of DNA. Desmoids represent a category of additional manifestations suffered by some APC and Gardner's syndrome patients (Smith, 1958; 1959). Loss of heterozygosity in such tumours has been reported even though they presumably have a different aetiology from adenomas being fibroblastic and largely comprised of collagen. All four tumours were constitutionally heterozygous for at least one of the chromosome 5 markers used and heterozygosity was retained in all cases. However, the sample size was very small and few conclusions can be drawn from the negative results obtained .

The epidermal cyst obtained from APC patient PS is an example of the extracolonic manifestations which are sometimes a feature of the disease. This patient, who is slightly mentally retarded, possesses a constitutional deletion of the region 5q22-q23.1 (Cross et al., 1991) on one copy of chromosome 5 which is known to include the markers YN5.48, EF544 and MCC i.e. the patient is hemizygous for these markers and the study was carried out in order to detect possible loss of the remaining allele in the

cyst DNA using these three markers and loss of heterozygosity with the markers which lie outside the critical region. No allele loss was apparent using the markers C11p11, ECB27 (patient constitutionally homozygous) or with the marker L562 which may or may not lie within the deletion. Using the markers YN5.48, MCC and EF544 (patient constitutionally hemizygous) the remaining allele was retained whilst retention of heterozygosity was apparent with the markers π227 and λMS8. The fact that one allele was retained with the markers known to lie within the deletion possessed by this patient therefore suggests that the remaining normal allele is not lost in the epidermal cyst by means of a deletion although this does not rule out the possibility of a more subtle change on the retained chromosome.

How applicable then is Knudson's hypothesis to the tumorigenic mechanisms involved in the genesis of colon cancer ? The lack of evidence for loss of alleles on chromosome 5q has been used by some workers (Solomon et al. 1987; Vogelstein et al. 1988) to deny the applicability of Knudson's hypothesis to colorectal carcinoma even though loss of 5q markers in sporadic adenomas has been shown to be a comparatively frequent event (Vogelstein et al. 1989). However there is another possible interpretation of these findings which could be further argument in favour of Knudson's theory. In APC the inherited mutation at the APC gene locus is sufficient to give rise to small adenomas of the class I type generally seen in APC (Vogelstein et al., 1988) perhaps by some threshold growth effect (Bodmer et al., 1987) or induction of epithelial hyperproliferation (Vogelstein et al., 1988), but a further somatic mutation on the homologous chromosome is necessary to give rise to the larger, more villous adenomas with a greater malignant potential. This explanation is backed up by the observation that in any one APC patient the greatest number of cancers seen is 7 (Bussey, 1975) even though there may be more than 5000 adenomas. This suggests that not all APC adenomas have the potential to progress to malignancy and that further somatic events are necessary to bestow this extra malignant potency.

In light of this explanation, it is not unreasonable to assume that adenomas of the class II and III types (i.e. non-APC) (Vogelstein et al., 1988) have already undergone this secondary change at the APC locus and already possess the capability of

progressing further along the carcinogenic pathway. There appears to be no reason to assume that the familial and sporadic adenoma-carcinoma sequences to be different and the chance finding of chromosome 5 deletions in the small proportion of APC adenomas which posses them has been demonstrated in certain cases (Rees et al., 1989; Sasaki et al., 1989). It is obvious in the case of colorectal carcinoma that several more mutational events are necessary to bestow fully-fledged malignancy including changes on chromosomes 17 and 18 but Knudson's hypothesis may still hold true for formation of adenomas with a higher malignant potential.

APC is unusual in that heterozygosity gives rise to local growth excesses via a threshold growth effect produced by fluctuating levels of the normal gene product (Solomon et al., 1987). The smallest adenomas may therefore be a manifestation of this hyperproliferation as they show no evidence of a secondary change at the APC locus (Law et al., 1988). Post-colectomy regression of rectal polyps has been observed (Feinberg et al., 1988b), suggesting that no irreversible genetic change has taken place but in the case of the adenomas which exhibit chromosome 5 allele loss, a secondary change at the APC locus may cause an inherent growth advantage which may, in turn, make other changes more likely. It has been observed that villous adenomas are more common in sporadic patients than in patients with inherited polyposis and these are generally regarded as having a higher malignant potential (Muto et al., 1975). Therefore, loss of chromosome 5 sequences may be a feature of those sporadic and familial adenomas in which malignant transformation is more likely.

4.2.2. Allele loss on chromosome 17

Involvement of chromosome 17p in colorectal carcinogenesis has been demonstrated both karyotypically (Muleris et al., 1985) and at the molecular level (Vogelstein et al., 1988). Loss of sequences on chromosome 17p13 has been shown in up to 75% of carcinomas and also in sporadic adenomas (Vogelstein et al., 1988) indicating the presence of tumour suppressor sequences in this region. This tumour suppressor gene has been identified as the p53 gene and allele loss together with point mutations in the remaining allele have been demonstrated (Baker et al., 1989). For

these reasons polymorphic DNA probes in the 17p13 region were used to detect allele loss.

4.2.2.1. Chromosome 17p allele loss in carcinomas

From table 3.13, it can be seen that all 12 samples investigated were informative for at least one of the markers used. Unfortunately the p53 gene probe (pProsp53) was uninformative in all cases). A total of 8/12 (66%) of carcinomas exhibited allele loss and this is within the range detected by other workers (Vogelstein et al., 1988). All tumours showing loss in this region were Dukes' B or C (134 was a metastasis). In contrast tumour 161 (Dukes A) showed no loss although it did show loss on chromosome 5. Even though no direct correlation was apparent between the occurrence of such loss and the Dukes' stage of the tumour, it has been suggested by other workers that colorectal carcinomas with 17p loss are more aggressive than those without (Kern et al., 1989). Of the 7 carcinomas which were informative for more than one marker, differential LOH was observed in 5 (112, 119, 131, 134, 145) whereas two showed loss of both markers for which they were informative. This suggests that, in the majority of cases, loss of the whole short arm does not occur. However, this observation is not supported by the karyotypic data of Muleris et al. (1985) who frequently observed 17p loss and formation of an iso17q. The sporadic carcinoma, HB, showed loss with both markers with which this patient was informative and this correlates with the karyotypic data (figure 3.3) showing there to be only one copy of chromosome 17 in the tumour. It would be useful to be able to order the probes used within band p13 to check that the markers detecting deletions were adjacent as it has been suggested that in the case of breast cancer allele loss on 17p occurs at two separate loci (Coles et al., 1990). It is unfortunate that the p53 gene probe was uninformative in these samples but it is probable that the majority of allele loss in this region has been detected by the other markers used as all were highly polymorphic VNTR probes.

4.2.2.2. Chromosome 17 allele loss in adenomas

In contrast with the uninformativeness of the chromosome 5 probes used, with the exception of the p53 gene probe, most adenoma patients were informative for at least two of the 17p13 markers used (table 3.14). All patients were constitutionally homozygous with the p53 probe. No cases of allele loss were seen in any of the adenomas looked at from patients 145, 146, 147, 136, 139 or 57. In contrast to the chromosome 5 situation in which 4/10 adenomas from patient 145 showed allele loss but not the carcinoma, using the MS228 probe the carcinoma showed reduction in intensity of the smaller allele (figure 3.10) but none of the adenomas showed this change which is not surprising since 17p allele loss is a much later event in carcinogenesis occurring near the transition from benign to malignant state (Baker et al., 1990b; Sasazuki et al., 1991). As is the experience of other workers, this study shows a stark contrast between 17p allele loss in carcinomas (~70%) and those in adenomas (0% - this study) although a higher proportion of 17p allele loss is seen in larger sporadic adenomas (Vogelstein et al., 1988).

4.2.3. Allele loss on chromosome 18q

Whole chromosome loss of 18 has been seen in a high percentage of carcinomas (Muleris et al., 1985; Monpezat et al., 1988) and is a feature of the tumour karyotype presented in figure 3.3 (HB). This observation has provided a starting point for the elucidation of a tumour suppressor gene sequence at 18q21.3 (the DCC gene) which has recently been isolated and shown to be deleted in the majority of sporadic colorectal carcinomas (Fearon et al., 1990). A detailed investigation of this region was therefore carried out to establish the involvement of this gene in familial carcinomas and adenomas.

4.2.3.1. Chromosome 18q allele loss in colorectal carcinomas

Six polymorphic DNA probes were used which map to the long arm of chromosome 18 (appendix 11.3). Three of these markers are cDNA probes specific for the DCC gene and were invaluable in establishing the region of most common loss. Of

the 10 carcinomas which showed reduction in intensity of one of the allelic fragments with these probes, 7/10 exhibited loss with one of the DCC probes indicating that this is the most common region of involvement in the aetiology of these tumours. The highest percentage loss (75%) was seen using the Sam1.1 DCC gene probe compared with 33% and 38% respectively for the other DCC probes p15-65 and Josh4.4 (figure 3.12). It is known that the p15-65 probe recognises the N-terminus of the DCC gene and that the other probes correspond to exonic sequences but their exact orientation with respect to each other and to the other 18q probes is not clear. Analysis of the deletion patterns seen in the tumours suggest the orientation shown in figure 3.12 for the three DCC markers. Marker OS-4 maps to 18q21.3-qter and the deletion data suggests that it may lie between Sam1.1 and Josh4.4 as tumour sample 130 shows LOH with Sam1.1 and OS-4 but retention of heterozygosity with Josh4.4 and p15-65. The observation of LOH in patient 119 using the OLVIIE10 but the retention of the DCC markers may at first sight point to the non-involvement of the DCC gene in this tumour. However, the OLVIIE10 locus is known to be very close to the DCC gene and was used to isolate it (Fearon et al., 1990). The DCC gene covers an area of ~370kb and it is possible that the OLVIIE10 locus lies within it (Fearon et al., 1990).

No significance could be attached to the Dukes' staging of the carcinomas with respect to 18q loss although, as previously mentioned, the sample size is small. However, as with the 17p observations, tumour sample 161 (Dukes' A) showed no evidence of loss whereas LOH was detected on chromosome 5 suggesting that 17 and 18 allele loss represent later events.

Figure 3.13 illustrates the results on PstI digestion of carcinoma samples HB and 158 following hybridisation to the DCC gene probe Josh4.4. In the case of HB, the normal DNA sample is heterozygous for alleles A1 (15kb) and A2 (10+5kb) whereas the tumour DNA is homozygous for a novel band of ~7-8kb. This tumour sample also exhibited LOH with the p15-65 but in this case no rearrangement was seen. The data with respect to allele loss is consistent with the karyotypic data which shows that the tumour cells possess only one copy of chromosome 18. The rearrangement is more difficult to explain because it is not clear which allele is retained. The most likely

scenario is that the A2 allele (10+5kb fragments) has been lost and the novel band represents a rearrangement of the 15kb allele. A similar situation has probably occurred in carcinoma 158 in which the normal sample is homozygous for the A1 allele, whereas the tumour sample is homozygous for a novel band of the same size as the one observed in HB. The most obvious explanation is that loss of one 15kb homologue has occurred whilst the other copy has undergone a rearrangement. This observation is supported by studying the intensities of the respective bands. In both cases, an equal amount of DNA has been loaded but in the normal track the homozygous band is much more intense than the novel band in the tumour track thus suggesting deletion of one allele.

4.2.3.2. Chromosome 18 allele loss in adenomas

All APC patients were heterozygous for at least one of the DCC markers (table 3.16). In two cases (146 and 139) all three were informative and no LOH was detected in any of the adenomas examined from these patients. Two patients were heterozygous for two of the DCC markers (145 and 57) but again no allele loss was seen in the adenomas. It is interesting to note that loss of the 15kb Josh4.4 allele was seen in the carcinoma of patient 145 but was retained in the adenomas (figure 3.14) (some of which showed LOH on chromosome 5) again suggesting that chromosome 18 changes occur later. Patients 136 and 147 were only informative for one of the DCC markers and no loss was observed. Similar retention of alleles was seen in both desmoids, and the epidermal cyst.

4.3. K-*ras* mutations

4.3.1. K-*ras* mutations in colorectal cancer cell lines

These cell lines were chosen because (a) they were available and (b) several were known to possess K-ras mutations and were useful as positive controls, particularly in the ASO dot-blot assay. At first it was thought that all these cell lines would contain mutations in the K-ras gene as a cellular clone with a selective growth advantage would

grow preferentially in culture. Of the 9 lines used , 4 were positive in the dot-blot assay (figure 3.17; table 3.17) and of these, 3 were already known to possess a mutation. These mutations were confirmed in all cases. The line SW480 was known to be homozygous for a mutation at position 2 of codon 12 involving a G -> T base change resulting in the substitution of valine for the normal glycine (Orita et al., 1989). This mutation was confirmed both by ASO analysis and by direct sequencing. Because there is only one copy of chromosome 12, the cell line is homozygous for the mutation (figure 3.18). Similarly the mutations previously described in the JW2 (Farr et al., 1988b) and KMS-4 (Yuasa et al.,1986a) APC-derived cell lines were also confirmed, both of which were heterozygous for their respective mutations. Only one other cell line was found to possess such a mutation which had not previously been reported, involving a glycine to alanine amino acid change at codon 12. No correlation was obvious between the Dukes' stage and the occurrence of ras mutations and supports the observations of Laurent-Puig et al. (1990). SW480 was isolated from a Dukes' carcinoma whereas several lines originating from more advanced tumours did not contain mutations (HT29; COLO320). Previous studies in our laboratory (S.H.Rider, Ph.D thesis, 1986) found KMS-4, LIM1899, LIM 1215 and HCA-7 to be positive with respect to transfection in NIH3T3 cells. In the case of KMS-4 and LIM 1899 this is probably due to the presence of the codon 12 K-ras mutation whereas it is possible that the other two lines contain mutations at other codons (59 or 61) or in the N-ras gene but these are much less frequent than those of K-ras. (Vogelstein et al., 1988) However, it has been suggested that such transfection studies are not entirely representative (Forrester et al., 1987; Bos et al., 1987) as the large size of the K-ras gene means that it rarely remains intact.

4.3.2. K-*ras* mutations in colorectal carcinomas

In line with previous investigations (Bos et a., 1987; Forrester et al.., 1987; Vogelstein et al., 1988), the frequency of K-ras mutations found was 43.8% and 41.7% for sporadic and APC carcinomas respectively. Although the sample sizes are relatively small, the results do, therefore, appear to be representative of other larger

surveys. More than 90% of mutations found were at codon 12, base position 2. No correlation was apparent between the Dukes staging of the tumours and the frequency with which K-ras mutations were detected as many well-established carcinomas (Dukes' C1 and C2) were found not to contain a mutation and this has been the experience of other workers (Forrester et al., 1987; Bos et al., 1987). Recently, however, Miyaki et al. (1990) have found correlation between increasing frequency of K-ras mutations and the transition from moderate (11%) to severe (36%) adenoma but found no significant difference between severe adenomas and carcinomas. None of the sporadic carcinomas which were positive in this study show any signs of allele loss with the λMS8 probe (table 3.1) but this may be due to the large distance between this marker and the APC locus. Direct sequencing of the pcr products in 3 of these sporadic carcinomas revealed two to be glycine to aspartic acid substitutions, whilst the other was a glycine to alanine substitution. All tumours were heterozygous for their respective amino acid change i.e. they all retained one wild-type allele which from the sequence data shown in figure 3.20 is usually stronger, probably representing contamination of the tumour with normal tissue.

Of the four APC carcinomas which were found to contain mutations, three were positive on ASO analysis all hybridising strongly to the mutant oligonucleotide probe cocktail 12.2.Sequencing these three samples showed them to contain a glycine to aspartic acid substitution at codon 12 (figure 3.21). Sample 147 also appeared to contain a codon 13.2 mutation which would also result in a glycine to aspartate amino acid change. This was not shown on ASO analysis as sample 147 did not hybridise to the 13.2 probe cocktail and it is always possible that this could be the result of misincorporation by Taq polymerase. However, if this result is genuine, it may point to the presence of different cellular clones within the tumour sample. Sequencing of sample 161 which was not investigated using the dot-blot approach, revealed a Gly -> Ser amino acid change at codon 13 due to a base transition at position 1. Interestingly the sequence data from this tumour sample also revealed a base substitution at position 3 of codon 13 but because of the degeneracy of the genetic code this will not result in an amino acid change. In general there is a great deal of variation between the intensities

of the mutant bands which could reflect normal tissue contamination, the presence of different clones within the tumour or may merely be an artefact of this method of sequencing, as there is variation between the band intensities seen on some gels using this approach. Again, in the case of the familial cancers, comparison of K-ras mutations with Dukes' staging is not conclusive and probably requires a larger sample size.

4.3.3. K-*ras* mutations in colorectal adenomas

Of the 82 adenomas examined, 5 (6%) were found to contain a mutation, all at codon 12 position 2 (figure 3.22). Sequence analysis was carried out in four of these (table 3.19), three of which contained an aspartic acid substitution resulting from a G-> A base change. The remaining sample (139) showed a very slight band indicating the presence of a G -> C base change (Gly -> Ala) probably in a subset of the tumour cells only. It is interesting to note that of the 12 adenomas which were investigated in this patient, this sample (no. 4) was the only one to grow in culture suggesting the possibility that the K-ras mutation had bestowed a selective growth advantage on the cells involved. Unfortunately the culture only grew sufficiently to enable routine chromosome analysis (karyotype normal) otherwise it might have been possible to extract DNA from the cultured cells to establish whether or not the mutation was present in a greater proportion of the cultured cells than in the primary tumour. If this was true it would lend credence to the view that adenomas arise as a result of successive waves of clonal expansion (Fearon and Vogelstein, 1990). All adenomas from this patient were of the tubulovillous type ranging in size from 10-30mm and exhibiting mild to moderate degrees of dysplasia.

Of the 10 adenomas examined from patient 145, only 1 contained a K-ras mutation and this was a 9mm tubular adenoma originating in the descending colon and exhibiting only mild dysplasia. A larger (15mm) moderately dysplastic adenoma from this patient did not contain a K-ras mutation neither did a carcinoma. There appears, therefore to be no obvious correlation between tumour size,degree of dysplastic. and the presence of K-ras mutations. Two of the five adenomas from patient 70 were also positive in the dot blot assay and were confirmed by direct sequencing. The larger of these (VLP table

3.19) was a 7mm villous adenoma which also exhibited a rearrangement with the λMS32 probe (1q) (table 3.2).

By far the most common mutation appears to be the Gly->Asp substitution resulting from a G->A base substitution at codon 12 position 2, in accordance with the results of Vogelstein et al (1985) and thus may be more advantageous to growth in colorectal carcinomas (Miyaki et al., 1990), although a range of different mutations has been the experience of other workers (Burmer and Loeb, 1989; Laurent-Puig et al., 1990). These differences may therefore represent variations between samples and may be representative of the different geographical origins of the tumours used. This may be particularly relevant with respect to the Japanese surveys as Japanese polyposis patients are known to have a higher occurrence of gastric adenomas and carcinomas (Utsinomiya, 1990) which may reflect dietary influence.However, it is thought that subsitution of the normal glycine at codon 12 with any other amino acid would compromise the nucleotide-binding capacity of the protein (Wierenga and Hol, 1983) and therefore any base change at this codon would have severe implications fro the control of signal transduction. It has recently been suggested that an aspartic acid substitution at codon 12 leads to retention of the normal allele whereas other mutations allow loss of the remaining normal allele (C. Marshall, pers. comm.). However, in the present study the normal allele appeared to be retained at all times with the exception of the SW480 cell line which is known to contain only one copy of chromosome 12 (Orita et al., 1989) The LIM1899 and KMS-4 cell lines both of which contain mutations other than aspartic acid both retain the wild-type allele as do carcinomas 193 and 161 and adenoma sample 4 from patient 139. It is possible that contamination of the tumour with normal cells would lead to a masking of allele loss but this is obviously not the case for the cell lines which represent a pure tumour cell population.

4.4. Correlation of allele loss and K-*ras* mutations

As previously suggested by Fearon and Vogelstein (1990), even though a hypothetical time scale and sequence has been proposed for the various genetic changes

which occur in colorectal carcinogenesis, it seems that this sequence is not rigid and that the accumulation of these changes is more important than the order in which they occur. However, it appears that changes at the DCC gene locus on chromosome 18 and of the p53 gene on chromosome 17 are, on the whole, later events than the changes on chromosome 5 or mutations in the K-ras gene. This view is supported by the present study in which allele loss on chromosomes 17 and 18 was not observed in any of the adenomas investigated whereas chromosome 5 loss and K-ras mutation, although infrequent, were observed in these samples. A recent publication suggests that chromosome 17 involvement begins with a mutation of the p53 gene on one homologue and is followed rapidly by loss of the remaining normal allele in up to 75% of carcinomas (Baker et al., 1990b) and that this occurs just prior to full malignant transformation. In light of this fact, it is probable that all carcinomas in the present study in which chromosome 17 allele loss has been demonstrated, possess a mutation on the retained chromosome.The involvement of the p53 gene in colorectal (Baker et al., 1989; Rodrigues et al., 1989) and other cancers (Nigro et al., 1989) seems unequivocal and it is probably one of the main factors in determining malignant transformation. Other studies have found no correlation between K-ras activation, overexpression of the p53 gene (presumably a consequence of the increased half-life of the mutated protein product) and Dukes' staging of carcinomas (Laurent-Puig et al., 1990). Even though allele loss at this locus is not evident in all colorectal carcinomas it is possible that mutation without allele loss is the prevailing situation in some. In such cases, expression of the mutant p53 could still exert a pseudo-dominant effect by dimerising with the normal gene product whereby considerably lengthening its half-life. It has been suggested that inactivation of both alleles is not always necessary for the loss of phenotypic effects of a tumour suppressor and that "dominant -ve and dosage effects need to be integrated into the conceptualisation of suppressor gene action" (Fearon and Vogelstein, 1990).

4.4.1. Carcinomas

Table 4.1 is a compilation of the data obtained for the panel of 13 carcinomas studied with respect to allelic changes on chromosomes 5, 17 and 18 and mutations of the K-ras gene. The number of changes observed is illustrated graphically in figure 4.1(a), with 92% of carcinomas showing at least one of these changes; 77% two or more; 23% three or more and 1 carcinoma (8%) showed all four changes.

Table 4.1 Correlation of K-ras activation and allele losses in colorectal carcinomas.

Sample	K-ras mutation	LOH Chr.5	LOH Chr.17	LOH Chr.18
APC				
72	+	-	+	+
112	+	+	+	+
119	-	-	+	+
130	-	-	-	+
131	+	-	-	+
134	-	+	+	-
141	-	-	+	+
145	-	-	+	+
147	+	-	-	+
150	-	-	-	-
158	-	-	-	+
161	+	+	-	-
sporadic				
HB	-	+	+	+

No obvious conclusions could be drawn between the various changes observed and the Dukes' staging of the tumours although it is important to realise that the sample size

is relatively small and the histopathological data available was quite limited. Also, it has been suggested that the Dukes' staging is not a reliable indication of prognosis (Kerr et al., 1986) and it may be that detailed molecular genetic data will reflect more accurately the severity of the neoplasia and the risk of recurrence as has been suggested by measurement of fractional allelic loss (Vogelstein et al., 1989).

4.4.2. Adenomas

The only changes observed in the adenoma samples investigated were K-ras mutations and chromosome 5 allele loss. No evidence of deletions on chromosomes 17 or 18 was seen indicating that, as a rule, these changes occur later in the pathway of tumour progression (figure 4.1b). The four adenomas exhibiting chromosome 5 allele loss from patient 145 were the largest of the 10 looked at from this patient and one of these also contained a K-ras mutation (sample 10, table 3.7). Of the two adenomas from patient 57 and the adenoma from patient 146 which exhibited LOH on chromosome 5, neither showed K-ras activation.It is possible that there may exist a degree of correlation between the occurrence of K-ras mutations and adenoma size but no obvious correlation exists between K-ras activation and degree of dysplasia. It is interesting that even though adenoma 146 was very large (~50mm) it was of the tubular type and only mildly dysplastic (table 3.6). The complete allele loss seen in this adenoma using the chromosome 5 probes (figure 3.8b) suggests that this loss occurred early in terms of adenoma formation as there is no evidence for the existence of more than one cellular clone. However, no ras activation was detected suggesting that further genetic changes are not a consequence of chromosome 5 loss. Possibly methylation status plays a role in subsequent tumour development (Goelz et al., 1985). It seems certain that chromosome 5 loss alone is not sufficient for the development of a more aggressive, villous-type adenoma. Unfortunately, no material was available from the cancer with which this patient presented. This data illustrates that it is possible for a tumour to grow large but remain benign due to the absence of further genetic aberrations.

4.4.3. Accumulation rather than order?

It has been suggested by previous studies that at least 4 independent genetic events are a necessary prerequisite for malignant transformation of the colorectal epithelium (Vogelstein et al., 1988) and that certain changes are more often associated with a particular stage of tumour development (Fearon and Vogelstein, 1990). The observation that genetic changes involving chromosome 5 and activation of the K-ras gene by point mutation generally precede those changes on chromosomes 17 and 18 is a hypothesis which is supported by the results presented in this thesis (figure 4.1b). No evidence for allele loss on chromosomes 17 and 18 preceding those on chromosome 5 or K-ras has been established. Larger studies, however, have revealed that certain changes are not restricted to a particular stage of tumour development and that the overall accumulation of these changes is more important than the order in which they occur (Fearon and Vogelstein, 1990). It seems that the process of adenoma progression is a continual one resulting from "successive waves of clonal expansion" involving at various times the genetic events previously discussed.

4.5. New mutations and genetic heterogeneity

A recent retrospective survey in Cleveland, Ohio, has estimated that spontaneous mutation is responsible for the symptoms of APC in around 22% of families (Rustin et al., 1990). If this were true and these patients followed a normal reproductive pattern, the incidence of APC could be expected to increase exponentially. This study examined 5 "new" mutation pedigrees initially to determine whether or not non-paternity was a likely cause of this apparently high mutation rate. Hybridisation of DNA from patients and parents to a panel of highly polymorphic DNA minisatellite probes was carried out and revealed that in all cases there was good agreement between the allelic patterns observed in the parents and the inheritance of these alleles in the patients (figures 3.24-3.27). The parents of these patients (except family -patient 146) have been examined by sigmoidoscopy and found to be clear. It seems likely therefore that all five represent cases of "new" or spontaneous mutations at the APC locus.

The pattern of expression of the APC gene in terms of its phenotypic implications was examined as far as possible in these kindreds. Symptomatic data was available for the patients 145, 146, 57 and 417II1and 409II1. All apparently exhibited early onset of the disease. Patient 145 presented with two cancers at age 23, patient 146 with a cancer and lymphatic metastases at age 29, patient 417II1 with a Dukes' B carcinoma at 23 and patient 57 presented with symptoms and underwent a colectomy at 15.Patient 409II1 presented with an enormous desmoid at the age of 24, along with multiple sebaceous cysts and osteomas and was reported to have suffered with various "lumps" since a small child. Superficial study of the symptoms in these patients suggests that they possibly possess a more severe allelic form of the disease and the reason for there not being an exponential increase in patient numbers maybe due to early onset of cancer precluding reproduction. Observations of the symptoms suffered by patient 409II1 supports the theory that extracolonic manifestation is higher in spontaneous mutation cases than in the general APC population (Rustin et al., 1990).The study of Rustin et al. (1990) supports the hypothesis that these patients harbour a more severe form of the disease and a greater proportion present with both colorectal cancer and extracolonic cancer.The idea that there exists heterogeneity within the APC locus has been mooted for some time and there exists the possibility that different ethnic populations have different disease aetiologies. Chromosome 22 involvement in Japanese APC patients (Sasazuki et al., 1989) appears to occur more often than in other populations (Vogelstein et al., 1989) and the increased incidence of upper GI cancers in Japanese patients supports this theory (Utsinomiya, 1990). The observations that some patients present with Gardner's syndrome (Gardner, 1951), osteomas (Gardner and Plenk, 1952), desmoids (Smith, 1958; 1959) and CHRPEs (Blair and Trempe, 1980) suggests that there are several allelic possibilities at the APC locus responsible i.e. a spectrum of possible changes all linked to the same locus which can only be explained by the existence of a range of mutated alleles with varying effects on cellular hyperproliferation. It seems that Gardner's syndrome and APC patients can be classified into two groups with either sparse or profuse polyposis. Gardner's syndrome is much more common in the group with sparse polyposis (Utsinomiya, 1990)

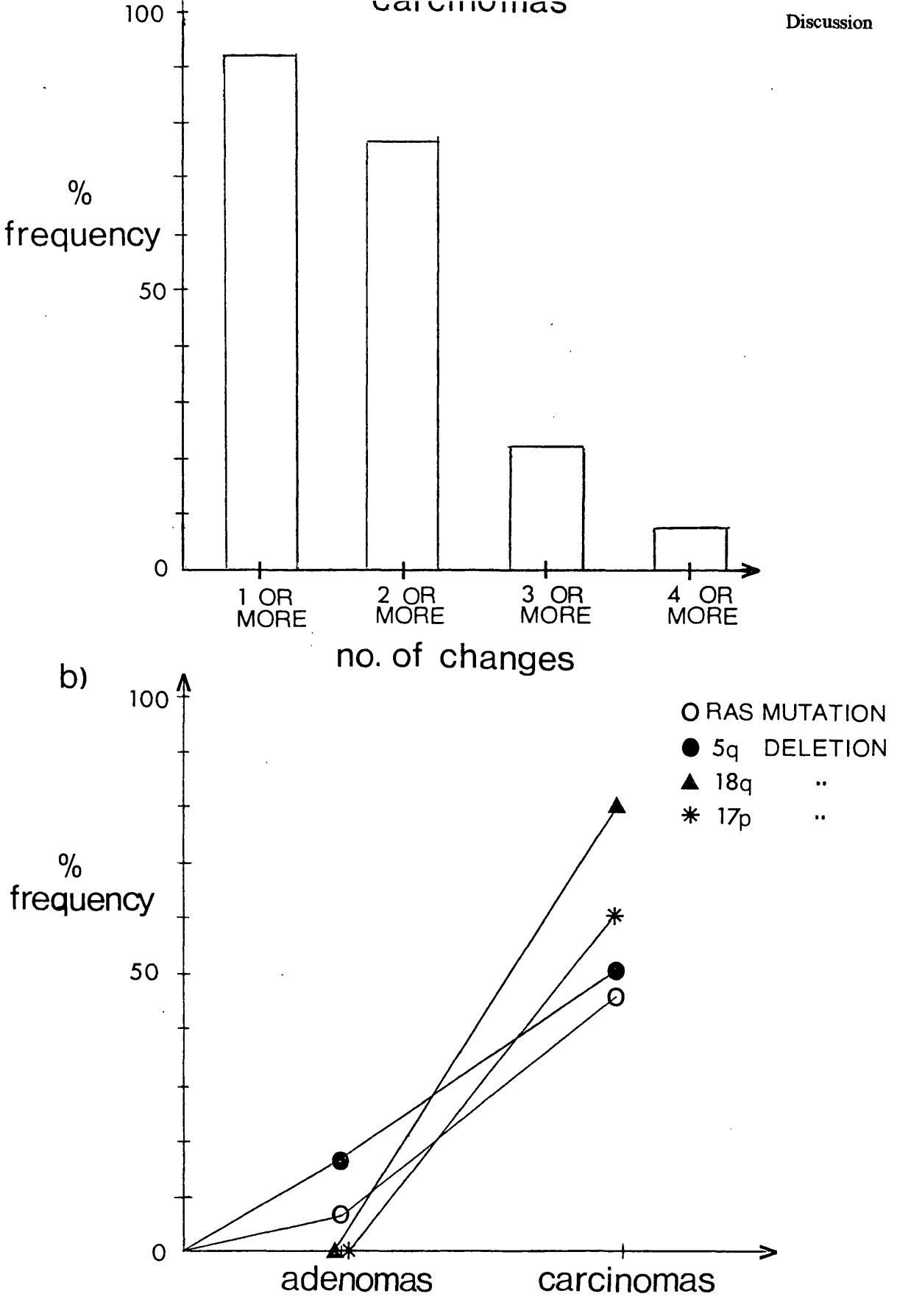


Figure 4.1a) Accumulation of genetic events in a panel of 13 colorectal carcinomas.**b)** Occurrence of genetic alterations in APC adenomas and carcinomas

suggesting that different mutated alleles are responsible for the differences seen with respect to frequency of extracolonic manifestations.

One of the most striking observations of this study is that although generally very infrequent in APC adenomas (Law et al., 1988), chromosome 5 allele loss is much more frequent in the adenomas of patients who have undergone spontaneous germline mutations at the APC locus. Several adenomas from new mutation patients 57, 145 and 146 were studied and shown to exhibit LOH with chromosome 5 markers in contrast to the results seen in the majority of our patients (Rees et al., 1989) and the experience of other workers (Solomon et al., 1987; Vogelstein et al., 1988; Law et al., 1988) suggesting that mutated alleles which arise spontaneously are of a type which encourage loss of the remaining normal allele perhaps leading to a higher malignant potential.

The parental chromosome on which the new mutations arose was determined in patients 145 and 146 (figures 3.28 and 3.29) by examining the inheritance of the lost and retained alleles. Presumably, if the APC gene represents a true tumour suppressor i.e. one which acts recessively at the cellular level, the lost allele will represent the normal APC homologue whilst the one which is retained will harbour the mutation. In the case of patient 145, the paternally-derived MS8 allele was lost suggesting that the mutation arose on the chromosome 5 inherited from the mother. Of course this analysis is dependent upon the assumption that allele loss detected at 5q35-qter is representative of proximal events at the APC locus which has been established in the case of patient 57. Ideally other informative markers are necessary to establish the extent of the chromosome 5 deletion seen in the material from patient 145 and a search for such markers is currently underway. The involvement of the APC locus in the deletion observed in patient 146 is unequivocal, being demonstrated by the markers ECB27 (centromeric to APC) and YN5.48 and EF544 (telomeric to APC) (Dunlop et al., 1990). Figure 3.29 demonstrated that in all cases the lost allele was inherited from the mother indicating paternal derivation of the chromosome 5 on which the mutation occurred. Unfortunately, no blood was obtained from the parents of patient 57 so such an analysis could not be carried out. The observation that the mutation arose on a

maternally-derived chromosome in one case and a paternally-derived chromosome in the other case could be interpreted as evidence that the phenomenon of imprinting does not occur in these cases of spontaneous mutations although it has been shown in other cancer predisposition syndromes (Wilkins, 1988).

4.6. Conclusions.

The results of the present study indicate that in general chromosome 5 deletions and point mutations of the K-ras oncogene occur earlier in the pathway of colorectal tumour progression than do changes associated with the DCC or p53 genes. Even though chromosome 5 allele loss is not a common occurrence in APC adenomas, it occurs with a much greater frequency in "new" mutation patients. This indicates that the constitutional mutation is much more destabilising and leads to a more severe manifestation of the disease pointing to a range of possible mutations within the APC locus which are responsible for the spectrum of changes seen in Gardner's syndrome and APC patients. It seems likely that the gene responsible is one with pleiotropic effects involving skin and colonic epithelium (Dunlop, 1990) and isolation of the gene will prove extremely interesting with respect to the analysis of mutations present in patients with different disease manifestations. It will be interesting to see if, like the NF-1 gene, the APC gene product will have homology to GAP and possibly interact with the $p21^{ras}$ gene product which in turn has been shown to cooperate with p53 (Parada et al., 1984). It is possible that the MCC gene = APC gene although recent doubt has been cast on this proposal and initial studies of MCC gene status have revealed no detectable alterations at the Southern level in the new mutation pedigrees.Sequencing of the MCC gene in these families using a pcr strategy is underway in Bert Vogelstein's laboratory although preliminary results have revealed no mutations. More recently, another promising candidate gene has been isolated and it remains to be seen whether or not there will be evidence for germline transmission of a mutation at this locus in APC individuals. Only when the APC gene has finally been isolated will any progress be made in understanding its complex expression and the various manifestations of mutations within its sequence.

5. APPENDIX

5. Appendix

A.1 Standard Buffers

TE	1mM Tris pH8
	0.1mM EDTA (disodium salt)
STE	150mM NaCl
	10mM Tris pH8
	10mM EDTA (disodium salt)
20xSSC	3M Nacl
	0.3M Na_3 citrate
	pH to 7.0
TAE- working strength	0.04M Tris.Hcl pH 7.9 - 8.1
	0.002M Na_2 EDTA
	0.02M NaOAc
	6.25% (^{V}/v) glacial acetic acid
TBE - working strength	0.089M Tris.HCl pH8
	0.089M Boric acid
	0.002 EDTA
20xSSPE	200mM Na_2PO_4 pH7
	3.6 M NaCl
	20mM EDTA (disodium)
Agarose gel	40% sucrose
loading buffer (10x)	0.025% ^{W}/v bromophenol blue.
	0.025% ^{W}/v xylene cyanol

A.2 Genomic DNA Extractions

Phenol Preparation : 0.1% (by weight) hydroxyquinolone was added to 1kg solid phenol in 1 litre 0.1M EDTA (disodium) and the mixture melted at 42°C. The resulting solution was shaken vigorously to form an emulsion and the top layer removed. The remainder was extracted once with 1 litre 0.1M Tris; 10mM EDTA and once with 1 litre 10mM Tris; 1mM EDTA. Both times the top layer was syphoned off and discarded. Finally the top layer was replaced with 100ml TE buffer.

Chloroform preparation : mixed in the ratio 24 parts $CHCl_3$ to 1 part isoamyl alcohol.

Hanks Balanced Salt Solution :

	g/l
NaCl	8
KCl	0.2
Na_2HPO_4 (anhydrous)	1.15
KH_2PO_4	0.2
Phenol red	0.015

The above were autoclaved at 15psi 121°C for 30 minutes and neutralised by the addition of 6% $NaHCO_3$ dropwise.

Versene : 0.2g EDTA (disodium salt) dissolved in 100ml sterile Hanks BSS working strength solution. 5ml 1% trypsin added before use and neutralised by addition of 6% $NaHCO_3$.

PBS : 10xPBS (Gibco) diluted to working strength with sterile deionised water and neutralised with sterile 1M NaOH.

A.3 Bacterial Culture Media and Antibiotic Concentrations

L-Broth	g per 100ml
Bacto tryptone	1.0
Bacto Yeast Extract	0.5
NaCl	0.5
Glucose	0.1

L-Agar	
Bacto tryptone	1.0
Bacto Yeast Extract	0.5
NaCl	0.5
Bacto Agar Noble	1.5

Antibiotic	working conc^{n}	stock conc^{n}	Solvent
	$\mu g\ ml^{-1}$	$\mu g\ ml^{-1}$	
Ampicillin	50	5	Water
*Tetracycline	15	1.5	Ethanol

* light sensitive - stored wrapped in aluminium foil.

A.4 Solutions for Large Scale Plasmid Preparations

Solution I

50mM Glucose

25mM Tris HCl pH8

10mM EDTA

Filtered C through 0.45µm filter.and stored at 4°

Solution II

0.15 - 0.2g NaOH

18ml ddH_2O

2ml 10% SDS

Made up fresh every time.

Solution III

3M potassium acetate

11.5% (V/v) glacial acetic acid

A.5 Standard Hybridisation Solutions

Southern Prehybridisation Mix (Genescreen Plus):

1M NaCl

5 - 10% $^{W}/_{v}$ dextran sulphate (Pharmacia LKB, Uppsala, Sweden)

1% SDS

Made up to required volume with sterile deionised water.

Southern Prehybridisation Mix (Hybond N+)

5xSSPE

5x Denhardt's solution

0.5% $^{W}/_{v}$ SDS

Salmon Sperm DNA :

Sigma type III DNA from salmon testes was made up to 10mg ml^{-1} with sterile deionised water and then sheared by passing through a 1.1mm needle, boiled for 10 minutes and then frozen at -20°C. Before use the appropriate amount was added to the labelled probe, boiled for 5 minutes, quenched on ice for 5 minutes and added to the prehybridisation mix.

Southern Hybridisation Mix :

As for prehybridisation mix, but with the addition of 100µg ml^{-1} sheared, denatured salmon sperm DNA and 50 - 100ng denatured radiolabelled probe.

Denhardt's Solution :

	g/100ml
Fraction V, Sigma BSA	0.4
Pharmacia Polyvinyl pyrrolidone	0.4
Pharmacia Polyethylene Glycol 400	0.4

A.6 Solutions for Radiolabelling DNA

Labelling Solution	DTM / OL / Hepes
Buffer (LS)	25 : 7 : 25

DTM

100μM dATP

100μM dGTP

100 μM dTTP

Each in 250mM Tris HCL pH8, 25 mM $MgCl_2$, 50mM β-mercaptoethanol filtered and stored at -20°C.

OL

90 OD units ml^{-1} hexanucleotides in 1mM Tris HCl pH7.5, 1mM EDTA. Pharmacia $d(N)_6$ (500.D. units) dissolved in 550μl Tris HCl. Filtered and stored at -20°C.

Hepes

1M pH6.6 autoclaved.

BSA

10mg ml^{-1} (acetylated to remove nucleases e.g. BRL).

10x Kinase buffer

0.7M Tris pH8

0.1M $MgCl_2$

50mM Dithiothreitol (DDT - Sigma).

A.7 Solutions for PCR

10x PCR buffer (Cetus):

100mM Tris.HCl pH8.4

500mM KCl

15mM $MgCl_2$

10x PCR buffer (Promega):

100mM Tris.HCl pH9.0

500mM KCl

15mM $MgCl_2$

0.1% $^{W}/_{v}$ gelatin

1% Triton X-100

Deoxynucleotides were obtained as 50 mg solid from Boehringer Mannheim and were dissolved in sterile ddH_2O, neutralised with sterile 0.1M Tris and made up to the appropriate stock concentration:

15mM dATP M_R 589.2 50mg in 5.66 ml

15mM dCTP M_R 511.1 50mg in 6.52 ml

15mM dGTP M_R 551.2 50mg in 6.05 ml

15mM dTTP M_R 570.2 50mg in 5.85 ml

Dideoxynucleotides for sequencing were obtained as 4μM solids (ddATP, ddCTP, ddGTP) and 10μM solid (ddTTP) from Pharmacia and were similarly made up to the required volume.

A.8 K-*ras* Exon 1 Amplification Primers

Primer K1 (sense)

5'.....ATGACTGAATATAAACTTGTG.....3'

Complementary to the non-coding strand, bases 1 - 21

Primer K2 (antisense)

5'.....CTCTATTGTTGGATCATATTC.....3'

Complementary to the coding strand, bases 90 - 111

A.9.1 Oligonucleotide Probe "Cocktails"

(antisense sequences)

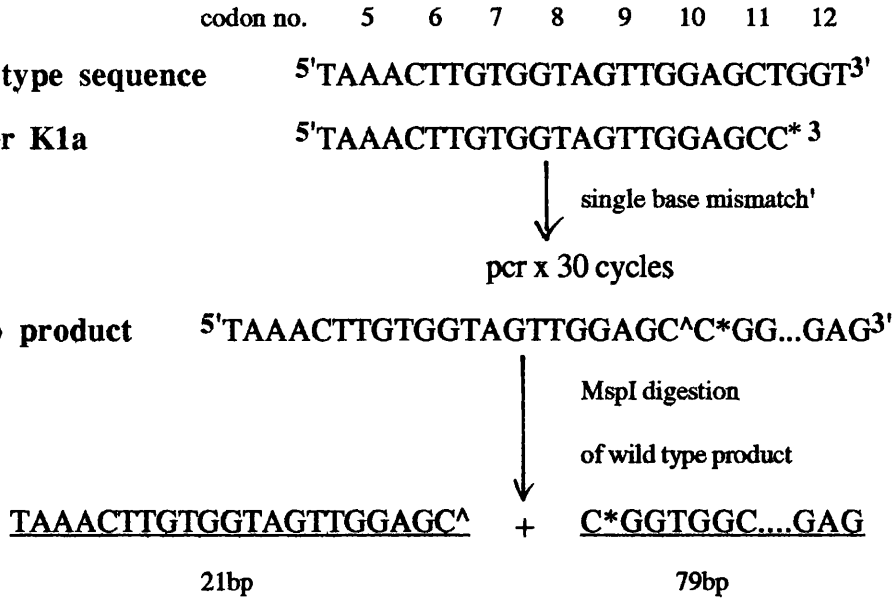
	13 12
Wild-type sequence:	TTGCCTAGCCACCAGCTCC.

Mutant cocktails

Codon 12.1	TTGCCTAGCCAC$^{A}G_{T}$AGCTCC
Codon 12.2	TTGCCTAGCCA$^{A}G_{T}$CAGCTCC
Codon 13.1	TTGCCTAGC$^{A}G_{T}$ACCAGCTCC
Codon 13.2	TTGCCTAG$^{A}G_{T}$CACCAGCTCC

A.9.2 Modified pcr of K-*ras* exon 1

(using primer K1a to introduce an artificial MspI site).



A point mutation at either position 1 or 2 of codon 12 will result in the MspI site not being created and therefore the product will remain undigested. In the case of a sample heterozygous for such a mutation, one copy will be digested and the other will not digest.resulting in the detection of both the 100bp product and the 79bp digested product. (Under normal conditions of electrophoresis the 21bp product will not be detectable)

A.10. Sequencing gel preparation

6% acrylamide; 7M urea 0.4mm thickness Made up in 0.5x TBE:

Volumes given are for 21 x 50 Biorad sequencing apparatus

5x TBE	5ml
urea	23g
40% acrylamide*	7.5ml
ddH_2O	19ml
total	40ml

The sequencing mix was incubated at 37°C with occasional mixing to completely dissolve the urea. 60µl of a 25% W/V solution of ammonium persulphate (Sigma) was added followed by 60µl Temed (Biorad). The solution was swirled briefly to mix and poured quickly into the gel mould using a 25ml syringe avoiding air bubbles and allowed to set (approximately 1 hour).Prior to sample loading the gel was prerun for an hour in 0.5x TBE buffer at a constant voltage of 2000V until the temperature reached 50-55°C.This is the temperature at which the best resolution of sequence is achieved. 5µl samples were denatured (together with 3µl of a formamide loading buffer**) at 95°C for 3 minutes and loaded onto the gel using a 24-well, 0.4mm sharkstooth comb. The gel was run under the same conditions for a further 2 hours and then removed and autoradiographed overnight at -70°C using Fuji X-ray film.

* 40% stock solution of 19:1 acrylamide:bis-acrylamide (Sigma) made up in sterile,deionised water and stored at 4°C.

** Formamide loading buffer:

98% deionised formamide

10mm EDTA (pH 8.0)

0.025% xylene cyanol

0.025% bromophenol blue

A 11.1 Polymorphic chromosome 5 probes used for Southern hybridisation

Location (HGM10)	Symbol (HGM10)	Probe name	Vector	Site	Insert (kb)	Antibiotic resistance	RFLP digest	Constant bands (kb)	Alleles (kb)	Freq.	PIC	Reference
5p	D5S110	MS621	pUC13	EcoRI/ HindIII		ampicillin	MboI AluI HinfI		hypervariable >10 alleles		0.92	
5q21	D5S37	П227	ПAN7	EcoRI/ HindIII	0.9	ampicillin	BclI		A1 8.6,1.2 A2 3.0 A3 1.8,1.2	0.20 0.37 0.43	0.56	Stewart et al., 1987
							BstXI		B1 2.7 B2 2.3	0.30 0.70	0.33	
							PstI		C1 4.3 C2 3.0	0.16 0.84	0.23	
							MboI		D1 0.55 D2 0.45	0.44 0.56	0.37	
5q14-q21	D5S71	C11p11	pUC8	EcoRI	3.6	ampicillin	TaqI	5.7, 5.4	A1 4.4 A2 3.9	0.08 0.92	0.14	Bodmer et al., 1987
5q15-q21	D5S98	ECB27	pUC18	SalI	2.8	ampicillin	BglII		A1 11.9 A2 10.5	0.38 0.62	0.36	Varesco et al., 1989
5q21-22	D5S81	YN5.48	pUC18	TaqI	2.4	ampicillin	TaqI		A1 6.0 A2 3.6			Nakamura et al., 1988
							MspI		B1 9.0 B2 8.0	0.5 0.5	0.38	
5q21-22	D5S84	MC5.61	pUC18	TaqI	3.0	ampicillin	MspI		A1 5.5 A2 5.0	0.49 0.51	0.37	Nakamura et al., 1988
							TaqI		B1 20 B2 18	0.54 0.46	0.37	
5q21-22		EF544	pUC18	Eco/Hind	1.9	ampicillin	MspI		A1 2.9 A2 2.1	0.18 0.82		Dunlop et al., 1990
5q21-22		L562	pUC18	Eco/Hind	1.2+ 0.9+ 0.7+ 0.65+ 0.6	ampicillin	BglII		A1 9.0 A2 5.5	0.93 0.07		Dunlop et al., 1990
5q33-q35	CSFIR	v-fms	pUC9	EcoRI	3.1	ampicillin	EcoRI		A1 29 A2 16,13	0.14 0.86	0.21	Xu et al., 1985
5q35-qter	D5S43	pMS8	pUC13	Eco/Hind	1.0	ampicillin	HinfI		Hypervariable	VNTR	0.90	Wong et al., 1987
5q22	MCC	MCC40	BS			ampicillin	EcoRI	many	A1 4.3 A2 4.1			B. Vogelstein, pers. comm.

A 11.2 Chromosome 17 probes

Location (HGM10)	Symbol (HGM10)	Probe name	Vector	Site	Insert (kb)	Antibiotic resistance	RFLP digest	Constant bands (kb)	Alleles (kb)	Freq.	PIC	Reference
17p13.3	D17S5	pYNZ22	pBR322	BamHI	1.7	ampicillin	RsaI	VNTR	>10		0.86	Nakamura et al.,
									1.3-2.3			1988
							MspI	VNTR	>10		0.86	
									1.5-3.0			
17p13	D17S34	p144D6	pSP65	BamHI	5.5	ampicillin	RsaI	VNTR	A1 5.3	0.08	0.86	Kondoleon et
									A2 3.8	0.02		al.,
									A3 3.5	0.16		1987;
									A4 3.4	0.02		Nakamura et al.,
									A5 3.3	0.02		1988
									A6 3.2	0.05		
									A7 3.1	0.16		
									A8 3.05	0.05		
									A9 3.0	0.05		
									A10 2.8	0.02		
									A11 2.55	0.02		
									A12 2.5	0.02		
17p13		pProsp53	pSP65	BamHI		ampicillin	BglII		A1 12.0			Hoyheim et al.,
									A2 10.0			1989
17p13-pter	D17S134	MS228	pUC13	BamHI	5.7	ampicillin	AluI	VNTR	0.8 -> 12		0.94	Armour et al.,
							MboI		0.6 ->5.9			1989

A 11.3 Chromosome 18

Location HGM10	Symbol HGM10	Probe name	Vector	Site	Insert (kb)	Antibiotic resistance	RFLP digest	Constant bands (kb)	Alleles (kb)	Freq.	PIC	Reference / Source
18q21.3	D18S8	OLVIIE10	pTZ18R	Eco/Hind	0.8	ampicillin	MspI		A1 11.0 A2 8.2 A3 7.4	0.26 0.28 0.46	0.57	Marlhens et al., 1987
18q21.3	DCC	Sam1.1	BS SK	EcoRI	1.1	ampicillin	EcoRI		A1 9.0 A2 1.1	0.5 0.5		B. Vogelstein
18q21.3	DCC	p15-65	BS KS	MspI	2.5	ampicillin	MspI		A1 10.5 A2 9.7 A3 7.8 A4 7.0	0.17 0.04 0.49 0.30		B. Vogelstein
18q21.3	DCC	Josh4.4	BS SK	EcoRI	4.4	ampicillin	PstI		A1 15 A2 10+5	0.5 0.5		B. Vogelstein
18q21.3-qter	D18S5	OS-4	pBR322	HindIII	1.0	ampicillin	TaqI		A1 8.6 A2 6.7	0.42 0.58	0.37	Tateishi et al., 1986
							PstI		B1 7.5 B2 4.8	0.65 0.35	0.35	
18q	D18S31	MS440	pUC18	BamHI		ampicillin	MboI AluI HaeIII		Hypervar-iable VNTR >10		0.72	Armour et al., 1990

A.11.4 Other minisatellite probes

Chromosome	Location HGM10	Symbol HGM10	Probe Name	% heterozygosity	Reference
1	p33-p35	D1S7	λMS1	99.4	Wong et al., 1987
1	q42-q43	D1S8	λMS32	97.5	"
7	q36-qter	D7S22	pλg3	97.4	"
7	p22-pter	D7S21	λMS31	98.0	"

A.12 Colorectal Cancer Cell Lines

CaCO2	Fogh et al., 1977
COLO320	ATCC Catalogue no. 220.1
HCA-7	Kirkland, 1985
HT29	Fogh and Trempe, 1975 Fogh et al., 1977.
JW2	Paraskeva et al., 1984
KMS-4	Namba et al., 1983
LIM1215	Whitehead et al., 1985
LIM1899	R.Whitehead, pers. comm.
SW480	Leibovitz et al., 1976

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Chromosome 5 allele loss in familial and sporadic colorectal adenomas

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Summary DNA extracted from familial and sporadic colorectal neoplasms was compared with constitutional DNA using a range of hypervariable locus specific probes to assess the extent of allele loss during conversion to malignancy. Chromosome 5 allele loss was observed in 23% of carcinoma samples, as previously found by others. However, we have been able to show for the first time loss of the D5S43 locus on chromosome 5 in adenomas from three patients, two of whom had the precancerous condition adenomatous polyposis coli (APC). These results suggest significant genetic changes involving chromosome 5 are occurring in benign adenomas. Probes for chromosome 1 (loci D1S7 and D1S8) and for chromosome 7 (loci D7S21 and D7S22) revealed no notable alterations in the adenoma samples. Complete loss of alleles for loci on chromosome 7 was not observed in carcinomas but reduced intensity of one parental allele was found in three specimens one of which was known to have multiple copies of this chromosome. Results using probes for chromosome 1 suggest that deletion of the D1S7 or D1S8 loci is not a common event in colorectal carcinogenesis. Loss of chromosome 5 alleles in adenomas from APC patients provides evidence in support of Knudson's hypothesis.

The observed characteristics of the autosomal dominant condition, adenomatous polyposis coli (APC–Human Gene Mapping 9, 1987), are hyperproliferation of epithelial and mesenchymal tissues (Bussey, 1975; Bulow, 1987) and widespread spontaneous chromosome instability (Gardner *et al.*, 1982; Delhanty *et al.*, 1983). Expansion of the proliferative compartment of the colonic crypts and shift of this region to the mucosal surface (Lipkin, 1988) results in the production of hundreds of adenomatous polyps by the second decade of life. Without surgical intervention progression to malignancy occurs in all cases (Muto *et al.*, 1977).

The gene for APC (also called FAP–familial adenomatous polyposis) has been mapped to chromosome 5, region 5q21-22 by virtue of close linkage to the probe C11p11 (Bodmer *et al.*, 1987; Leppert *et al.*, 1987). According to Knudson's hypothesis (Knudson, 1971), exemplified classically by retinoblastoma (Cavenee *et al.*, 1983), inheritance of one mutant form of the gene should be followed by loss or inactivation of the normal allele in tumorigenesis. Loss of chromosome 5 alleles relative to non-malignant tissue has indeed been found in three out of five informative APC cancers (Okamoto *et al.*, 1988). Following the retinoblastoma model, in sporadic cases of colorectal cancer reduction of heterozygosity for loci on chromosome 5 should also be demonstrable; evidence for this in at least 20% of cases has been gathered (Solomon *et al.*, 1987; Okamoto *et al.*, 1988). However, in these previous studies investigation of adenomas from APC patients revealed no loss of DNA restrictions fragments from chromosome 5 compared with normal tissue. We wish to report the first examples of such loss in adenomas from polyposis patients and from a normal individual, shown by the use of highly informative locus specific minisatellite probes (Wong *et al.*, 1987).

Materials and methods

Tissue samples

Tissue was obtained from 26 sporadic colorectal carcinomas, three sporadic adenomas from two patients, 48 adenomas from 21 APC patients, two colorectal cancers and a desmoid tumour from APC patients, together with corresponding normal mucosa or blood in all cases. With certain exceptions, the material came from patients at St Mark's Hospital, London; carcinoma specimens had been flash frozen in liquid nitrogen, adenomas were received fresh. Samples from patients 26, 49 and 52 were from Ashington Hospital, Northumberland, no. 29 came from the Royal Victoria Infirmary, Newcastle upon Tyne and adenomas from patient 50 were received from the Royal Naval Hospital, Plymouth; all these samples came as fresh tissue.

In addition, cells were cultured from a colon carcinoma cell line established from an APC patient, no. 27 (Paraskeva *et al.*, 1984); the corresponding normal fibroblasts were grown from a skin biopsy in this laboratory.

Adenomas from the majority of APC patients were 5 mm or less in diameter – the exceptions are listed in Table II. The two sporadic adenomas were 5 mm and 1 cm in size. None had any macroscopic evidence of malignant change.

DNA extraction and hybridisation

DNA was prepared from tissue samples and cultured cells by standard methods (Maniatis *et al.*, 1982). Samples were digested with the appropriate restriction endonuclease and were size fractionated by electrophoresis through 1% agarose gels. The DNA was transferred to Gene Screen Plus hybridisation membrane (NEN, Dupont) according to the manufacturer's specifications. DNA probes were radiolabelled with α-^{32}P-dCTP (3,000 Ci mmol^{-1}) by the random hexanucleotide primer method (Feinberg & Vogelstein, 1983) to a high specific activity. Hybridisations were performed at 65°C in 1% SDS, 1M NaCl and 5% dextran sulphate (w:v) for 16 h. Filters were washed to a stringency of 2 × SSC and were autoradiographed at −70°C using Fuji RX-L X-ray film.

DNA probes

The locus-specific hypervariable DNA probes used (obtained from ICI Diagnostics) were: λMS1, chromosome 1 (p33-p35), λMS8 (5q35-qter), λMS31 (7p22-pter), pλg3 (7q36-qter), all of which show polymorphisms with HinfI restrictions digests of genomic DNA, and λMS32 (1q42-q43) which requires AluI digests.

Results

The great advantage of the minisatellite probes is that they detect extremely variable loci with heterozygosites ranging from 90 to 99% (Wong *et al.*, 1987). However, if the locus detected by the probe is not close to the critical region of interest (as is the case for chromosome 5) loss of the whole or a substantial part of the chromosome will be detected but not small deletions which may allow expression of recessive

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Patient no.	Probe and chromosome				
	λMS1 1p33-p35	λMS32 1q42-q43	λMS8 5q35-qter	λMS31 7p22-pter	pλg3 7q36-qter
Sporadic cases					
1	1.2	–	–	1.2	1.2
2	1.2	1.2	1.2	–	1.2
3	1.2	1.2	1.2	1.2	1.2
4	1.2	1.2	1.2	–[b]	1.2
5	1.2	1.2	1.2	1.2	1.2
6	1.2	1.2	1.2	1.2	1.2
7	**1.2**[a]	1.2	–	–	1.2
8	1.2	**1.2**[a]	1.2	1.2	–
9	1.2	1.2	1.2	1.2	1.2
10	1.2	1.2	1.2	–	1.2
11	1.2	**1.2**[a]	–	1.2	1.2
12	1.2	1.2	**1.(2)**	1.2	**(1).2**
13	1.2	1.2	–	1.2	1.2
14	1.2	1.2	1.2	1.2	1.2
15	1.2	1.2	**2**	1.2	–
16	–	1.2	1.2	1.2	1.2
17	1.2	–	1.2	1.2	1.2
18	1.2	1.2	–	1.2	1.2
19		1.2	**(1).2**		
20		1.2	1.2		
21		1.2	1.2		
22			1.2		
23	1.2	1.2	–	**(1).2**[c]	1.2
24	1.2	1.2	1.2	**1.2**[c]	1.2
25	1.2	1.2	**2**	1.2	1.2
26	1.2	1.2	**(1).2**	–	**1.(2)**
APC					
27		**1**	–	1.2	1.2
28	1.2	1.2	1.2	1.2	–
29	1.2		1.2	1.2	1.2

Homozygosity in the constitutional DNA is indicated as a dash; where the normal tissue was informative the tumour genotype is shown in the table. Heterozygosity is indicated by 1.2 even though some probes recognise multi-allelic systems. The continued presence of the larger allelic restriction fragment is indicated by '1' and '2' indicates continued presence of the smaller allelic restriction fragment. Reduction of intensity is indicated by ( ). Absence of an entry indicates not tested or no result. [a]Altered band size in cancer DNA; [b]decreased intensity of band in cancer; [c]additional band(s) in cancer.

Table II Allele changes in familial and sporadic colorectal adenomas

Patient no.	No. of adenomas	Size[a]	Probe and chromosome				
			λMS1 1p33-p35	λMS32 1q42-q43	λMS8 5q35-qter	λMS31 7p22-pter	pλg3 7q36-qter
APC							
30	3	6mm max.	1.2	1.2	–	1.2	1.2
31	3		1.2	1.2	1.2	1.2	1.2
32	5	largest 2cm	1.2	**1.2**[b]	1.2	1.2	1.2
33	1		–	1.2	1.2	–	1.2
34	3	6mm max.	1.2	1.2	**(1).2**[c]	1.2	1.2
35	3		1.2	1.2	–	1.2	1.2
36	3		1.2	1.2	–	1.2	–
37	3		–	1.2	1.2	1.2	1.2
38	2		1.2	1.2	1.2	1.2	1.2
39	2		1.2		1.2		
40	1				**1**		1.2
41	2	6mm max.		1.2	1.2	1.2	
42	1	7mm			1.2		
43	3		1.2	1.2	1.2	1.2	1.2
44	2		–	1.2	1.2	1.2	–
45	2		–	1.2	1.2		1.2
46	2		1.2	1.2	1.2		–
47	1				1.2		
48	3		–		1.2		1.2
49	1		1.2	–	–		1.2
50	2	1.5cm; 1cm	1.2	1.2	1.2	1.2	1.2
Sporadic cases							
51	2	1cm	1.2	1.2	1.2	1.2	1.2
52	1				**1**		
Desmoid (APC)							
53			1.2	1.2	1.2		1.2

Homozygosity in the constitutional DNA is indicated as a dash; where the normal tissue was informative the tumour genotype is shown in the table. Heterozygosity is indicated by 1.2 even though some probes recognise multi-allelic systems. The continued presence of the larger allelic restriction fragment is indicated by '1' and '2' indicates continued presence of the smaller allelic restriction fragment. Reduction of intensity is indicated by ( ). Absence of an entry indicates not tested or no result. [a]Adenomas ≤ 5 mm diameter unless otherwise stated; [b]altered band size DNA from largest polyp; [c]reduced intensity of larger allele in two separate polyp DNA samples.

mutations in the APC gene. Hence the number of changes detected may be a gross underestimate.

The results obtained by hybridisation of the probes to the matched normal and carcinoma pairs are shown in Table I and those for the adenomas in Table II. A total of 23 carcinoma patients were informative for the probe λMS1, which recognises the locus D1S7 on chromosome 1; all the cancer samples retained heterozygosity. Thirteen adenoma patients (11 of them APC) were also informative with this probe; none showed any changes with adenoma formation. Heterozygosity for the second chromosome 1 probe, λMS32 (locus D1S8), was revealed in 22 carcinoma patients; clear allele loss was found in one case (no. 27), the cancer cell line derived from an APC patient, while DNA from two sporadic cancers (nos. 8 and 11) showed different sized bands compared with the normal counterpart (Figure 1). Of eleven informative adenoma patients (10 of them APC) a single adenoma from a total of five from one APC patient (no. 32) showed an altered band size; this specimen was a 2 cm diameter sessile villous polyp (Figure 1).

With the chromosome 5 probe, λMS8 (for locus D5S43), 22 carcinoma patients proved informative and allele loss was seen in two cancers (nos. 15 and 25) with decreased intensity of one allele in a further three (nos. 12, 19 and 26); all these were sporadic cancers (Figure 2a). Among 19 informative adenoma patients (17 of them APC) three gave evidence of allele loss in DNA from adenoma tissue. A clear reduction in intensity of the larger allele was seen in two of the three adenomas examined from one APC patient (no. 34). DNA extracted from a single adenoma from a second APC patient (no. 40) had complete loss of the smaller allele, while DNA from the sporadic polyp of patient 52 showed a similar loss (Figure 2b).

Two probes were available for chromosome 7. A total of 20 carcinoma and 10 adenoma patients were informative with λMS31 (D7S21). One of the carcinomas (no. 23) exhibited reduced allele intensity together with the appearance of two new bands (Figure 3a); additional bands were also seen in a further carcinoma (no. 24). No obvious changes were detected in the adenomas. Finally, the pλg3 probe (D7S22) detected heterozygosity in 22 carcinoma patients, of which two (12 and 26, both sporadic cases) showed a definite reduction in intensity of one allele in cancer DNA (Figure 3b). Chromosomes prepared from a short-term culture of the cancer from patient 26 revealed four copies of chromosome 7 in diploid cells. With this probe the 15 informative adenoma patients remained heterozygous in all samples tested.

The desmoid tumour (a benign neoplasm of mesenchymal origin) from an APC patient (no. 53) who was informative at one locus for each tested chromosome showed no change from the constitutional type.

Reduced intensity of one of a pair of allelic fragments rather than complete loss probably reflects the presence of normal stromal tissue in the neoplasm, the coexistence of more than one clone, or duplication of one allele at the expense of the other.

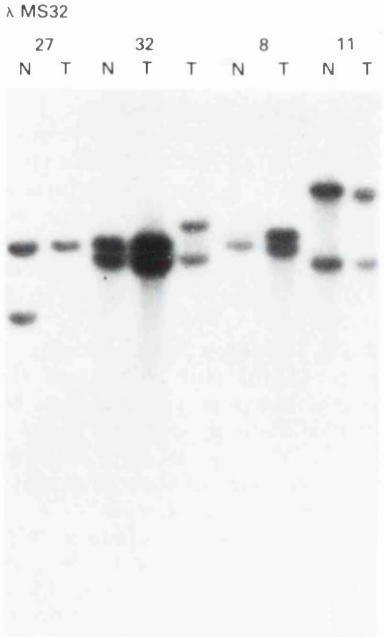

Figure 1 Autoradiograph of Southern hybridisation with λMS32. Patient numbers are indicated above the tracks. N = constitutional (normal) DNA, T = tumour DNA. For tumour classification see Tables I and II. In the case of patient 32, DNA from two adenomas was examined, of which one showed a change in allele size. Changes in allele size are thought to represent somatic mutation which is not necessarily involved in tumorigenesis.

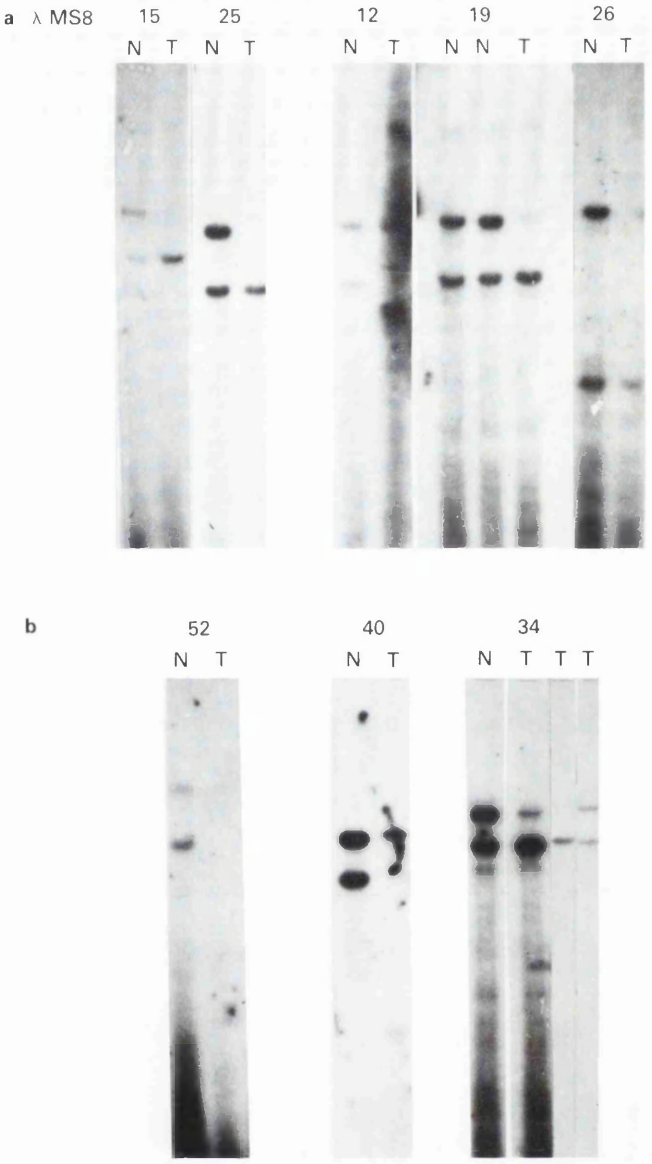

Figure 2 Autoradiographs of Southern hybridisation with λMS8; (**a**) carcinoma patients; (**b**) adenoma patients. Patient numbers are indicated above the tracks. N = constitutional DNA, T = tumour DNA. Patient nos. 15, 25, 40 and 52 show allele loss, whereas patients 12, 19, 26 and 34 show reduced allele hybridisation intensity. In the case of patient 34 changes were detected in DNA from two adenomas while a third retained the constitutional type.

Discussion

We have compared DNA extracted from a number of colorectal neoplasms with constitutional DNA using a range of highly informative locus specific probes.

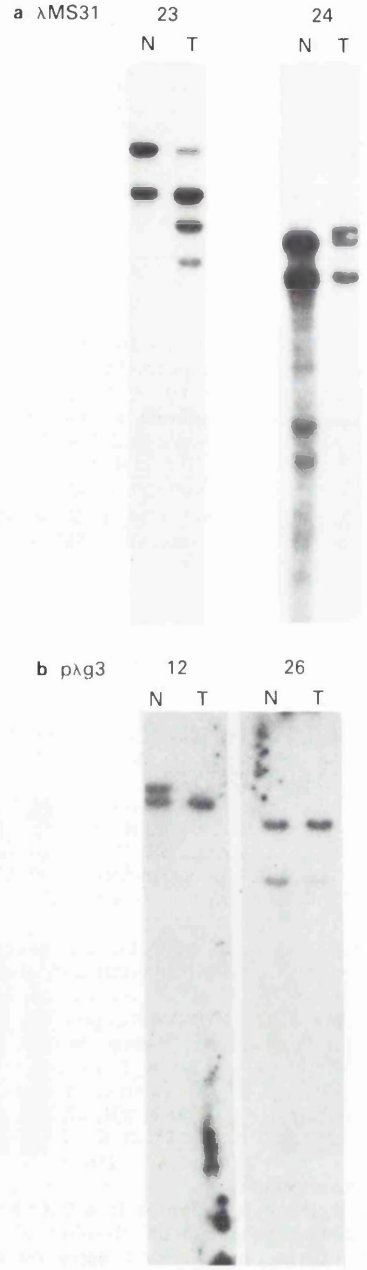

Figure 3 Autoradiographs of Southern hybridisations from carcinoma patients with (**a**) λMS31 and (**b**) pλg3. N = constitutional DNA, T = tumour DNA.

In accordance with the findings of Solomon *et al.* (1987) and Okamoto *et al.* (1988) chromosome 5 allele loss was observed with λMS8 (D5S43) in 23% of carcinoma samples. However, we have been able to show for the first time loss of the D5S43 locus in adenomas from familial polyposis patients (Table III), demonstrating probable APC allele loss in not only the APC precancerous condition, but also in an adenoma from a non-APC individual. These results suggest that significant genetic changes involving chromosome 5 are occurring in benign adenomas, whereas the other probes tested revealed no notable alterations in the adenoma samples. In view of the number of polyps typically present in APC patients, an average of just over 1,000 in counted colectomy specimens, the rate of conversion to malignancy is low (Bussey, 1975). Allele loss in three of 38 informative adenomas thus appears to be significant. While this paper was in preparation Law and colleagues published the results of their study on non-syntenic allelic loss in colorectal carcinomas and adenomas (Law *et al.*, 1988). They found no allelic loss from chromosome 5 in 40 adenomas from APC

Table III Summary of results obtained with locus-specific probes

Probe	*No. informative patients*	*Allele loss*	*Decreased allele intensity*	*Altered allele size*
Carcinomas				
λMS1	23	–	–	1
λMS32	25	1	–	2
λMS8	22	2	3	–
λMS31	20	–	1	2
pλg3	22	–	2	–
Adenomas				
λMS1	13	–	–	–
λMS32	16	–	–	1
λMS8	19	2	2[a]	–
λMS31	13	–	–	–
pλg3	15	–	–	–

[a]Two adenomas from one patient.

patients who were informative for at least one chromosome 5 probe; no information on size of the adenomas was given. The difference between their findings and ours may simply be due to sampling from the multitudes of polyps available or may reflect differences between patients. Two of the losses we observed were from adenomas of a single patient, a 15-year-old with exceptionally well developed adenomas considering his age. Chromosomes prepared from a 48-h culture of a smaller adenoma from this same patient showed random loss or gain (sometimes both) of chromosomes in 11 of 26 cells analysed.

Trisomy of chromosome 7 in colorectal carcinogenesis has been reported previously (Reichmann *et al.*, 1985). In this study, while complete loss was not seen, reduced intensity of alleles on this chromosome was observed in DNA from three separate carcinomas, one of which was known to have multiple copies of chromosome 7. The DNA results indicate duplication of one parental chromosome at the expense of the other in the latter case.

Increased copy number of this chromosome is thought to be important in carcinogenesis of solid tumours in general (Van Der Berghe, 1987). The various proto-oncogenes mapped to chromosome 7 are obvious candidates for a role in this process (Human Gene Mapping 9, 1987).

In common with most malignancies, chromosome 1 structural alterations are frequent in colorectal cancer (Reichmann *et al.*, 1984). Before this study we had evidence for loss of expression of the α-fucosidase gene (located at 1p34) in two of six informative colorectal cancers, although phosphoglucomutase 1 (at 1p22) expression remained, suggesting loss or deletion of part of chromosome 1p (our unpublished observations using isoenzyme analysis, S.H. Rider, M.B. Davis & J.D.A. Delhanty). Use of the hypervariable probe λMS1 in 23 informative colorectal cancers failed to detect allele loss in the region 1p33-p35 in this larger sample.

The appearance of additional or altered sized bands in the samples when probed with both λMS32 and 31 may be due to the high somatic mutation rate known to be detected in this type of material with these probes (J.A.C. Armour, I. Patel, S.L. Thein, M. Fey & A.J. Jeffreys, manuscript submitted). The significance of such mutations with respect to oncogenesis is unknown at present.

Loss of a normal gene product is thought to play a critical role in the generation of several embryonal tumours (Cavenee *et al.*, 1983; Koufos *et al.*, 1984; Orkin *et al.*, 1984) and certain adult cancers (Koufos *et al.*, 1985; Fearon *et al.*, 1985; Kok *et al.*, 1987). APC is unusual in that heterozygosity for the deficiency gives rise to local growth excesses, possibly through a threshold effect produced by fluctuating levels of gene product (Solomon *et al.*, 1987). The smallest adenomas in this condition can be viewed as simply a manifestation of hyperproliferation. Post-colectomy regression of rectal polyps has been observed (Feinberg *et al.*,

1988), which suggests that no irreversible genetic change has occurred. Demonstration of the clonal origin of these adenomas (Fearon *et al.*, 1987) is not incompatible with this hypothesis since the colonic crypts are known to be maintained by a single stem cell (Griffiths *et al.*, 1988). Larger adenomas would be expected to have undergone one or more genetic changes of a clonal nature; we have provided evidence for this in three adenomas from two polyposis patients. In view of the multistage nature of carcinogenesis it is probable that large adenomas will have undergone several gene or chromosome mutations before reaching the fully malignant state.

In normal people loss or mutation of one copy of their two normal alleles of the APC gene would be expected to be an early event to initiate the requisite hyperproliferation for adenoma formation. Loss of chromosome 5 alleles would thus be expected in some small sporadic adenomas, of which we have one example. Chromosome instability would presumably be conferred by the heterozygous state, providing a mechanism for further genetic change by means of deletion or somatic crossing over leading to homozygosity or functional hemizygosity for critical loci on chromosome 5 or on chromosomes 17, 18 and 22. The latter chromosomes have recently been implicated in colorectal cancer by cytogenetic (Muleris *et al.*, 1987) and molecular data (Fearon *et al.*, 1987; Okamoto *et al.*, 1988). Use of polymorphic DNA probes which are closer to the critical region of chromosome 5 as well as those assigned to chromosomes 17, 18 and 22 will enable us to obtain a more complete picture of the genetic events leading from adenoma to carcinoma in both polyposis patients and normal individuals.

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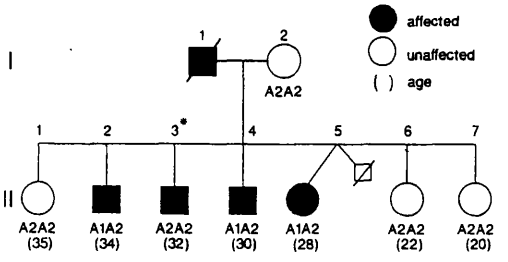
FAMILIAL POLYPOSIS COLI

SIR,—The gene for familial polyposis coli (adenomatous polyposis coli [APC]) is linked to the probe C11p11 (D5S71), which maps to chromosome 5 in the region q21-q22.[1,2] No recombinants have been reported. We have investigated the linkage relation between C11p11 and the APC gene in seven previously untested families from the polyposis registry at St Mark's Hospital, London, and have found an example of paternal recombination.

DNA was extracted from blood or normal colonic mucosa by standard methods and Southern blot analyses were done.[1] C11p11 reveals a *Taq1* polymorphism with alleles A1 at 4·4 kb and A2 at 3·9 kb. The frequencies for A1 and A2 were initially estimated as 0·08 and 0·92, respectively.[1]

Two families were informative; one has at least one recombinant, II 3 in the figure. The C11p11 typing was confirmed in more than one tissue for this individual. Hence the probe is not as closely linked to the disease gene as was originally hoped.



Pedigree of APC family.

A1 and A2 = 4·4 and 3·9 kb alleles of probe C11p11. * = the recombinant.

Initial use of the probe in polyposis patients in this laboratory resulted in a heterozygote frequency approximately double that expected from reported allele frequencies.[1] Subsequent investigation of randomly ascertained individuals revealed an almost identical allele frequency of 0·2 for A1 and 0·8 for A2 in the two groups. The 60 controls and 26 patients examined were mainly caucasian. These gene frequencies agree with those reported from the United States.[2] The probe C11p11 is thus likely to be more informative in Northern European families than at first predicted.

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CLOBAZAM FOR EPILEPSY

SIR,—We agree with Dr Scott (Aug 6, p 337) that clobazam is a useful drug for the treatment of chronic intractable epilepsy, but have found tolerance to be a drawback. In a study of 41 chronic drug-resistant adolescent or adult patients, 25 (61%) showed an initial dramatic response (over 90% reduction of seizures) at one month of follow-up.[1] However, 45% of these responders were tolerant at 6 months and 64% at a year of follow-up. Nevertheless, for 9 patients (22% of the study group) to be seizure-free, or nearly so, after a year of treatment is a considerable achievement. Patients with a known cause of their epilepsy, with complex partial seizures without secondary generalisation, and without mental retardation were more likely to have a sustained striking response to the drug.

We also agree that clobazam treatment in epilepsy is not associated with the usual features of dependency. Withdrawal seizures occur, as with all antiepileptic drugs, but no more so in patients who have become tolerant than in those whose epilepsy remains well controlled. Tolerance to the antiepileptic action of the drug is not therefore related to dependency, which seems to undermine the theory, supported by Dr Feeley and Dr Haigh (June 25, p 1460), that tolerance and dependency result from the same adaptive mechanisms.

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USE OF MIDAZOLAM IN CHILDREN

SIR,—Your July 16 editorial on midazolam calls attention to its side-effects, especially in elderly patients. We frequently use midazolam as an anxiolytic and sedative in children before bone marrow biopsy and other invasive procedures. It provides good sedation, causes less local irritation with intravenous injection, and has a shorter elimination half life (1–4 h)[1,2] than other benzodiazepines. Furthermore, its antegrade amnesia effect confers no recollection of the unpleasant and painful procedures.

Children are, however, resistant to the action of midazolam, and there is considerable individual variation in the dose requirement, which is age-related and may partly be affected by emotional state. But its rapid onset of action following intravenous administration allows titration of the dose to achieve the optimum effect. Total doses for adequate sedation range from 0·2 to 1·25 mg/kg.[3] We followed these requirements in our children and most of them needed 0·3–0·5 mg/kg. Although the dose is greater than that recommended by the manufacturer, it is much the same as that commonly used for anaesthetic premedication.[1]

We have not seen complications such as arrhythmia or cardiorespiratory suppression on more than 100 occasions in the past two years. Therefore we think that midazolam is a safe and effective agent for sedation in children. However, we agree that precautions should include careful monitoring of the patient's condition and the provision of facilities for resuscitation. Great care should also be taken when giving the drug to patients in respiratory distress or who have impaired conscious states.

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GLYCERYL TRINITRATE IN SKIN NECROSIS CAUSED BY EXTRAVASATION OF PARENTERAL NUTRITION

SIR,—Skin necrosis due to extravasated intravenous infusions, which cause intense local vasoconstriction, causes morbidity in infants treated in special care units and is a common reason for litigation by their parents. Where skin loss occurs over a wide area or over a joint, grafting may be necessary.

In a newborn baby with extravasated parenteral nutrition solution we applied glyceryl trinitrate, a powerful local vasodilatator,[1,2] to an area about 2 × 3 cm on the dorsum of the foot, which still appeared white and ischaemic 2½ h after removal of the intravenous cannula. A patch containing glyceryl trinitrate 25 mg (releasing 5 mg per 24 h) was applied for an hour. On removal, the superficial epidermis sloughed but the area beneath appeared healthy and well perfused and has healed without scarring. A small