Abnormalities of Chromosome 9p and MYC in Lymphoid Malignancies

A thesis submitted for the Degree of Doctor of Philosophy in the Institute of Cancer Research at the University of London

by

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ABSTRACT

The aim of this thesis was to determine the genes involved in the recurrent chromosome 9p abnormalities seen in lymphoid malignancies. In particular, the frequency and extent of deletions involving the \( \textit{p16}^{\text{INK4A}} \) tumour suppressor gene at 9p21 in haematologic tumours was investigated. The involvement of \textit{MYC} at 8q24 with candidate genes at 9p13.3 in the translocation t(8;9)(q24.1;p13.3) was also studied.

Deletion of the cyclin dependant kinase inhibitor \( \textit{p16}^{\text{INK4A}} \) had been implicated in leukaemogenesis by limited studies on cell lines. Analysis here of 231 primary tumours showed that biallelic deletions of \( \textit{p16}^{\text{INK4A}} \) occurred in 18% of B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) and in some high grade and transformed non-Hodgkin’s lymphomas (NHL).

Abnormalities of \( \textit{p16}^{\text{INK4A}} \) were shown to correlate with the ability of BCP-ALL to grow in SCID mice. Deletions of \( \textit{p16}^{\text{INK4A}} \) were frequently undetectable cytogenetically, and their extent was variable, although most also involved the closely linked gene \( \textit{p15}^{\text{INK4B}} \) which may also function as a tumour suppressor gene. Analysis of heterozygosity indicated that a third lymphoid tumour suppressor gene may exist centromeric to \( \textit{p15}^{\text{INK4B}} \) on chromosome 9p.

The recurrent translocation t(8;9)(q24.1;p13.3) has been reported in high grade B-cell NHL and mature B-cell leukaemias. Two cell lines which possessed this translocation showed overexpression of \textit{MYC} mRNA. Pulsed field electrophoresis showed that in one cell line the breakpoint was within 70 kb 3’ of the \textit{MYC} gene, but this could not be pinpointed by conventional electrophoresis with the available probes for the region 3’ of \textit{MYC} nor with probes for candidate genes from 9p21.

Finally, allelic restriction of the expression of \textit{MYC} was investigated in a B-NHL cell line showing duplication of one \textit{MYC} allele and translocation of the other. Sequence analysis of PCR amplified genomic DNA and mRNA showed that only the translocated allele was transcribed.
Acknowledgements

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I am extremely grateful to all those who supplied me with cell lines or tumour material used in this thesis, and to Dr. Robert Huddart for the donation of fluorescent labelled primers for amplification of polymorphic repeats. I would also like to thank Dr. Janet Shipley and Dr. Ellie Nacheva for providing the FISH data and karyotype analysis discussed here.

Finally, special thanks to Nadine, Carol, Matt and Dave.

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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>adenosine</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukaemia</td>
</tr>
<tr>
<td>B-ALL</td>
<td>B-cell acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>B-CLL</td>
<td>B-cell chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>B-NHL</td>
<td>B-cell non-Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>B-PLL</td>
<td>B-cell prolymphocytic leukaemia</td>
</tr>
<tr>
<td>BCP-ALL</td>
<td>B-cell precursor acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt’s lymphoma</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment tool (version 1.4)</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CDI</td>
<td>cyclin dependant kinase inhibitor</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependant kinase</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myeloid leukaemia</td>
</tr>
<tr>
<td>CML-LBC</td>
<td>lymphoid blast crisis of chronic myeloid leukaemia</td>
</tr>
<tr>
<td>CML-OBC</td>
<td>myeloid blast crisis of chronic myeloid leukaemia</td>
</tr>
<tr>
<td>CR</td>
<td>complete remission</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxy adenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxy cytosine triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxy guanidine triphosphate</td>
</tr>
<tr>
<td>DLCL</td>
<td>diffuse large cell lymphoma</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxy thymidine triphosphate</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FL</td>
<td>follicular lymphoma</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in-situ hybridisation</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3 phosphate dehydrogenase</td>
</tr>
<tr>
<td>G</td>
<td>guanidine</td>
</tr>
<tr>
<td>HCL</td>
<td>hairy cell leukaemia</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>HGMP</td>
<td>Human Genome Mapping Project</td>
</tr>
<tr>
<td>IGH</td>
<td>immunoglobulin heavy chain</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D- thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LGL</td>
<td>large granulocytic lymphoma</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>M-phase</td>
<td>mitosis (of cell cycle)</td>
</tr>
</tbody>
</table>
Mbp megabase pair
MCL mantle cell lymphoma
MIF MYC inhibitory factor
MOPS 3-[N-Morpholino]propanesulphonic acid
MZL marginal zone lymphoma
NHL non-Hodgkin’s lymphoma
NS subtype not specified
NT not tested
PCR polymerase chain reaction
PEG polyethylene glycol
RFLP restriction fragment length polymorphism
RNA ribonucleic acid
S-phase synthesis phase (of cell cycle)
SCF stem cell factor
SCID severe combined immuno-deficiency/deficient
SDS sodium dodecyl sulphate
SLVL splenic lymphoma with villous lymphocytes
STS sequence tagged site
T thymidine
T-ALL T-cell acute lymphoblastic leukaemia
TCP-ALL T-cell precursor acute lymphoblastic leukaemia
TdT terminal transferase
TGF-β transforming growth factor beta
T-PLL T-cell prolymphocytic leukaemia
UV ultra violet light
WCC white cell count
XGAL 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YAC yeast artificial chromosome

Cytogenetic Nomenclature - some examples

del(9)(p11p22) a deletion of the short arm of chromosome 9 involving bands 11 to 22
inv(7)(p13-p22) an inversion of bands 13-22 on the short arm of chromosome 7
iso(9q) an isochromosome consisting of two long arms of chromosome 9 joined by a centromere
t(8;9)(q24;p13) a reciprocal translocation involving band 24 of the long arm of chromosome 8 and band 13 of the short arm of chromosome 9
tdic(9;12) a non-reciprocal translocation between chromosome 9 and chromosome 12 resulting in a dicentromeric chromosome
1. Chapter One: Introduction

1.1 General Introduction

The unrestrained growth that is the hallmark of cancer cells, is the result of accumulated somatic mutations of genes involved in the control of growth, differentiation and programmed cell death pathways. In many tumours these aberrations can be seen at the cytogenetic level as gross chromosomal alterations. These cytogenetically visible chromosomal abnormalities include the exchange of DNA between chromosomes (known as translocations), as well as inversions, large deletions and amplifications. Although damage to chromatin was first proposed as a causal event in the cancer process almost 100 years ago, this idea was not generally accepted until much later when banding techniques allowed the identification of consistent recurring chromosomal changes in tumour cells.

Techniques for chromosome banding have revealed that distinct recurring chromosomal abnormalities are consistently associated with specific subtypes of disease in nearly all malignancies, in particular in leukaemias, lymphomas and sarcomas. These aberrations have often proved to be useful diagnostic and prognostic indicators, and molecular analysis of the breakpoint regions has allowed the identification of many of the critical genes involved in tumourigenesis.

Proto-oncogenes are defined as genes whose alteration has been shown to be involved in malignant transformation. Translocations may result in the activation of proto-oncogenes by juxtaposing them to new regulatory
sequences, or they may fuse two proto-oncogenes to create a chimaeric protein with novel functions. A common motif in tumours of mature lymphoid cells, is the translocation of proto-oncogenes into antigen receptor gene loci, the immunoglobulin and T-cell receptor genes. These loci undergo somatic rearrangement during normal lymphoid development and translocation may occur as a result of mistakes in this recombination mechanism. Translocated oncogenes are then expressed in a deregulated fashion, in part as a consequence of powerful lymphoid-specific enhancers within the antigen receptor loci. For example, the MYC and BCL2 oncogenes, which will be discussed in more detail in this thesis, are frequently deregulated in this way in lymphoid malignancies.

In contrast, examination of recurring regions of chromosomal deletion has allowed isolation of tumour suppressor genes that restrict neoplastic growth. Loss of tumour suppressor gene function typically requires two genetic events in order to inactivate both alleles, for example an inactivating point mutation of one allele and deletion of the second. A region of loss of heterozygosity (LOH) at loci surrounding tumour suppressor genes is common and reflects the deletion of one copy of this region.

Analysis of the role that oncogenes and tumour suppressor genes play in both normal and neoplastic lymphoid cells has lead to a better understanding of normal cell growth and differentiation, especially in relation to the development of blood cells from their precursors. It has also aided the diagnosis and management of these diseases and may eventually permit the development of tumour specific therapies.
Work presented in this thesis characterised some of the recurrent abnormalities of the short arm of chromosome 9 seen in lymphoid malignancies, and investigated the hypothesis that these were associated with disease progression, especially of some non-Hodgkin's lymphomas (NHL) in association with MYC translocation. Abnormalities of 9p in NHL include both recurrent translocations and interstitial deletions \(^{16}\), which may indicate that several genes involved in the pathogenesis of lymphoid tumours, are located in this region. Particular emphasis was placed on the frequency, extent and mechanism of deletions of the putative tumour suppressor gene \(p16^{INK4A}\) \(^{17}\) in lymphoid tumours and lymphoid cell lines. This gene encodes the cyclin dependant kinase inhibitor p16 and is located at 9p21. Expression studies of a B-cell specific transcription factor \(PAX-5\) at 9p21 were also performed in lymphoid tumours and cell lines.

Progression of low-grade follicular lymphoma possessing translocation of \(BCL2\), to high grade disease, is associated in some cases with translocation of the \(MYC\) oncogene \(^{18}\). This thesis investigated the molecular events leading to \(MYC\) deregulation in three leukaemic cell lines with \(BCL2\) translocations and/or deregulation of \(BCL2\). Molecular analysis of the recurring translocation t(8;9)(q24.1;p13.3) was undertaken, along with analysis of an unusual B-NHL cell line with three \(MYC\) alleles.

1.2 Lymphoid Malignancies

Non-Hodgkin's lymphomas (NHL) and the lymphoid leukaemias are cancers arising from B and T-lymphocytes. Leukaemias predominantly
manifest themselves in the blood and bone marrow, whilst the NHL generally involve the lymph nodes or other lymphoid tissues.

During the normal process of B-cell maturation, B-cell precursors in the bone marrow rearrange dispersed immunoglobulin gene segments to produce functional immunoglobulin genes. An enormous diversity of antibody molecules can be encoded in this way, allowing recognition of all possible antigens. B-cells expressing surface immunoglobulin then migrate to the follicle centre of the lymph nodes, where B-cells that produce antibodies that bind antigen are selected. B-cells that do not bind antigen in the follicle centres die by apoptosis. Leukaemias and lymphomas vary widely in their clinical behaviour, phenotype and response to therapy. This diversity has its biological basis in the complexity of the lymphoid system itself, but also results from the deregulation of specific combinations of genes within specific subsets of disease. A comparison of the B-cell maturation pathway with recognised subgroups of disease is shown schematically in Figure 1.1.

1.2.1 Classification of Lymphoid Malignancies

The phenotype of most lymphoid tumours appears to correspond to some point in the development of normal lymphoid cells, and most classifications of lymphoid malignancies attempt to relate the tumour cells to their closest normal counterparts. Lymphoma and chronic lymphoid leukaemia cells almost always retain their lineage specificity, whilst acute lymphoid leukaemias, which have a more immature phenotype, may occasionally show phenotypic characteristics of more than one haemopoietic lineage and may therefore be termed biphenotypic.
The classification of lymphomas and lymphoid leukaemias has been, and continues to be, based primarily on the morphology of the tumour cells. It has, however, evolved to incorporate information on cell surface antigens and more recently rearrangements of antigen receptor genes and oncogenes. Many classifications of lymphoid malignancies exist. A recently proposed classification based on morphology, cell surface antigens and oncogene rearrangements is outlined in Table 1.1.

Figure 1.1 B-cell differentiation pathway

Figure 1.1 Bars represent the nearest corresponding normal stage of B-cell differentiation of various B-cell malignancies with respect to the typical marker phenotype of the tumour cells. Abbreviations: μ = cytoplasmic immunoglobulin heavy chain molecules; Y = surface immunoglobulin molecules; BL = Burkitt's Lymphoma; B-NHL = B-cell non-Hodgkin's lymphoma; BCP-ALL = B-cell precursor acute lymphocytic leukaemia; B-ALL = B-cell acute lymphocytic leukaemia; B-CLL = chronic B-cell lymphocytic leukaemia; B-PLL = B-cell prolymphocytic leukaemia; HCL = hairy cell leukaemia.
Table 1.1. The REAL Classification of B-cell malignancies

<table>
<thead>
<tr>
<th>Disease</th>
<th>Postulated normal precursor</th>
<th>Immunophenotype</th>
<th>surface IG+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor B lymphoblastic leukaemia (BCP-ALL)</td>
<td>Bone marrow derived precursor B-cell</td>
<td>TdT+ CD19+ CD22+ CD10+</td>
<td>-</td>
</tr>
<tr>
<td>B-Cell Chronic Lymphocytic Leukaemia (B-PLL)</td>
<td>Recirculating CD5+ CD23+ peripheral B-cell</td>
<td>CD5+ CD19+ CD20+ CD23+</td>
<td>weak+</td>
</tr>
<tr>
<td>Lympho-plasmacytoid Lymphoma/Immunocytoma</td>
<td>CD5- plasma cell</td>
<td>CD5- CD10- CD19+ CD20+ CD22+</td>
<td>+</td>
</tr>
<tr>
<td>Mantle Cell Lymphoma (MCL)</td>
<td>CD5+ CD23- peripheral B-cell of inner follicle mantle</td>
<td>CD5+ CD19+ CD20+ CD22+ CD23-</td>
<td>+</td>
</tr>
<tr>
<td>Follicle Centre Cell Lymphoma (FL)</td>
<td>Germinal centre B-cells</td>
<td>CD5- CD10+/-CD19+ CD20+ CD22+</td>
<td>+</td>
</tr>
<tr>
<td>Marginal Zone Lymphoma (MZL)</td>
<td>Marginal zone B-cell of nodal or extranodal type</td>
<td>CD5- CD10-CD19+ CD20+ CD22+ CD23-</td>
<td>+</td>
</tr>
<tr>
<td>Splenic Marginal Zone Lymphoma (SMZL)</td>
<td>Peripheral B-cell with differentiation in part to a splenic marginal zone cell</td>
<td>similar to MZL</td>
<td>+</td>
</tr>
<tr>
<td>Hairy Cell Leukaemia (HCL)</td>
<td>Peripheral B-cell of unknown differentiation stage</td>
<td>CD5- CD10-C11c+ CD19+ CD20+ CD22+ CD23- CD25+ CD103+</td>
<td>+</td>
</tr>
<tr>
<td>Plasmacytoma/ Plasma Cell Myeloma</td>
<td>Plasma cell</td>
<td>CD19- CD20- CD22- CD38+ CD45+/-</td>
<td>- but clG+</td>
</tr>
<tr>
<td>Diffuse Large B-cell Lymphoma (DLCL)</td>
<td>Proliferating peripheral B-cell</td>
<td>CD5+/- CD10+/- CD19+ CD20+ CD22+</td>
<td>+/-</td>
</tr>
<tr>
<td>Burkitt's Lymphoma (BL)</td>
<td>B-cell of unknown differentiation stage</td>
<td>CD5- CD10+ CD19+ CD20+ CD22+</td>
<td>+</td>
</tr>
<tr>
<td>High-grade B-Cell Lymphoma, Burkitt's Like</td>
<td>Proliferating peripheral B-cell</td>
<td>CD5- CD10- CD19+ CD20+ CD22+</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Table 1.1 Abbreviations: clG = cytoplasmic immunoglobulin; sIg = surface immunoglobulin; TdT = terminal deoxynucleotide transferase.

1.2.2 Immunophenotyping

Leukocyte cell surface molecules can be identified by specific antibodies, catalogued with a cluster of differentiation number (CD) assigned when a single antigen molecule binds two or more different monoclonal antibodies. This system does not include immunoglobulin components, T-cell receptors or histocompatibility antigens. By using a panel of antibodies against CD antigens and immunoglobulin and T-cell receptor molecules, a
more precise diagnosis of lymphoid malignancies can be obtained as has been recently reviewed \(^{21}\).

CD19, CD20 and CD22 are pan-B cell markers which identify most B-cell lymphomas and further markers such as CD5, CD10 and CD23 can be used to obtain a more specific diagnosis. For example, CD10 is typically present in some B-cell lymphomas of germinal centre origin, including FL and some cases of BL, but absent from MCL which is typically CD5 positive.

In conclusion, a combination of morphological features and immunophenotypic markers can be used to identify haematological malignancies and their subgroups.

### 1.3 Molecular Cytogenetics of B-cell Malignancies

Within a specific disease, molecular genetic and cytogenetic studies are helpful in the identification of subgroups with specific translocations associated with a specific prognosis. For example, BCP-ALL with the t(9;22)(q34.q11) has a poor prognosis in comparison with other subgroups of BCP-ALL \(^{22, 23, 24}\). Comparable prognostic subgroups have also been observed in B-CLL \(^{25}\) and B-NHL \(^{26}\).

In acute leukaemias, single translocations are seen, which frequently involve transcription factors and result in the expression of novel chimaeric proteins by the fusion of two genes \(^4\). In contrast, lymphomas often exhibit multiple translocations of the type that deregulate oncogene expression by juxtaposition to immunoglobulin or T-cell receptor loci (see Table 1.2).
Table 1.2. Examples of chromosomal abnormalities in B-cell malignancies and correlation with disease subgroup.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Cytogenetic abnormality</th>
<th>Genes Involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor B lymphoblastic leukaemia (BCP-ALL)</td>
<td>t(1;19)(q23;p13.3)(^{27})</td>
<td>PBX1/E2A</td>
</tr>
<tr>
<td></td>
<td>t(9;22)(q34;q11)(^{28})</td>
<td>BCR/ABL</td>
</tr>
<tr>
<td></td>
<td>translocations of 11q23 including</td>
<td>MLL and</td>
</tr>
<tr>
<td></td>
<td>t(4;11)(q21;q23)(^{29})</td>
<td>AF4</td>
</tr>
<tr>
<td></td>
<td>t(9;11)(p22;q23)(^{30})</td>
<td>AF9</td>
</tr>
<tr>
<td></td>
<td>del 9p21(^{31})</td>
<td>p16(^{\text{INK4A}})/p15(^{\text{INK4B}})</td>
</tr>
<tr>
<td>B-cell chronic lymphocytic leukaemia (B-CLL)</td>
<td>del 13q14(^{32})</td>
<td>DBM(^{33})</td>
</tr>
<tr>
<td></td>
<td>t(11;14)(q13;q32.3)(^{34})</td>
<td>BCL1/IGH</td>
</tr>
<tr>
<td></td>
<td>t(14;19)(q32.3;q13.1)(^{35})</td>
<td>IGH/BCL3</td>
</tr>
<tr>
<td>Lympho-plasmacytoid Lymphoma/ Immunocytoma</td>
<td>translocations of 9p13-9p22(^{16})</td>
<td>?</td>
</tr>
<tr>
<td>Mantle Cell Lymphoma</td>
<td>t(11;14)q13;q32.3(^{36})</td>
<td>BCL1/IGH</td>
</tr>
<tr>
<td>Follicle Centre Cell Lymphoma (FL)</td>
<td>t(14;18)(q32.3;q21.3)(^{37})</td>
<td>IGH/BCL2</td>
</tr>
<tr>
<td></td>
<td>17p(^{-26}), 6q(^{-26})</td>
<td>p53 , ?</td>
</tr>
<tr>
<td>Splenic Marginal Zone Lymphoma</td>
<td>t(11;14)(q13;q32.3)(^{38})</td>
<td>BCL1/IGH</td>
</tr>
<tr>
<td>Diffuse Large B-cell Lymphoma (DLCL)</td>
<td>t(3;14)(q27;q32.3)(^{39})</td>
<td>BCL6/IGH</td>
</tr>
<tr>
<td></td>
<td>t(8;14)(q24.1;q32.3)(^{40})</td>
<td>MYC/IGH</td>
</tr>
<tr>
<td></td>
<td>t(14;18)(q32.3;q21.3)(^{41})</td>
<td>IGH/BCL2</td>
</tr>
<tr>
<td>Burkitt’s Lymphoma (BL)</td>
<td>t(8;14)(q24.1;q32.3)(^{42,43})</td>
<td>MYC/IGH</td>
</tr>
<tr>
<td>High-grade B-Cell Lymphoma, Burkitt’s Like</td>
<td>t(14;18)(q32.3;q21.3)(^{44})</td>
<td>IGH/BCL2</td>
</tr>
<tr>
<td></td>
<td>no MYC translocation</td>
<td></td>
</tr>
</tbody>
</table>
1.3.1 Proto-oncogenes

Some proto-oncogenes have been implicated in many cancers, for example, \textit{MYC} and members of the \textit{RAS} family. These genes are involved in growth mechanisms that are common to all cells. Many proto-oncogenes have been shown to be involved in pathways that stimulate the cell cycle. However the mechanisms by which they are altered may be specific to the tumour type. For example, \textit{MYC} over-expression in lung carcinoma is associated with amplification of the gene \cite{45}, whilst in Burkitt's lymphoma \textit{MYC} is almost always juxtaposed to the immunoglobulin locus by the translocation t(8;14)(q24.1;q32.3)\cite{42,43}.

Some genetic events are characteristic of particular malignancies. These disease-specific events may be caused by aberrations in lineage specific growth and development pathways and may also subvert these pathways. For example, the translocation t(14;18)(q32.3;q21.3) is seen in 70-80\% of follicular lymphomas (FL) \cite{46,47} and in approximately 20\% of aggressive diffuse large cell lymphomas \cite{48}. This abnormality may result from mistakes in the normal \textit{IGH} recombination mechanism, and causes inappropriate expression of \textit{BCL2} in B-cells by its juxtaposition to transcriptional enhancers of the immunoglobulin heavy chain locus \cite{49}. Bcl2 protects cells against some programmed cell death or apoptosis pathways which eliminate the large majority of normal B-cells during their development \cite{50,51}.

Some genes identified at translocation breakpoints are involved with multiple translocation partners. For example \textit{BCL6} at 3q27 is frequently involved in reciprocal translocations with the immunoglobulin loci in diffuse
large cell lymphomas (DLCL) and other extranodal lymphomas, but is also commonly involved with genes at several other recurrent chromosomal loci in the same diseases. Similarly \textit{MYC} has been shown to have several translocation partners. Most \textit{MYC} translocations involve \textit{IGH} at 14q32.3, however rearrangements of the immunoglobulin light chain loci at 2p11-12 and 22q11 and the T-cell receptor alpha/delta locus at 14q11.2 are also seen. \textit{BCL4} and \textit{BTG1}, located at 17q22 and 12q22 respectively, are both involved in lymphoid cell growth and differentiation and were isolated from the breakpoint regions of translocations involving \textit{MYC}. Other translocation breakpoints involving \textit{MYC} remain to be characterised, but analysis of these breakpoint regions may reveal more genes that are regulated in a lymphoid specific manner. Work presented here aimed to use this approach by analysing cases with a recurrent translocation t(8;9)(q24.1;p13.3) in order to identify the critical gene at 9p13.3 involved in translocations in lymphoid malignancies.

1.3.2 Tumour Suppressor Genes

Tumour suppressor genes may be defined as growth inhibitory genes which are deleted or inactivated at both alleles in tumour cells. Examples of tumour suppressor genes include the retinoblastoma susceptibility gene (\textit{RB1}), the adenomatous polyposis gene (\textit{APC}), the \textit{p53} gene, and the Wilms' tumour gene (\textit{WT-1}). Mutations or deletions of the germline of one allele of these genes were shown in affected members of families showing an inherited predisposition to these tumours. At a cellular level, loss of function mutations of tumour suppressor genes are
normally recessive to the normal allele. However, the individual is at greater risk of developing cancer because loss of function of the second allele, in any cell, may lead to tumour development when associated with other genetic events. Thus such mutations are inherited in a dominant fashion.

Classical loss of function mutations include deletions, chain terminations, frameshifts and exon-skipping mutations, and lead to the production of inactivated protein, or no protein at all. These mutations are common in RB1, p53 and APC genes in both the familial cancers in which they were first characterised and numerous types of sporadic cancers. Indeed, mutation of p53 is a frequent event in a diverse range of human cancers. However, p53 is unusual in that some mutations of this gene show a dominant oncogenic effect at the cellular level. Deletions of RB1 are also seen in many human tumours, including small cell carcinomas of the lung and 30% of bladder cancer cell lines.

In lymphoid tumours loss of known tumour suppressor genes appears to be rare as a primary mechanism of tumour development. Deletion of the RB1 gene at 13q14 has been reported in BCP-ALL at a frequency of between 11% and 29% of cases possessing the t(9;22)(q34;q11) and in 21% of B-CLL, but a second unidentified tumour suppressor in this region may be the main target of these deletions in B-CLL. Between 10 and 20% of BCP-ALL show mutation of p53 and these cases appear to be restricted to BCP-ALL with translocation of MYC or MLL oncogenes. B-CLL also show mutation of p53 in around 15% of cases. In B-NHL, tumour suppressor loss of function has been infrequently reported, with the exception of Burkitt’s
lymphoma in which \textit{p53} is mutated in 33\% of cases \textsuperscript{71}. Nevertheless, a proportion of high grade B-cell lymphomas show \textit{p53} mutations which may be associated with tumour progression \textsuperscript{72, 73, 74, 75, 76}.

Several chromosomal loci are frequently deleted in lymphoid malignancies and are as yet uncharacterised. These regions may contain lymphoid tumour suppressor genes and include chromosomal arms 6q \textsuperscript{77, 78} and 9p \textsuperscript{31}.

\subsection*{1.3.3 Multistep Events in Tumour Formation}

A combination of several genetic alterations must occur for the initiation and progression of most types of human cancer. This can be clearly seen in studies of colon cancer in which the clinical course of the disease has been correlated with the alteration of specific genes \textsuperscript{79}. In colorectal carcinoma it appears that the accumulation of genetic abnormalities is more important for tumour progression than the order of genetic events. However, in other cancers it may sometimes be important for genetic changes to occur in a defined order. For example expression of the \textit{MYC} proto-oncogene can lead to cell proliferation in growth factor stimulated cells, or cell death by apoptosis in the absence of growth factor stimulation \textsuperscript{80}. This apoptotic pathway can be blocked by over-expression of \textit{BCL2} \textsuperscript{50, 81}, and in some tumours \textit{BCL2} deregulation may be a necessary first event before \textit{MYC} overexpression can give the tumour cell a proliferative advantage \textsuperscript{18}. 
1.3.4 Tumour Progression

It has been observed that, over time, all types of tumour may become more clinically aggressive and less responsive to chemotherapy as a result of the accumulation of multiple genetic alterations. Clinically advanced cancers generally have more chromosomal aberrations than early stages of malignant disease. In some tumours, specific genetic alterations have been associated with progression. For example, the progression of gliomas has been associated in some cases with the deletion of sequences on the short arm of chromosome 9.

Low grade NHL frequently transforms into a more aggressive disease and rarely to a leukaemic phase after a variable period of time. Follicular lymphoma is a relatively indolent B-cell malignancy characterised by deregulation of the $BCL2$ proto-oncogene as a consequence of the $t(14;18)(q32.3;q21.3)$ translocation. Between 5-10% of follicular lymphoma per year progress to more aggressive disease, which is usually chemotherapy resistant and rapidly fatal. Progressed follicular lymphomas may have the phenotype of either high grade diffuse B-NHL or, more rarely, acute leukaemias of mature B-cells. In some cases deregulation of the $MYC$ proto-oncogene by the $t(8;14)(q24.1;q32.3)$ translocation has been shown to be concomitant with disease progression. As already discussed, $BCL2$ can act synergistically with $MYC$ by protecting against $MYC$ induced apoptosis, which causes cell death when $MYC$ is expressed in the absence of stimulating growth factors. De novo acute leukaemias with both $t(14;18)(q32.3;q21.3)$ and $t(8;14)(q24.1;q32.3)$ are rare and have a highly
aggressive clinical course with a poor prognosis\textsuperscript{87, 88}. It is not known if these cases are truly \textit{de novo} leukaemias or if they arise from clinically undetected follicular lymphomas.

Whilst \textit{MYC} deregulation may be associated with the progression of some cases of follicular lymphoma, this only accounts for around 10\% of cases and in the remaining group \textit{MYC} translocation appears to be absent\textsuperscript{86}. The molecular basis for disease progression of NHL is heterogeneous. Levine and colleagues analysed sequential karyotypes in NHL and reported several secondary abnormalities including rearrangements of chromosomes 1, 2 and 14 (band q32), and loss of 17p\textsuperscript{89}. Whang-Peng \textit{et al.} noted that progression of follicular lymphoma in four cases was associated with the acquisition of der(18), +7, and/or +12, all abnormalities which are associated with high grade disease or short survival\textsuperscript{78}. Other studies have found frequent losses of 9p sequences in subtypes of high grade lymphoma\textsuperscript{90, 91}, but have not shown that these were acquired as the disease progressed from low grade lymphoma. As discussed above, several groups have reported a high frequency of abnormalities of the \textit{p53} tumour suppressor gene in high grade NHL\textsuperscript{73, 74, 75} and an association of \textit{p53} mutation with progression of follicular lymphoma in 25-30\% of cases\textsuperscript{76}.

\section*{1.4 Chromosome 9p Abnormalities in Lymphoid Malignancies}

Recurrent abnormalities of chromosome 9 have been observed in cytogenetic studies of both BCP-ALL and TCP-ALL\textsuperscript{92} and NHL\textsuperscript{93}, and have
been correlated with histological, immunophenotypical and clinical features of subsets of these cancers. These abnormalities include a variety of recurrent and sporadic translocations involving different bands on either arm of chromosome 9, inversions, and loss of chromosome 9 material. The losses include interstitial deletions of either chromosomal arm, isochromosomes 9q and 9p, monosomy 9 and unbalanced translocations.

Abnormalities of 9p have been detected by cytogenetic studies in 7-12% of childhood ALL and in around 10% of adult ALL. In one study of childhood ALL approximately 65% of these abnormalities were deletions, 23% were unbalanced translocations and 12% were apparently balanced translocations of 9p. Murphy et al. noted an association between 9p abnormalities and a high tumour load and poor prognosis, and other groups have reported a correlation between 9p abnormalities and a T-cell phenotype with lymphomatous features, although more recent studies have not confirmed this finding. De novo acute leukaemias with t(14;18)(q32.3;q21.3) and deregulation of the MYC proto-oncogene are rare, but a high incidence of chromosome 9 abnormalities in these cases has also been reported.

In NHL cytogenetic abnormalities of chromosome 9p have been observed in 6.5% of karyotypically abnormal cases, and of these 94% were translocations and 6% were deletions. In contrast, myeloid disorders have a very low frequency of cytogenetic abnormalities of 9p (<1%).
1.4.1 Translocations Involving Chromosome 9p

In translocations of 9p in NHL, the most frequent breakpoint on chromosome 9 is 9p13, which has been observed in greater than 5% of NHL. This band has been reported to be translocated to numerous partner chromosome bands (see Table 1.3), but is most commonly seen in association with band 14q32.3. The translocation t(9;14)(p13.3;q32.3) correlates with an indolent clinical course and plasmacytoid features in NHL. One t(9;14)(p13.3;q32.3) breakpoint has been cloned from a diffuse large cell lymphoma cell line, which revealed an actively transcribed sequence on chromosome 9 which was fused with the immunoglobulin heavy chain gene on chromosome 14. Diffuse large non-cleaved cell lymphomas have also been observed with the t(9;12)(p13;q21) translocation and in follicular lymphomas rearrangement of 9p13 by the t(9;9)(p13;q13) and t(9;12)(p13;q13) translocations has been reported. None of these breakpoints have yet been cloned.

In high grade B-cell malignancies, loci reported to be involved in translocations to 9p13 include the MYC locus at 8q24.1. The translocation t(8;9)(q24.1;p13.3) has been observed in three cases of high grade NHL and in three cases of aggressive B-ALL in association with t(14;18)(q32.3;q21.3). The recurrent translocation t(9;20)(p13;q11) has been described in BCP-ALL.

Some 2% of childhood BCP-ALL show involvement of the 9p11 band in the non-random translocation tdic(9;12)(p11;p12), which is associated with a good prognosis. Other sporadic translocations involving
9p11 and different partner chromosomes have also been reported in other lymphoid malignancies. However, preliminary studies have indicated that these breakpoints are not tightly clustered on 9p11. Probes derived from the breakpoint region of a t(9;14)(p11;q32) in the MAL cell line, derived from a case of lymphoplasmacytoid lymphoma, showed that the breakpoints in the t dic(9;12)(p11;p12) translocation of BCP-ALL in 3 cases were between 100 and 300 kb distant from the original breakpoint cloned. In a fourth case the breakpoint was within a repetitive region 3-18 kb from the MAL breakpoint. In sporadic translocations involving 9p11, rearrangement could only be shown in one of two cell lines derived from cases of T-ALL, and this was 100-130 kb distant from that of the MAL cell line.

Reciprocal translocations involving the MLL gene at chromosome band 11q23 and various partner chromosomes, including 9p22, are particularly common in paediatric myeloid leukaemias, but also seen in some childhood lymphoblastic leukaemias (see Table 1.2), and are associated with poor prognosis. The t(9;11)(p22;q23) translocation is often seen in cases of myelomonocytic leukaemia and has been reported in approximately 4% of monocytic leukaemia of all age groups. The gene involved on chromosome 9, AF9, is a nuclear protein with serine-rich and proline-rich regions. The C terminal domain of AF9 is highly homologous to the transcriptional activation domain of AF19 that forms an in frame fusion with the N terminal region of MLL.
Table 1.3. Recurrent chromosome 9p translocation breakpoints in lymphomas and leukaemias

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>t dic(9;12)(p11;p12)</td>
<td>B-ALL</td>
<td>Carroll et al 1987&lt;sup&gt;102&lt;/sup&gt;</td>
</tr>
<tr>
<td>t(9;14)(p11;q32)</td>
<td>lymphoplasmacytoid lymphoma</td>
<td>Pellet et al 1990&lt;sup&gt;103&lt;/sup&gt;</td>
</tr>
<tr>
<td>t(8;9)(q24.1;p13.3)</td>
<td>B-ALL and diffuse large cell B-NHL</td>
<td>Levine et al 1989&lt;sup&gt;99&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nacheva et al 1993&lt;sup&gt;98&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lillington et al 1994&lt;sup&gt;100&lt;/sup&gt;</td>
</tr>
<tr>
<td>t(9;9)(p13;q13)</td>
<td>FL</td>
<td>Offit et al 1990&lt;sup&gt;16&lt;/sup&gt;</td>
</tr>
<tr>
<td>t(9;12)(p13;q13)</td>
<td>FL</td>
<td>Offit et al 1990&lt;sup&gt;16&lt;/sup&gt;</td>
</tr>
<tr>
<td>t(9;12)(p13;q21)</td>
<td>DLCL</td>
<td>Offit et al 1990&lt;sup&gt;16&lt;/sup&gt;</td>
</tr>
<tr>
<td>t(9;14)(p13;q32)</td>
<td>lymphoplasmacytoid lymphoma DLCL</td>
<td>Offit et al 1992&lt;sup&gt;110&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ohno et al 1990&lt;sup&gt;97&lt;/sup&gt;</td>
</tr>
<tr>
<td>t(9;20)(p13;q11)</td>
<td>high risk ALL</td>
<td>Slater et al 1992&lt;sup&gt;101&lt;/sup&gt;</td>
</tr>
<tr>
<td>t(9;11)(p22;q23)</td>
<td>ALL (but mostly AML)</td>
<td>Iida et al 1993&lt;sup&gt;30&lt;/sup&gt;</td>
</tr>
<tr>
<td>t(9;11)(p22;p15)</td>
<td>biphenotypic ALL</td>
<td>Ha et al 1994&lt;sup&gt;111&lt;/sup&gt;</td>
</tr>
<tr>
<td>t(9;19)(p22;q13)</td>
<td>NHL</td>
<td>Offit et al 1991&lt;sup&gt;93&lt;/sup&gt;</td>
</tr>
<tr>
<td>t(7;9)(p15;p23-24)</td>
<td>ALL</td>
<td>Raimondi et al 1991&lt;sup&gt;112&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
1.4.2 Deletions of Chromosome 9p

Recurrent deletions of a localised region of the short arm of chromosome 9 (9p13-p22) have been reported in a wide variety of tumours including melanomas, gliomas, non-small cell lung carcinomas and bladder tumours as well as leukaemias and lymphomas. Intensive efforts to map genetic markers within this region, combined with the results of loss of heterozygosity studies and deletion mapping indicated the presence of at least one, and possibly two, tumour suppressor genes on 9p.

In acute leukaemias the most common abnormalities involve deletion of the short arm of chromosome 9, seen in around 10% of both BCP-ALL and TCP-ALL. Diffuse large cell lymphomas (DLCL) also exhibit frequent cytogenetic deletions of 9p, and the smallest common region of deletion in both cases has been mapped to 9p21-22. Another tumour suppressor gene is thought to be located on 9q.

1.4.3 Candidate Genes at 9p13-21

The nature of many of the critical genes on chromosome 9p, involved in both the deletions of BCP-ALL and the translocations of B-NHL and other B-cell malignancies, remain unknown. The translocations and deletions may affect two or more separate gene loci. Although one t(9;14)(p13;q32) translocation breakpoint has been cloned from a B-NHL cell line, the derived probes have not detected breakpoints in other cases (see Chapter 5). Other genes have been mapped to 9p13-9p21 which are involved
in B-cell growth and differentiation and are possible candidates as targets for translocations in lymphoid malignancies. **PAX-5** is a transcription factor located at 9p13 which is expressed in B-cell precursors but down-regulated in plasma cells. **PAX** (paired box) genes are a family of transcription factors that are involved in the regulation of development in mice and humans. They have been shown to induce transformation both in vivo and in vitro, and one member of the family, **PAX 3**, has been shown to be directly involved in the t(2;13)(q35;q14) translocation of solid tumour alveolar rhabdomyosarcoma. DNA binding sites for **PAX-5** have been located upstream of several immunoglobulin switch regions and down-regulation of this gene has been shown to inhibit class switching in one cell line. However, class switching also requires cell division, which is inhibited in B-cells by low **PAX-5** levels. **PAX-5** has binding sites 5' of the B-cell differentiation antigen, CD19, and may regulate its expression.

An orphan immunoglobulin ε (epsilon) pseudogene has also been mapped to 9p which may be a possible target for translocations in B-cells, as a result of mistakes in immunoglobulin rearrangement or class switching mechanisms. However, it is located very close to the telomere at 9p24 and distant from most lymphoid translocation breakpoints.

In most of the cases analysed, deletions of 9p include the interferon (IFN) gene cluster **IFNA** and **IFNB** at 9p22, and extend centromeric to the 5' methyl thioadenosine phosphotase gene locus, or **MTAP**. It has been suggested that loss of the interferon genes may play a role in leukaemogenesis as a result of their antiproliferative action. Leukaemic
cells show an altered expression of IFN subtypes when compared to normal leukocytes, and some malignant T-cells with intact IFN genes have defects in other aspects of the IFN system. Paediatric cases of ALL with deletion of IFN genes, or loss of heterozygosity for all RFLP within the IFN locus have a poorer prognosis than heterozygotes and are more likely to relapse.  

RFLP analysis of leukaemic cells indicated that deletions of IFN genes in ALL may be far more frequent than are observed using Southern blotting techniques alone, and may occur in up to 30% of primary ALL clones. However, the smallest region of deletion of 9p in a variety of malignant diseases maps immediately centromeric to the IFNα gene cluster and includes two recently identified putative tumour suppressor genes p16INK4A, (also known as CDKN2, MTS1 or CDK4I) and p15INK4B (also known as MTS2). Whilst it seems likely that inactivation of these genes plays a major role in tumourigenesis of clones with 9p deletions, this does not exclude the possibility that deletion of the IFN genes may contribute to development of the malignant phenotype in some cases.

1.4.4 Deletions of p16INK4A in neoplasia

P16 is a cyclin-dependent kinase 4 inhibitor, which prevents progression through the cell cycle when bound to cdk4. The discovery that p16INK4A was located within the smallest common region of deletion, on 9p, in a large percentage of tumour cell lines, lead to the proposal that p16INK4A corresponds to the tumour suppressor gene thought to exist on chromosome 9p. This hypothesis was reinforced by the discovery of point mutations in the
coding region of $p16^{INK4A}$ in melanoma cell lines $^{132}$ and subsequently in several types of primary tumours $^{133, 134, 135}$. Furthermore, introduction of a full length $p16^{INK4A}$ cDNA into glioma cell lines with $p16^{INK4A}$ deletions causes G$_1$ growth suppression $^{136}$. This means that cells arrest in G$_1$, do not pass the restriction point R, and fail to express late G$_1$ proteins. Most cell lines in which $p16^{INK4A}$ was deleted also had deletion of $p15^{INK4B}$, an immediately adjacent homologous gene. However in a few cases only $p16^{INK4A}$ was deleted, leading to the supposition that $p16^{INK4A}$ was the critical deleted gene $^{131}$.

However, preliminary studies in bladder tumours, astrocytomas and other cancers $^{136, 137}$ showed that $p16^{INK4A}$ deletions occurred at much lower frequencies in primary tumours than in cell lines. This raised the possibility that the high frequency of deletions observed in cell lines represented an artefact of in vitro culture, for example a secondary event that is selected for in the establishment of cell lines $^{138}$.

In haematological malignancies, high resolution cytogenetics has revealed recurrent interstitial deletions and translocations of 9p. In particular, deletion of 9p21-22 was seen in 7-12% of childhood BCP-ALL $^{31, 92}$. Deletions of $p16^{INK4A}$ were reported in 25% to 64% of leukaemic cell lines $^{17, 131}$, but these studies were based on relatively small sample sizes (n=4 and 14) and did not investigate deletions in uncultured tumours. This thesis investigated deletions of $p16^{INK4A}$ in lymphoid malignancies, in both cell lines and uncultured tumour samples. Since completing this work, several reports of the extent of $p16^{INK4A}$ and $p15^{INK4B}$ deletions in lymphoid malignancies have
been published. These data are reviewed in Chapter 6 and discussed with respect to results reported here in Chapters 3 and 4.

Evidence that a cyclin dependant kinase inhibitor is deleted in many tumours has highlighted the importance of loss of normal cell cycle control in neoplasia. Recent research has identified many of the key proteins involved in the control of cell cycle progression, and the genes that encode them. Some of the critical genes identified from translocation breakpoints and regions of deletion in lymphoid malignancies have been shown to be integral components of cell cycle regulation. The next section briefly discusses what is known of the complex interplay of gene products throughout the cell cycle with emphasis on those genes which are involved in lymphoid malignancies.

1.5 Cell Cycle Control and Lymphoid Malignancies

Cell division is a tightly controlled process that responds to a complex array of external signals such as growth factor stimulation and internal signals such as DNA damage. Many proto-oncogenes have been identified that are components of the signal transduction pathways that link growth factor signals to cell division. For example, \textbf{C-KIT} encodes the tyrosine kinase receptor for stem cell factor (SCF). SCF stimulates growth of multipotential haematopoietic stem cells via the signalling intermediates, p21ras and myc, products of the \textbf{H-RAS} and \textbf{MYC} proto-oncogenes \cite{139}. Tumour suppressor genes may also directly influence the cell cycle (Fig 1.3). For example, the retinoblastoma gene product inhibits transition from G\textsubscript{1} to S
phase\textsuperscript{140} and DNA damage induces expression of p53, which can cause cells to arrest in G\textsubscript{1}, or die by apoptosis\textsuperscript{141}.

### 1.5.1 Cell Cycle Checkpoints

At all stages in the cell cycle, feedback controls ensure that each step is completed before proceeding to the next phase. Once cells are committed to cell division there is a short gap phase, called G\textsubscript{1}, before DNA is replicated in S phase. A second gap, G\textsubscript{2}, precedes mitosis, M phase, and cells can arrest at this point for considerable periods. However, when cells leave the cell cycle, they arrest in a specialised G\textsubscript{1} phase known as G\textsubscript{o}.

Transitions between each step in the cell cycle are regulated by checkpoints so that, for example, M phase is directly coupled to the completion of S phase. These checkpoints are mediated by the assembly and activation of multimeric proteins known as cyclin dependant kinase complexes. These are composed of a catalytic kinase subunit (CDK) and a positive regulatory subunit known as a cyclin, and may include a negative regulatory subunit or kinase inhibitor (CDI). Cyclin levels fluctuate throughout the cell cycle as a result of their accumulation and periodic destruction, whilst CDK levels remain relatively constant. In mammalian cells, different cyclins activate the different CDKs at different phases in the cell cycle. Each activated kinase complex phosphorylates a distinct group of proteins required for the progression of the cell through a specific stage of the cell cycle. At least 11 cyclins (A to G, including B1 to B3 and D1 to D3) and 7 CDKs (1 to 6
plus cdkX) have been identified in mammalian cells and these may have different roles in different tissues.

Figure 1.2 Simplified schematic view of interactions between some of the cyclin-cdk complexes controlling the mammalian cell cycle. Positive and negative mitogenic signals are transduced by cyclins and their related regulatory factors into discrete phosphorylation events that control expression of the genes necessary for initiation and progression of the cell cycle. Multiple cyclin-cdk complexes show some functional redundancy in order to accommodate the diversity of factors that influence cell division. Abbreviations: Rb = retinoblastoma gene product; PCNA = proliferating cell nuclear antigen; P = phosphate groups; CDK = cyclin dependant kinase.
Of the many mammalian CDKs the best characterised are, cdk1, cdk2 and cdk4. Cdk1 interacts with B-type cyclins to regulate mitosis. Cdk2 complexes first with cyclin E to regulate the G₁/S checkpoint and then with cyclin A as cells leave G₁ and enter S phase. Cdk2/cyclin A complexes appear to be required for DNA replication. Cdk4 interacts exclusively with the D-type cyclins and acts at the restriction point (R) in G₁ where cells become committed to DNA replication. Once cells have passed this point they become independent of external growth factors until the cell cycle is complete. Deregulation of this checkpoint may be important in oncogenesis by allowing cells to replicate in isolation from external cues. Thus overexpression of positive regulators such as cyclins, or the loss of negative regulators such as CDIs, both of which have been observed in tumour cells, may contribute to the transformed phenotype. Several leukaemic and solid tumour cell lines have been demonstrated to express cyclin E and/or cyclin B1 at inappropriate points in the cell cycle, and this may reflect the loss of cell cycle regulatory mechanisms. However, the D type cyclins are the most commonly deregulated cyclins in lymphoid tumours.

1.5.2 D type Cyclins

D type cyclins are differentially expressed, but most cells express cyclin D3 in conjunction with either cyclin D1 or D2. T-cells express predominantly cyclin D3 with some cyclin D2 and no cyclin D1. Expression of D type cyclins is rapidly induced by the addition of serum to quiescent cells.
and their continued transcription requires the presence of growth factors. This, in conjunction with their short half life of about 30 minutes, suggests that the D-type cyclins provide a link between transduction of growth factor signals and the cell cycle, and therefore are implicated in oncogenesis. D-type cyclins are thought to regulate the cell's decision to enter the cell cycle, by activating cdk4 or cdk6 which then phosphorylates the retinoblastoma protein. In normal cells that express D-type cyclins, synthesis is highest during mid-late G1 and lowest during S phase. In isolation, both cyclin D1 and D2 may be rate limiting for progression from G1 to S phase.

Cyclin D1 was one of the first components of the cell cycle to be identified as a proto-oncogene, being identified as the critical gene at the inv(11)(p15;q13) breakpoint in parathyroid tumours as PRAD1, and at the t(11;14)(q13;q32) in B-cell malignancies as BCL1. Cyclin D1 (CCND1) was also identified as an expressed gene within the amplified region on 11q13 in some carcinomas. Rearrangement of the BCL1 locus occurs in at least 80% of mantle cell lymphoma, approximately 15% of splenic lymphoma with villous lymphocytes and in many cases of B-cell pro-lymphocytic leukaemia and myeloma. Oesophageal, breast and gastric carcinomas also have frequent amplification of a 1 Mbp amplicon at 11q13 which includes the cyclin D1 gene, and this is associated with over expression of cyclin D1. In cultured cells, overexpression of cyclin D1 slightly shortens G1 and causes the retinoblastoma protein to be phosphorylated earlier than in the normal cell cycle.
Juxtaposition of the cyclin D1 gene to the \textit{IGH} locus by the translocation t(11;14)(q13;q32.3), causes it to be inappropriately expressed in B-cells. However, even in malignant B-cells with this translocation cyclin D1 protein levels continue to fluctuate throughout the cell cycle\textsuperscript{162}. Cyclin D1 is normally expressed in macrophage lineage cells but not in lymphoid cells. Consequently, T and B cells rely on other D-type cyclins to mediate progression through the G\textsubscript{1} phase of the cell cycle\textsuperscript{163}. Antibody and antisense-mediated inactivation of cyclin D1 in B-cell tumour lines with t(11;14)(q13;q32.3), showed that the aberrant cyclin D1 expression was necessary for progression from G\textsubscript{1} to S phase in these tumours\textsuperscript{162}. Thus, inappropriate cyclin D1 expression in B-cell malignancies overcomes both positive and negative normal G\textsubscript{1} controls and may free cells from dependence on positive growth factor signals for replication.

The two other D type cyclins have also been implicated in oncogenesis although less directly. The cyclin D2 gene, \textit{CCND2} at 12p13, was amplified in one colorectal carcinoma\textsuperscript{164} and in mice is the integration site of a murine leukaemia provirus in a mouse T-cell leukaemia\textsuperscript{165}. Cyclin D2 has also been shown to be over-expressed in B-CLL\textsuperscript{166}. The chromosomal band containing the cyclin D3 gene, 6p21, is rearranged in several leukaemias and lymphomas\textsuperscript{167}. However the \textit{CCND3} gene has not been shown to be rearranged in lymphoid malignancies. Furthermore, the 6p21 band is also the locus for p21 or \textit{WAF1} (see below) a cyclin dependant kinase inhibitor.
Cdk4, the kinase partner most frequently found complexed with cyclin D1 and the other D type cyclins, may also have a role in oncogenesis. In osteosarcomas, neuroblastomas and gliomas an amplicon from 12q13-14 containing CDK4 as well as several other genes has been reported. Whether CDK4 is the critical gene is not known, but of the genes characterised in this region it is one of the most frequently amplified, and its amplification is associated with strong cdk4 overexpression. However other genes within the amplicon may also play a part in the neoplastic phenotype, for example MDM2 which inactivates the tumour suppressor gene product, p53.

When complexed, cyclin D1 and cdk4 act to phosphorylate the retinoblastoma protein at the end of G₁, which releases bound transcription factors such as E2F. These factors are then free to activate genes necessary for S phase. Many early response genes such as MYC and DHFR have E2F enhancer elements within their promoters.

In cells which produce functional retinoblastoma protein, the hypophosphorylated form activates transcription of cyclin D1 in G₁ by binding to retinoblastoma control elements (RCE) within the BCL1 promoter. Subsequent cyclin D1 synthesis and cdk4 activation leads to retinoblastoma phosphorylation which according to this model would shut off cyclin D1 expression in late G₁ via a negative feedback loop. Correspondingly, low levels of cyclin D1 and D3 expression have been associated with inactivation of the retinoblastoma protein in tumour cell lines. In the absence of
functional retinoblastoma protein, cyclin D1 is dissociated from cdk4 and anti
cyclin D1 antibodies fail to prevent progression from G1 to S phase \(^{174,175}\).

1.5.3 Cyclin Dependant Kinase Inhibitors

Understanding of the role of cell cycle checkpoint controls in
oncogenesis has greatly improved with the recent discovery of small protein
inhibitors of the G1 cyclin-CDK complexes (CDIs). To date at least seven of
these inhibitors p15 \(^{17,176}\), p16 \(^{17,177}\), p18 \(^{178}\), p19 \(^{179}\), p21 \(^{180}\), p27 \(^{181}\) and p57 \(^{182}\)
have been identified and cloned by various routes. These genes could
potentially act as tumour suppressor genes by inhibiting the kinase activity of
CDK-cyclin complexes and so slowing or blocking progression through
different phases of the cell cycle.

P21 and p27 are 42% identical in their N-termini and inhibit a wide
variety of cyclin/CDK complexes in vitro, including cdk4-cyclin D, and cdk2-
cyclin E or A \(^{183}\). Inhibition appears to require binding of more than one p21 or
p27 molecule, and in normal cells most cyclin-CDK complexes are associated
with p21 \(^{184}\). This association is often lost in transformed cells. Overexpression
of either of these two inhibitors has been shown to block progression into S
phase. P21 expression is induced by wild type p53 in response to DNA
damage in G1 phase and appears to be essential for p53 mediated cell cycle
arrest \(^{185}\). P27 on the other hand appears to be regulated by TGF-β or cell-

cell contact which causes its release from a sequestered state within the cell
\(^{181}\). However, whilst altered expression of both p27 and p21 has been
implicated in cellular transformation, neither of these genes has been seen to
be mutated in human cancers\textsuperscript{186}, with the exception of a mutant \textit{p21} isolated from one BL\textsuperscript{187} and a mutant \textit{p27} isolated from a case of ATL\textsuperscript{188}. \textit{P27} is located at 12p13, a locus that is deleted in a wide spectrum of lymphoid and myeloid malignancies, but was not mutated in 19 leukaemia cases analysed which had hemizygous deletions of \textit{p27}\textsuperscript{189}. The closely linked gene \textit{TEL} or another as yet unidentified gene may be the critical deleted gene at 12p13 in lymphoid malignancies\textsuperscript{190, 191}. \textit{P57} is closely related to both \textit{p27} and \textit{p21} although it has a unique domain structure. \textit{P57} is located at 11p15.5 and is expressed in a tissue specific manner\textsuperscript{182}.

Two other CDIs have been shown to be important in oncogenesis. \textit{P16\textsuperscript{INK4A}} and \textit{p15\textsuperscript{INK4B}} are located adjacent to each other at 9p21\textsuperscript{17, 131}, and are frequently deleted or rearranged in many human tumours, including leukaemias and lymphomas. These genes share a high degree of homology both at the nucleic acid and the protein level, and both bind to and specifically inhibit cdk4 and cdk6, the kinase partners of cyclin D1\textsuperscript{176, 177}. Both \textit{p16} and \textit{p15} have four ankyrin motifs, and share 44\% identity of the first 50 amino acids and 97\% identity of the remaining 81 amino acid sequence. The major differences between \textit{p15} and \textit{p16} appear to be in the regulation of their expression.

Hypophosphorylated retinoblastoma protein inhibits expression of \textit{p16}, and cells with no functional retinoblastoma protein show high levels of \textit{p16} expression\textsuperscript{192}. Hypermethylation of a CpG rich region which includes the first exon of \textit{p16\textsuperscript{INK4A}} also downregulates \textit{p16\textsuperscript{INK4A}} transcription\textsuperscript{193}. Furthermore an alternative first exon (exon 1\textit{β}) located upstream of exon 1

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can be transcribed from methylated or hypomethylated DNA. This transcript is translated in vitro but the protein product has not been found in cell lines, which may indicate that this mRNA has a regulatory role in \textit{p16^{INK4A}} transcription.

\textbf{P15} expression is induced 30 fold by TGF-β and is involved in TGF-β mediated inhibition of retinoblastoma phosphorylation and growth arrest. It has been speculated that p15 could displace p27 from a sequestered state bound to cyclin D1-cdk4/6 complexes, allowing p27 to inhibit cyclin E-cdk2 complexes preventing progression into S phase. Constitutive expression of \textbf{CDK4} in mink lung epithelial cells (Mv1Lu) renders them resistant to TGF-β growth inhibition, possibly by titrating out the effect of increased p15 expression induced by TGF-β, or by overcoming TGF-β induced inhibition of cdk4 synthesis.

\textbf{P18} and \textbf{p19} (19p13), show sequence homology with \textit{p16^{INK4A}} and \textit{p15^{INK4B}} within the ankyrin repeats and similarly bind and inhibit cyclin D/cdk4 or 6 complexes. \textbf{P18} has not been found to be deleted or mutated in studies of ALL or AML and studies of \textbf{p19} have yet to be undertaken in haematological malignancies.

Whilst all the CDIs mentioned here are potential tumour suppressor genes only \textit{p16^{INK4A}} and \textit{p15^{INK4B}} have been directly implicated in a large number of human malignancies by molecular genetic studies. This may be because \textit{p16^{INK4A}} and \textit{p15^{INK4B}} act at an early stage to prevent cell cycle initiation. Phosphorylation of the retinoblastoma gene product is the critical step in initiation of the cell cycle and many of the proteins involved in
this pathway have been implicated in the development of human tumours. Figure 1.3 shows schematically some of the protein interactions that occur in G1 and highlights those gene products that have been shown to be involved in oncogenesis.

Figure 1.3 Cell cycle regulators implicated in human cancer acting at the restriction point in G1

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Figure 1.3 Cdk4 or cdk6 is activated by binding to a D type cyclin and phosphorylates the retinoblastoma gene product, which then releases bound transcription factors necessary for the expression of late G1 proteins. This process can be inhibited by p16, p15 or p21. Overexpression of either the cyclin or the kinase subunits as a result of translocation or amplification may reduce responsiveness to negative regulatory factors and increase the rate at which the retinoblastoma gene product is phosphorylated so reducing the duration of G1. Loss of p16, p15 or p21 through deletion or mutation can eliminate inhibition of this process and release cells from negative regulation of the G1 phase. Finally, loss or inactivation of the key protein, the retinoblastoma gene product, can eliminate this cell cycle checkpoint. Abbreviations: P_\text{=} phosphate groups; CDK_\text{=} cyclin dependant kinase.
1.6 Deregulation of MYC in Lymphoid Malignancies

1.6.1 MYC Translocations in Burkitt’s Lymphoma

Whilst p16^{INK4A} and p15^{INK4B} are putative tumour suppressor genes, that negatively regulate the cell cycle, MYC is a proto-oncogene which is intimately associated with the cell cycle and stimulates cell division.

The precise role of the myc protein in the regulation of cell division remains unclear. MYC expression is rapidly activated by mitogenic stimuli and maintained throughout the cell cycle, indicating that it is necessary for the exit from G₀ to G₁ and also for continuous proliferation. Expression of MYC is sufficient for cells to enter G₁ without expression of other immediate early growth response genes, bypassing normal G₁ controls. On withdrawal of growth factors MYC expression is quickly down-regulated leading to cell cycle arrest.

The myc protein binds max to form a heterodimer which is a sequence specific transcriptional activator. Max also forms homodimers that are transcriptionally inactive, and it is thought that the relative ratio of myc-max and max-max complexes modulate expression of target genes.

In addition to stimulating cell division, MYC expression can induce programmed cell death or apoptosis in the absence of appropriate growth factors. This may be a mechanism by which tumour cells can be primed for death if they fail to down-regulate MYC and continue cycling independently of external cues. Bcl2, the product of an oncogene that inhibits apoptosis, can
act synergistically with myc in lymphoid tumours by blocking myc induced apoptosis \(^{50,81}\).

In normal lymphoid cells terminal differentiation is associated with \textit{MYC} down-regulation. \textit{MYC} has been shown to be deregulated in NHL and some lymphoid leukemias. The translocation t(8;14)(q24.1;q32.3) and its variants t(2;8)(p12;q24.1) and t(8;22)(q24.1;q11) are characteristic of an aggressive B-cell malignancy, Burkitt's Lymphoma (BL) \(^{205}\). All three translocations involve the oncogene \textit{MYC} at 8q24.1 and one of the immunoglobulin genes. In around 90% of BL cases \textit{MYC} is translocated to the immunoglobulin heavy chain locus (\textit{IGH}) at 14q32.3 and in the remaining 10% of BL cases to the \text{\textit{k}} or \text{\textit{\lambda}} light chain loci at 2p11-12 and 22q11 respectively. Breakpoints on chromosome 8 fall into three groups; those that occur within the first exon or intron of \textit{MYC}, those that occur immediately 5' of \textit{MYC} and those that occur at a large distance from \textit{MYC} either 3' or 5' of the gene \(^{206}\). In addition to BL, these translocations are also seen to occur in other rapidly growing lymphoid malignancies, for example, small non-cleaved lymphomas and some acute leukaemias of mature B-cells which have been associated with a rapidly progressive course \(^{207}\). In some cases, the leukaemic transformation of follicular lymphoma (FL) has been shown to be concomitant with the translocation of \textit{MYC} \(^{84,85,208}\).
1.6.2 Mechanisms of MYC Deregulation

1.6.2.1 Immunoglobulin Enhancers

Translocations of \textit{MYC} to immunoglobulin loci result in deregulation of \textit{MYC} expression in B-cells, in some cases as a result of its juxtaposition to the enhancers of the immunoglobulin loci, one of which (\textit{E\mu}) is located within the intron of the \textit{C\mu} switch region. In some translocations the breakpoint falls within the \textit{J} region, and \textit{E\mu} remains on the 14q+ derivative chromosome, adjacent to the translocated \textit{MYC} allele. However in other cases the chromosome 14 breakpoint is located in switch \textit{\mu} or downstream of \textit{C\mu}, and \textit{E\mu} is transferred to the derivative 8q- chromosome (see Fig 1.4). A putative enhancer 3' to \textit{C\alpha}, first described in rats and mice, may stimulate \textit{MYC} expression in these translocations. However distances between immunoglobulin enhancers and the translocated \textit{MYC} gene may be as large as 300-600 kb requiring the cis-acting function of the enhancers to be active over a considerable range. In some cases (especially translocations involving immunoglobulin light chains) breakpoints may be up to 300 kb 3' of the \textit{MYC} gene itself, and may involve another transcribed locus \textit{PVT1}. As yet not much evidence exists for the involvement of a \textit{PVT1} gene in human lymphoid malignancies. However in three murine plasmacytomas \textit{PVT1}/\kappa hybrid mRNA has been detected. The \textit{MYC} gene is still transcriptionally activated in these cases despite the large distances between the breakpoint and the \textit{MYC} gene itself.
Figure 1.4 Map of common translocation breakpoints of t(8;14), t(8;22), and t(2;8) relative to MYC exons 1 to 3

Figure 1.4 In sporadic cases of BL the MYC breakpoint is usually within the first exon or intron whilst in endemic BL the breakpoint usually occurs distant from MYC. The heavy chain locus breakpoints are frequently within the switch regions situated 5' of constant region genes but also sometimes occur within the joining region. IGH and MYC are in a head to head arrangement on the derivative chromosome 14. Translocations involving the light chain genes frequently break 3' of the MYC gene and result in a head to tail arrangement of MYC and IGK or IGλ on the derivative chromosome 8. Abbreviations: Eμ, = Enhancer; ?E, = Putative enhancer; E1-3, = MYC exons 1-3; □ = centromere.
IGH enhancers have variable activity when transfected into BL cell lines. This has lead to speculation that for many cell lines trans-acting regulators of MYC promoters are as important, or more important than IGH enhancers in MYC deregulation \(^{215}\). Some workers have reported an increased level of MYC expression in BL cells and derived cell lines, but in some cases it appears that MYC expression is no higher than normal physiological levels \(^{216, 217, 218}\). More specifically, MYC mRNA levels are higher than in normal peripheral B-lymphocytes that are not in cycle, but similar to levels in EBV immortalised lymphoblastoid cell lines. MYC is ubiquitously expressed in dividing cells, under tight cell cycle control \(^{219}\). In cells containing the t(8;14)(q24.1;q32.3) and its variants, it is the inappropriate expression of MYC throughout the cell cycle, or the inability to down-regulate MYC in cells in which it would normally be silent that contributes to the malignant phenotype.

1.6.2.2 Alteration of MYC 5' Regulatory Sequences

Other factors may also influence the expression and transforming activity of the translocated MYC allele. For example, loss or mutation of 5' negative regulatory sequences may allow inappropriate MYC expression.

In BL, the translocated MYC allele has frequently been shown to accumulate point mutations and small deletions or duplications, particularly within the first exon and intron, if they are present on the translocated allele \(^{220, 221, 222, 223, 224}\). These mutations appear to cluster in sequences that negatively regulate MYC expression, and may contribute to the deregulated expression of the translocated MYC allele. Mutation of the MYC transactivation domain may
also have a pathogenic role. Recent work has revealed mutations within the
coding regions of some translocated \textit{MYC} genes, clustering around codons
for residues that are phosphorylated \textit{in vivo}\textsuperscript{224, 225, 226}.

The mechanism by which these mutations are caused is unknown,
but the frequency and type of substitutions may be similar to the process of
somatic hypermutation of immunoglobulin genes\textsuperscript{227}. This introduces point
mutations into productively rearranged immunoglobulin genes of B-cells that
have encountered antigen, increasing the affinity of the antibody for antigen.
In normal lymphocytes somatic hypermutation is confined to a few kilobases
around VDJ, with the constant region genes remaining unaffected. However,
although the t(8;14)(q24.1;q32.3) may be incorrectly recognised as a
productive immunoglobulin rearrangement, mutations are not restricted to a
few kilobases but spread more widely. Similar mutations have been reported
in some translocated \textit{BCL2} genes and these mutations may be up to 300 kb
distant from the \textit{IGH} translocation breakpoint\textsuperscript{228}.

There is some evidence to suggest that in some cases the
mutation of translocated \textit{MYC} alleles is ongoing, at least in Daudi and Raji
Burkitt’s lymphoma cell lines and that they occur after translocation\textsuperscript{229, 230}.
However other workers have shown that, in Burkitt’s lymphoma, mutations in
the \textit{MYC} coding region are homozygous and appear to have occurred before
translocation\textsuperscript{224}.

The \textit{MYC} gene has four promoters P0, P1, P2 and P3 (see Fig
1.5). In normal cells 75-90\% of transcripts are produced from P2 with between
10 and 25\% from P1\textsuperscript{231, 232}. Less than 5\% of transcripts are initiated at P0\textsuperscript{233}.
which has no TATA box. P3 is a cryptic promoter within the first intron which also lacks a TATA box. In normal cells P3 yields about 5% of MYC mRNAs. However in some translocations where the MYC gene is decapitated the promoters P0 to P2 are lost and all transcription occurs from P3.

In the majority of cases examined so far, the untranslocated MYC allele remains silent, or is expressed at very low levels. This may be due to transcriptional controls that would normally inhibit MYC transcription in the equivalent untransformed cell, or it may be a result of negative feedback by MYC on its own promoter. The cell line Raji is a notable exception and transcribes both MYC alleles.

Figure 1.5 Arrangement of MYC promoters (P0, P1, P2, P3)

Figure 1.5 The MYC gene is transcribed from four promoters, with P1 and P2 producing the majority of transcripts in normal cells. Two proteins p67 and p64 are produced from two alternative initiation codons. They differ by 15 amino acids at the amino terminus but share the same reading frame in exons 2 and 3. Abbreviations: P0, P1, P2, P3, = transcription initiation sites; MIF1,2,3, = binding sites for MYC inhibitory factor; CTG, ATG, = translation start sites; Mae III, Pvu II, = restriction enzyme sites (not complete).
1.6.2.3 MYC Amplification

In some non-lymphoid malignancies, for example small cell lung cancer and breast cancer, deregulation of MYC is not associated with translocation but with amplification of the MYC locus \(^{236, 237}\). This can sometimes be seen by the presence of double minute chromosomes that hybridise strongly to MYC specific probes by FISH (fluorescence in situ hybridisation) or by an abnormally long q arm of chromosome 8. Amplifications of MYC are rare in lymphoid cells, but have been reported in at least one T- NHL cell line \(^{238}\) and one B-NHL cell line \(^{239}\). A few cases have also been documented in which a translocated MYC allele has been duplicated \(^{98, 240}\). Work presented in chapter 5 looked at the allele specific transcription of MYC in an unusual B-cell line, derived from a transformed FL, which possesses both a duplicated MYC allele and a MYC translocation.

1.7 Summary

Genetic abnormalities involving different combinations of oncogenes and tumour suppressor genes are key events in the pathogenesis of different lymphoid malignancies. This thesis has focused on the deletions and translocations of chromosome 9p in B-cell tumours and abnormalities of the MYC proto-oncogene in high grade B-NHL and B-ALL with BCL2 deregulation.
2. Chapter Two: Materials and methods

2.1 Tumour material and cell lines

2.1.1 Patient population

Diagnosis was made on the basis of combined morphological, immunophenotypic, cytogenetic and genotypic analyses, using previously defined criteria. DNA was isolated from a mononuclear fraction of peripheral blood, bone marrow, biopsied lymph node or splenectomy specimens after informed patient consent.

2.1.2 Cell lines

All cell lines were grown in RPMI 10% FCS under standard conditions. Cell lines were kindly provided by the following sources:

a) Burkitt’s lymphoma- derived cell lines and EBV-transformed normal B-cell lines were derived by Professor GM Lenoir.

b) Cell lines Karpas 231, 247, 353, 422, 620, 1106 & 1272:

Dr. A. Karpas, Department of Haematology, Cambridge University.

c) Cell lines Granta 86, 452, 519:

Dr. E. Nacheva

d) Cell lines UoCB1 and UoCB4:

Dr. SD. Smith, Department of Pediatrics, University of Chicago, USA.

e) Cell lines JVM-2 and JVM-3:

Professor D. Catovsky

f) Cell line DoHH2:

Dr. P. Kluin, University of Leiden, Netherlands

All other cell lines were obtained through Dr. H. Drexler at the German collection of Micro-organisms and Cell Cultures, Braunschweig, Germany.
Since they form the basis of much of the work discussed in this thesis further details on the following cell lines are provided:

Karpas 231 was derived from a 70 year old woman with TdT<sup>+</sup> (terminal deoxynucleotid transferase) B-cell ALL. Immunophenotyping showed strong expression of CD10, CD19, surface CD22 and surface IgM with no expression of any T-cell or myeloid differentiation antigens.

Two cytogenetic clones were detectable, both with a modal chromosomal number of 51, trisomy of chromosomes 7, 12, and 20 and the reciprocal translocations t(14;18)(q32.3;q21.3) and t(8;9)(q24.1;p13.3). The major clone had some additional structural abnormalities including an abnormal chromosome 17p13, an additional chromosome 13 with del13(q12.3-q21.2) and a t(1;3;11)(q42.3;q27.1;q23.1). Some cells of the major clone showed two copies of both derivatives of the t(8;9)(q24.1;p13.3) and duplication of the 18q- marker.

Karpas 353 was derived from a 60 year old woman with TdT<sup>-</sup> B-cell ALL. Limited immunophenotyping showed expression of CD19 and CD10. Studies of surface and cytoplasmic immunoglobulin were not performed. Cells were harvested, as above, for cytogenetic analysis. Cells had a modal chromosomal number of 47 with trisomy 12 and occasional loss of chromosome 8. Three balanced translocations were seen in all cells; a t(8;9)(q24.1;p13.3), a t(14;18)(q32.3;q21.3) and a t(1;3;7)(p32.1;q21.1;q22.1).

Granta 452 was derived from a 37 year old Caucasian male with TdT<sup>-</sup> B-cell ALL, arising subsequent to follicular centrocytic/centroblastic NHL. Immunophenotyping of Granta 452 showed expression of CD19, TdT and
surface IgM with a subpopulation (17% at presentation) also expressing CD10. No T-cell or myeloid differentiation antigens were seen to be expressed.

Cytogenetic analysis was performed at presentation and at relapse. Similar results were observed. Of 18 metaphases analysed at presentation only one exhibited a normal male karyotype. Two cells showed the following abnormalities: t(8;22)(q24;q11), t(1;18)(q36;q21), t(6;20)(q11;p13). In addition the markers 9p+, 9p+ and 14q- were observed. The remaining 15 cells had in addition to the abnormalities above, trisomy of chromosome 11 and an extra X chromosome.

2.1.3 SCID mice

Growth of B-cell leukaemias in SCID mice was performed by Dr. Paul Mitchell and Dr. Corinne de Lord (Academic Department of Haematology and Cytogenetics, Institute of Cancer Research, Sutton, Surrey). SCID mice were obtained from The National Institute of Medical Research (Mill Hill, London, UK). They were maintained in specific pathogen free environment, irradiated with 200 cGy and injected intravenously with $2 \times 10^7$ leukaemic cells from patients with BCP-ALL by tail vein injection: assessment of engraftment and transplantation of human leukaemic cells was performed as described. DNA was extracted from spleens with leukaemic infiltration containing greater than 95% leukaemic cells as assessed by flow cytometry.
2.2 Nucleic acids

2.2.1 Plasmid vectors

2.2.1.1 pBluescript II

Bluescript II phagemid vectors were derived from the pBS vector by replacement of the polylinker with a synthetic sequence containing 21 unique restriction sites. All probes derived in this laboratory were cloned into Bluescript SK+ (Stratagene).

2.2.1.2 pGEMT

Many thermostable polymerases add a non-template derived deoxyadenosine to the 3' end of PCR products, which decreases the efficiency of blunt ended cloning protocols. Vectors that have single 3'-T overhangs at the insertion site have increased efficiency of ligation to PCR products. For this study PCR products were cloned into pGEMT (Promega) which has a single 3' T overhang.

2.2.2 Oligonucleotides

2.2.2.1 P16\textsuperscript{INK4A} primers

Primers to amplify STS in the chromosomal region surrounding the P16\textsuperscript{INK4A} gene at 9p13 were derived from the paper by Kamb \textit{et al.} 1994\textsuperscript{17}.

Table 2.1A P16\textsuperscript{INK4A} primers and reaction conditions

<table>
<thead>
<tr>
<th>Primer Name \textsuperscript{exon}</th>
<th>Sequence 5' to 3'</th>
<th>Annealing temp (°C)</th>
<th>Special conditions</th>
<th>[Mg\textsuperscript{2+}] pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>P16\textsuperscript{INK4A} exon 1</td>
<td>GAAAGAAGAGGGGCTG GCGCTACCTGATTCCAATTC</td>
<td>58°C + 5% DMSO</td>
<td>1.5 mM pH 9</td>
<td></td>
</tr>
<tr>
<td>P16\textsuperscript{INK4A} exon 2</td>
<td>GGAAATTGGAAACTGGAAGC TCTGAGCTTTGGGAAGCTCC</td>
<td>50°C + 5% DMSO</td>
<td>1.5 mM pH 9</td>
<td></td>
</tr>
<tr>
<td>P15\textsuperscript{INK4B} exon 2</td>
<td>TGAGTTTAACCTGAAGGTGG GGGTGAAAATTGGGTAAG</td>
<td>60°C + 5% DMSO</td>
<td>1.5 mM pH 9</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1 \( P16^{ink4a} \) locus STS primers and reaction conditions

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’ to 3’</th>
<th>Annealing temp (°C)</th>
<th>Special conditions</th>
<th>([\text{Mg}^{2+}]) pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1063.7F</td>
<td>CCGTTTCAGCTTCATC</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>1063.7B</td>
<td>CCGACTGTCCTCGTATG</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>c18b.F</td>
<td>CAAAGACTTTATGGATGG</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>c18b.B</td>
<td>TCCATTCTCTGGCTTTGCT</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>c5.1F</td>
<td>GAAGTCTGTGCCTGAT</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>c5.1B</td>
<td>CTCTTCTGCACAACCTCAACT</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>RN3.1F</td>
<td>GGAATAGAGAATAGAAGA</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>RN3.1B</td>
<td>TCTGAGCTTTTGAAGCTCT</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>c5.3F</td>
<td>GTGTTAGAAGTGGACAG</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>c5.3B</td>
<td>CTGTTTAAGCCTTCATAGA</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
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<tr>
<td>R2.3F</td>
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<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>R2.3B</td>
<td>GGGACACACATTAATAACT</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>R2.7F</td>
<td>GAGAACAGGCTTGAGGCA</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>R2.7B</td>
<td>AACTAGACCTAGGGATAAGG</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>c1.bF</td>
<td>AAGCTTTCACACAAACTGCC</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
<tr>
<td>c1.bB</td>
<td>AATGCCCTTGGCATAGGCAG</td>
<td>60°C</td>
<td>touchdown PCR</td>
<td>1.5 mM pH 9</td>
</tr>
</tbody>
</table>

Note: In touchdown PCR the annealing temperature used is very stringent for the first few cycles and is progressively lowered. In this case an initial annealing temperature of 68°C was reduced by 2°C every four cycles until an annealing temperature of 60°C was reached. Subsequently, 30 cycles were performed with a 60°C annealing temperature. See Fig 4.1 for map.

**2.2.2.2 MYC primers and reaction conditions**

Table 2.2 MYC exon 1 primers and reaction conditions

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’ to 3’</th>
<th>Amplified region ( ^{253} )</th>
<th>Size of product</th>
<th>Annealing temp</th>
<th>pH</th>
<th>([\text{Mg}^{2+}])</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC1</td>
<td>ATGCGAGGGGCTCTGCTG</td>
<td>2303-2882</td>
<td>580 bp</td>
<td>60°C</td>
<td>9</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>HC2</td>
<td>TCCTGCCTCGAGAAGGCG</td>
<td>2388-2882</td>
<td>495 bp</td>
<td>60°C</td>
<td>9</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>HC3</td>
<td>AGCCGGTTTTTCGGGGTTTA</td>
<td>2466-2882</td>
<td>417 bp</td>
<td>60°C</td>
<td>9</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>HC4</td>
<td>AAGACCCTCCTAGGGGAGCT</td>
<td>2550-2882</td>
<td>333 bp</td>
<td>60°C</td>
<td>9</td>
<td>1.5 mM</td>
</tr>
</tbody>
</table>

Note: The 5’ primers HC1-4 were each used in conjunction with the 3’ primer HC0 to amplify different portions of the MYC exon 1 (see Fig 5.6 for map). The base numbering used here is taken from Gazin et al. 1984.\(^{253}\)
2.2.2.3 Primers for CA repeats on chromosome 9

Table 2.3 Primers for CA repeats on chromosome 9 and reaction conditions

<table>
<thead>
<tr>
<th>Locus Symbol</th>
<th>Primer Sequences</th>
<th>Reference</th>
<th>Annealing temperature</th>
<th>Heterozygosity</th>
<th>Size bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>D9S171</td>
<td>AGCTAAGTGAACCTCATCTCTGTC ACCCTAGCAGCTGTATAGCT</td>
<td>Gyapay et al. 1994</td>
<td>Touchdown PCR</td>
<td>0.79</td>
<td>159-177</td>
</tr>
<tr>
<td>D9S147E</td>
<td>AGTGTTCACCTAAATAAGCC CTCCCTGCACCCCTTCCATAA</td>
<td>Polymeropoulos et al. 1992</td>
<td>Touchdown PCR</td>
<td>0.78</td>
<td>189-201</td>
</tr>
<tr>
<td>D9S175</td>
<td>GTAATGTGCTAAATACCAGGTTG CCCCCCTTACGAGATGGGCC</td>
<td>Gyapay et al. 1994</td>
<td>Touchdown PCR</td>
<td>0.85</td>
<td>200-230</td>
</tr>
<tr>
<td>D9S118</td>
<td>TTGCTCTCAAAGGCCGGA GTGGCCTAGGATTGGTGA</td>
<td>Kwiatkowski et al. 1992</td>
<td>Touchdown PCR</td>
<td>0.83</td>
<td>69-93</td>
</tr>
<tr>
<td>D9S159</td>
<td>CTTTCTGAGCCGAGCCAGGTA GCTGGAATGATTGTCGGGG</td>
<td>Gyapay et al. 1994</td>
<td>Touchdown PCR</td>
<td>0.79</td>
<td>293-309</td>
</tr>
<tr>
<td>D9S158</td>
<td>TCTCAAGCGGACAAACATCA GATTTGGCTAAAATAGGCTCA</td>
<td>Gyapay et al. 1994</td>
<td>Touchdown PCR</td>
<td>0.69</td>
<td>213-231</td>
</tr>
</tbody>
</table>

Note: An initial annealing temperature of 62°C was lowered by 1°C per cycle until 55°C was reached. 28 cycles were performed with 55°C annealing temperature. The heterozygosity quoted for each primer pair is an estimate of the proportion of individuals in the population that are heterozygous at the amplified locus (1= 100%).

2.2.3 Probe derivation

Table 2.4 Immunoglobulin gene and control probes

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Insert size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH</td>
<td>IGH</td>
<td>2.5 kb Eco RI/ Hind III</td>
<td>Flanagan</td>
</tr>
<tr>
<td>Cκ</td>
<td>Iκκ</td>
<td>800bp Eco RI/ Hind III</td>
<td>Malcolm</td>
</tr>
<tr>
<td>GAPDH</td>
<td>-</td>
<td>1kb Pst I</td>
<td>Tso</td>
</tr>
</tbody>
</table>

Table 2.5 MYC locus probes

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Insert size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDIA</td>
<td>MYC cDNA</td>
<td>2 kb Eco RI</td>
<td>PH Hamlyn</td>
</tr>
<tr>
<td>MYC exon 2</td>
<td>MYC exon 2</td>
<td>415 bp Pst I</td>
<td>derived from cDIA by digestion with Pst I</td>
</tr>
<tr>
<td>8q264</td>
<td>40 kb 3' MYC</td>
<td>0.8 kb Bam HI/ Eco RI</td>
<td>R Zeidler</td>
</tr>
<tr>
<td>8q267</td>
<td>60 kb 3' MYC</td>
<td>0.7 kb Hind III/ Sal I</td>
<td>R Zeidler</td>
</tr>
<tr>
<td>8q290</td>
<td>&gt;60 kb 3' MYC</td>
<td>0.7 kb Bam HI/Sal I</td>
<td>R Zeidler</td>
</tr>
<tr>
<td>pPA0.2s</td>
<td>MYC</td>
<td>0.2 kb Sac I</td>
<td>L Sun</td>
</tr>
<tr>
<td>Y2</td>
<td>PVT1 cDNA</td>
<td>4.1 kb Eco RI</td>
<td>E Shtivelman</td>
</tr>
</tbody>
</table>
Table 2.6 BCL2 locus probes

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Insert size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFL1</td>
<td>BCL2 Major breakpoint region</td>
<td>1.5 kb Hind III / Eco RI</td>
<td>Cleary²⁶²</td>
</tr>
<tr>
<td>pFL2</td>
<td>BCL2 Minor breakpoint cluster</td>
<td>3.8 kb Hind III / Eco RI</td>
<td>Cleary²⁶³</td>
</tr>
<tr>
<td>pB16</td>
<td>5' cDNA</td>
<td>0.7 kb Eco RI</td>
<td>Tsujimoto²⁶⁴</td>
</tr>
</tbody>
</table>

Table 2.7 Chromosome 9p probes

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Insert size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16 exon 1</td>
<td>p16 exon 1</td>
<td>0.35 kb Eco RI</td>
<td>Kamb¹⁷</td>
</tr>
<tr>
<td>p16 exon 2</td>
<td>p16 exon 2</td>
<td>520 bp</td>
<td>Kamb¹⁷</td>
</tr>
<tr>
<td>p15 cDNA</td>
<td>p15 cDNA</td>
<td>2 kb Eco RI / Xho I</td>
<td>Hannon¹⁹⁵</td>
</tr>
<tr>
<td>pKIS</td>
<td>pKIS cDNA</td>
<td>1.7 kb Eco RI / Hind III</td>
<td>Ohno⁹⁷</td>
</tr>
<tr>
<td>pHBSAP-2a</td>
<td>PAX 5 cDNA</td>
<td>3 kb Xho I</td>
<td>Barberis²⁶⁵</td>
</tr>
</tbody>
</table>

2.2.3.1 DNA preparation and labelling

Solution I  50 mM Tris-HCl pH 7.5, 10 mM EDTA
Solution II 0.2M NaOH, 1% SDS
Solution III 2.55M Potassium acetate, pH 4.8

10xTAE  40 mM Tris, 2 mM EDTA, 20 mM sodium acetate, 20 mM sodium chloride, pH to 8.5 using glacial acetic acid

Probes that were received as plasmid DNA were transformed into competent XL1-blue by heat shock ²⁶⁶. Approximately 200µl of competent cells were incubated on ice for 30 minutes, in a 1.5 ml eppendorf tube with 1µl (20-50 ng) of plasmid DNA. The cells were briefly heated by placing the tube in a water bath at 42°C for 90 sec and then returned to ice for two minutes. Then 1 ml of Luria Broth (LB see below) was added and the cells were incubated at 37°C for 45 minutes to allow expression of the antibiotic resistance gene encoded by the plasmid. Between 50 and 200µl of this broth
were spread onto agar plates containing 60μg ml⁻¹ ampicillin using a sterile glass rod, and the plates were incubated overnight at 37°C. The following morning, a single colony was picked from the plate, using a sterile inoculating loop, into 30 ml LB containing 60μg ml⁻¹ ampicillin, in a sterile 50 ml tube. This was grown at 37°C with shaking for 6 hr, or until A₆₀₀ = 0.6.

2.2.3.2 Extraction of plasmid DNA

Cells were harvested by centrifugation at 3,000 rpm in a bench top centrifuge and washed briefly in 5 ml of Solution I. This removes culture medium that may contain bacterial cell wall components that inhibit the action of restriction endonucleases. Plasmid DNA was isolated using the alkaline lysis method described by Maniatis. Cells were resuspended in 500μl of Solution I and lysed by the addition of 500μl of Solution II. After addition of 500μl of Solution III bacterial cell walls and associated chromosomal DNA were removed by centrifugation in a microcentrifuge at 14,000 rpm for 5 minutes. The supernatant mixed with an equal volume of phenol/chloroform and spun briefly to separate the phases. The upper aqueous layer was decanted to a fresh eppendorf tube and the plasmid was precipitated by the addition of 0.6 volumes of isopropanol. After incubation at 21°C for 15 minutes and centrifugation at 14,000 rpm for 10 minutes the supernatant was discarded and the plasmid pellet dried briefly at 37°C. The DNA was then resuspended in 100μl sterile distilled H₂O and stored at -20°C.

To release the insert or probe, 2-5μg of plasmid DNA were digested for 1hr with 10 units of the appropriate restriction endonuclease in a final volume
of 100µl of the corresponding restriction buffer. The digested plasmid was electrophoresed on a 1% low melting point agarose gel, containing 0.1µg µl⁻¹ ethidium bromide, in 1x TAE buffer. Bands were visualised on a long wave UV transilluminator and the insert was excised from the gel using a sterile scalpel. Three microlitres of H₂O were added per milligram of gel slice and the agarose/water mixture was boiled for 5 minutes. Probes were stored at -20°C until labelled.

Probe DNA was labelled in the diluted gel slice, using the Amersham Rediprime™ system, by priming with random nonamers. This is a modification of the method described by Feinberg and Vogelstein. Between 20 and 30 ng of DNA in a final volume of 45µl were heated to 95°C for 5 minutes and added to one aliquot of dried labelling mix. Final concentrations of reagents were 200 mM HEPES, pH6.6, 50 mM Tris-HCl, 5 mM MgCl₂, 10 mM β-mercaptoethanol, 20 mM each dGTP, dATP and dTTP, 25 units ml⁻¹ pd(N)₉ nonamers and 1 unit of DNA polymerase 1 (Klenow fragment). Approximately 50 µCi of [α-³²P]dCTP were added (specific activity 3000 Ci mmol⁻¹) and the reaction incubated at 37°C for 15-30 minutes.

2.3 Bacterial strains

2.3.1 Preparation of glycerol stocks for long term storage

Aliquots of 850µl of overnight culture of different E.coli strains containing appropriate antibiotic selection, were mixed with 150µl of sterile glycerol and snap frozen on dry ice. Glycerol stocks were stored at -80°C.
2.3.2 XL1-blue

XL1-blue was grown on plates or in media containing 12.5μg ml⁻¹ of the antibiotic tetracyclin to maintain the F' episome required for single stranded rescue and α-complementation.

2.3.2.1 Preparation of competent cells for transformation with plasmid

Luria Broth (LB) 10 mg ml⁻¹ Bacto Tryptone, 5 mg ml⁻¹ yeast extract, 5 mg ml⁻¹ NaCl

LB Agar 1.5% w/v agar made up in LB and autoclaved to melt

TFB1 100 mM RbCl₂, 50 mM MnCl₂, 10 mM CaCl₂, 35 mM Sodium Acetate pH 5.9, 15% v/v glycerol

TFB2 100 mM MOPS pH6.8, 10 mM RbCl₂, 75 mM CaCl₂, 15%v/v glycerol

Glassware was rinsed with distilled H₂O to remove any traces of detergent which can reduce transformation efficiency. Competent cells for transformation were prepared by the method of Zieg by picking a single colony of XL1-blue from an agar plate into 5 ml of LB and growing at 37°C overnight. The next day 2 ml of overnight culture were added to 200 ml of LB and grown at 37°C in a shaking incubator until OD₆₀₀=0.3-0.4. The cells were harvested by centrifugation for 10 minutes at 4,200 rpm in a desk top centrifuge and the pellet was resuspended in 66 ml of ice cold TFB1. After incubation on ice for 20 minutes, cells were pelleted as before and gently resuspended in 10 ml of TFB2. These cells were stored frozen at -80°C in 200μl aliquots until use.
2.4 DNA manipulation and Southern analysis

2.4.1 DNA extraction

PBS 120 mM NaCl, 2.7 mM KCl, 10 mM NaH$_2$PO$_4$, (pH 7.4)

LSN 340 mM Lithium Acetate, 75 mM Lithium Dodecyl Sulphate, 10 mM EDTA (pH8.0), 100 mM Tris-HCl (pH8.0)

TE 10 mM Tris-HCl, 1 mM EDTA

Phenol/ chloroform 50% by volume phenol buffered with Tris-HCl (pH8.0), 50% by volume chloroform

2.4.1.1 From cell lines:

Cells were harvested from growth medium by centrifugation in sterile 50 ml tubes at 1,600 rpm in a bench top centrifuge. They were then washed twice by resuspension in ice cold PBS and collected by centrifugation as before. High molecular weight DNA was extracted from approximately $5 \times 10^8$ cells which were evenly resuspended in 500$\mu$l of TE and then lysed by the addition of 5 ml of LSN with gentle swirling. This lysate was then extracted three times with phenol/chloroform to remove proteins. An equal volume of phenol/chloroform was added to the lysate and the tube was gently agitated on a rocker for 15 minutes. The homogenate was then spun briefly at 2,600 rpm to separate the phases and the upper aqueous phase carefully removed to a clean 50 ml tube, taking care not to disturb the interface. DNA was precipitated from the aqueous phase by the addition of 0.1 volumes of 3M sodium acetate, pH5.5, and 2 volumes of 100% ethanol. The DNA was then spooled onto a glass rod, dried briefly in a 1.5 ml eppendorf tube at 37°C, and resuspended in 200-300$\mu$l H$_2$O at 37°C overnight. Yields of between 200 and 300$\mu$g were obtained from $5 \times 10^8$ cells using this method.
2.4.1.2 From peripheral blood:

Lymphocytes were extracted from heparinised blood by centrifugation through Histopaque 1077 (Sigma Diagnostics Ltd. UK). Ten ml of blood were first diluted with an equal volume of PBS and carefully layered onto an 8 ml cushion of Histopaque 1077 in a sterile 30 ml tube. This was spun in a bench top centrifuge for 30 minutes at 1,700 rpm at 21°C. Lymphocytes were harvested from the interface with a sterile pipette and then washed three times in ice cold PBS as described above. DNA extraction was then carried out, as described for cell lines.

2.4.1.3 From frozen samples:

Frozen aliquots of patient material or cell lines were thawed quickly in a waterbath at 37°C for 3 minutes. Cells were harvested by centrifugation at 1,200 rpm for 5 minutes and washed once in PBS in order to remove DMSO, before proceeding with DNA extraction as described for cell lines.

2.4.1.4 Extraction of plasmid DNA from bacterial cultures:

Plasmid DNA was extracted from bacterial cultures as described above, page 64.

2.4.2 Measurement of DNA concentrations

The concentration of the extracted DNA was estimated by absorbance spectrophotometry at 260nm using a path length of 1cm. The DNA concentration was calculated using the rule that the OD_{260} cm of a 50μgml^{-1} solution of double stranded DNA is 1. Oligonucleotides give an OD_{260} of 1
at a concentration of 20μg ml\(^{-1}\) for single stranded DNA and 30μg ml\(^{-1}\) for double stranded preparations. RNA and single stranded DNA give an OD\(_{260}\) of 1 at concentrations of 40μg ml\(^{-1}\).

2.4.3 **Restriction enzyme digestion of DNA**

All restriction enzymes were supplied by Promega, UK or Boehringer Mannheim, Germany. Typically 100 ng-1μg of plasmid or phage DNA was digested in a total volume of 20μl of the supplied buffer with 10 units of restriction enzyme at 37°C for 1-2 hr. For Southern blotting of genomic DNA 10-15μg of DNA were digested in a volume of 50μl of the supplied buffer containing 4 mM spermidine, 0.1μg μl\(^{-1}\) BSA and 100 units of restriction enzyme. Digests were incubated at 37°C for 4 hr.

2.4.4 **Gel electrophoresis**

10x TBE 0.9M Tris-Borate, 20 mM EDTA

Loading Buffer 0.25%w/v Bromophenol Blue, 40%w/v Sucrose

Ethidium Bromide 10 mg ml\(^{-1}\) ethidium bromide in H\(_2\)O

Gels were made up from powdered agarose (Sigma, UK) stirred into 1 x TBE buffer and dissolved by heating in a microwave oven. The solution was allowed to cool to 50°C and ethidium bromide was added to a final concentration of 0.05μg ml\(^{-1}\). Warm agarose was poured into a gel mould and allowed to set for 15-20 minutes at room temperature. Gels of 1-3% (w/v) were used to resolve DNA fragments of between 50 base pairs and 1 kb.
Digested genomic DNA samples were electrophoresed through 0.7% (w/v) agarose gels containing no ethidium bromide. Samples were mixed with 1/6 volume of loading buffer, and run in 1 x TBE buffer at 2V cm⁻¹ for 1-2 hr or 24-30 hr in the case of genomic digests. The apparatus used was Horizon 20, 25 or 58 (BRL, UK). Size markers used were 1 kb DNA ladder and/or lambda DNA digested with Hind III. After running, gels were stained for 20 minutes in 500 ml 1 x TBE buffer containing 50μl of ethidium bromide stock solution if none had been included in the gel. DNA was visualised as fluorescent orange bands or smears on an ultraviolet (UV) transilluminator and the gel was photographed using Polaroid film (type 553).

2.4.5 Blotting

Denaturing solution 1.5M NaCl, 0.5M NaOH

In order to facilitate the transfer of large fragments (>10 kb) DNA was partially depurinated by soaking the gel in 500 ml of 0.25M HCl for 15 minutes and then neutralised and made single stranded in denaturing solution for 20 minutes. DNA was transferred onto a nylon membrane (Hybond N+, Amersham, UK) by capillary blot in 0.4M NaOH. Blots were allowed to transfer for 10-16 hr.

2.4.6 Hybridisation

Hybridisation 0.5 M Na₂HPO₄, 7% Sodium Dodecyl Sulphate, 1 mM EDTA Buffer pH to 7.3 using Orthophosphoric Acid

The hybridisation oven used was an HB-1 (Techne, UK) with rotating cylindrical hybridisation chambers. Filters were prehybridised for 5 minutes at
65°C with 20 ml of hybridisation buffer. Between 20 and 30 ng of probe DNA were labelled as described above using 50μCi of deoxycytidine \([\alpha^{32}\text{P}]\) triphosphate (Amersham, UK). Labelled probe was separated from unincorporated nucleotides on Nuc Trap™ sephadex columns (Stratagene, UK) and heat denatured in boiling water for 5 minutes before being added to the prehybridisation buffer in the hybridisation cylinder. Filters were hybridised overnight at 65°C. After hybridisation filters were washed three times for ten minutes at 65°C with 2 x SSC. If necessary further washes at 65°C with 0.1 x SSC/0.1% SDS were made. Filters were sandwiched between two sheets of Saran wrap and any excess fluid carefully squeezed out onto paper towels. Blots were then placed in light tight cassettes (Genetic Research Instrumentation, UK) lined with intensifying screens (Kodak, UK) and exposed on X-ray film (Fuji, UK) at -80°C. Films were developed after 12-96 hr depending on the intensity of the probe signal.

2.5 Northern analysis

2.5.1 RNA extraction

To destroy RNases all solutions were made up with DEPC treated H₂O, and all glassware was baked for 8 hr at 150°C prior to use. Wherever possible sterile disposable plastic ware was used and disposable gloves were worn during RNA preparation and handling. Non-sterile glass and plastic ware that could not be baked were treated with 3% H₂O₂ for 10 minutes and rinsed in DEPC treated H₂O.
DEPC Treated H$_2$O 0.05% by volume Diethyl pyrocarbonate (DEPC) was added to distilled H$_2$O and incubated at 37°C overnight. The DEPC was then destroyed by autoclaving the solution for 30 minutes.

GTC 4 M Guanidinium Isothiocyanate, 1 M Sodium Citrate, 0.5% N-Lauryl Sarcosine, adjust to pH 7.0 with 1 M NaOH

CsCl 5.7 M Caesium Chloride CsCl, 10 mM EDTA adjust to pH7.0 with 1 M NaOH

This method is essentially that described by MacDonald. Cells were harvested and washed in PBS as described above. Aliquots of 1 x 10$^8$ cells were lysed on ice in 10 ml of GTC, and DNA was sheared by passing the solution repeatedly through a sterile 0.8 mm gauge needle attached to 10 ml sterile plastic syringe, until the solution was no longer viscous. The sheared lysate was then layered onto a 3.5 ml CsCl cushion in a 14 ml polyallomer tube (Beckman, UK) and RNA pelleted by centrifugation in a Beckman ultracentrifuge at 27,000 rpm for 20 hr at 15°C. The glassy pellet was resuspended in 400μl of DEPC treated H$_2$O and extracted with 400μl of phenol/chloroform in a sterile 1.5 ml eppendorf tube. The RNA was precipitated from the aqueous phase by the addition of 50μl 3M sodium acetate and 1 ml ethanol and incubation at -80°C for 30 minutes. After spinning at 14,000 rpm in a microcentrifuge the pellet was dried briefly at 37°C and resuspended in 100μl of DEPC treated H$_2$O and quantified by spectrophotometry at 260nm (OD$_{260} =1$ of 40μg ml$^{-1}$ RNA solution). Yields of between 400μg and 500μg of RNA were achieved using this method.
**2.5.2 Denaturing gel electrophoresis and blotting**

Sample loading mix 65% Formamide, 8.6% Formaldehyde, 0.032 μg μl⁻¹ Ethidium Bromide

Tracking dye 50% Glycerol, 0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 1 mM EDTA

10x MOPS buffer 200 mM MOPS (3-[N-morpholino]propanesulphonic acid), 50 mM Sodium Acetate, 10 mM EDTA, adjust to pH 7.0 with 10M NaOH

RNA was separated by electrophoresis at 8V cm⁻¹ through a 1% agarose gel containing 7.4% formaldehyde and 1 x MOPS buffer as described in Maniatis. Denaturing formaldehyde gels were prepared by mixing 1g of agarose with 73 ml of DEPC treated H₂O and heating in a microwave oven to dissolve. The agarose was allowed to cool to 60°C and 10 ml of 10x MOPS buffer and 16.5 ml formaldehyde were added. This solution was then poured into a medium sized casting tray (Biorad Horizon 11 14) and allowed to set. Between 10 and 15 μg of sample RNA, in a volume of 5μl, was added to 19.5 μl of sample loading mix in a sterile 1.5 ml eppendorf tube and incubated at 65°C for 5 minutes. Before loading, the tubes were cooled on ice for 30 seconds. Once run, gels were visualised on a UV transilluminator and photographed. RNA was transferred onto a Hybond N nylon membrane (Amersham, UK) by capillary blot in 20 x SSC overnight, and fixed to the membrane by 2 minutes UV irradiation on the transilluminator.

**2.5.3 Hybridisation**

Hybridisation was performed essentially as described above for DNA blots. However, all solutions were made up with DEPC treated H₂O and the
hybridisation cylinders were treated with 3% hydrogen peroxide for 10 minutes immediately before use, to destroy RNases.

2.6 Pulsed field gel electrophoresis

2.6.1 Preparation of DNA in agarose blocks

SE 75 mM NaCl, 25 mM EDTA
NDS 10 mM Tris base, 500 mM EDTA, 1% N-Lauryl Sarcosine, adjust pH to 9.5 with NaOH pellets
Proteinase K stock 20 mg ml\(^{-1}\) Proteinase K (Sigma, UK) in SE
Agarose 1% Seaplaque low melting point agarose in SE

Cells were harvested and washed in PBS as described above, quantified on a Coulter Counter (Coulter, UK) and resuspended in SE to 3 x 10\(^7\) cells ml\(^{-1}\). Equal volumes of molten agarose and cells were mixed and pipetted into a perspex mould (1.5 mm x 6 mm x 9 mm). Once set, the blocks were gently pushed into 10 ml NDS, 1 mg ml\(^{-1}\) proteinase K and incubated at 50°C overnight. Blocks were then transferred to fresh NDS/proteinase K solution and incubated at 50°C for a further 24-48 hr. Blocks were stored in NDS at 4°C after rinsing twice in NDS for 2 hr.

2.6.2 Digestion of DNA in agarose blocks

TE(1) 10 mM Tris-HCl, 1 mM EDTA
TE(0.5) 10 mM Tris-HCl, 0.5 mM EDTA
PMSF 100 mM PMSF (Phenylmethylsulphonyl Fluoride) in isopropanol
BSA 10 mg ml\(^{-1}\) BSA (Bovine Serum Albumin)
Spermidine 200 mM spermidine
Stop buffer 20 mM EDTA, 0.25% Orange G, 0.5 x TBE(0.5) (see below)
Before digestion blocks were incubated overnight in 20 x volume TE(1) containing 0.1 mM PMSF in order to inactivate any residual proteinase K. They were then washed once for 30 minutes in TE(1) at 4°C, and then twice for 30 minutes in TE(0.5) at the same temperature. In a clean petri dish, using a sterile scalpel, blocks were cut into two equal pieces lengthways and then placed into 500μl of an appropriate restriction buffer in a sterile microcentrifuge tube to equilibrate for 30 minutes at 4°C. The DNA was digested in a volume of 120μl of fresh restriction buffer containing 4 mM spermidine, 100μg ml⁻¹ BSA and 40-50 units of restriction endonuclease, at 37°C or 50°C for 4-16 hr. The reaction was stopped by placing the blocks into 100μl of stop buffer, for 10 minutes at 21°C.

2.6.3 Pulsed field gel electrophoresis and blotting

10 x TBE (0.5) 0.9M Tris-borate, 10 mM EDTA, pH8.2

Blocks were carefully loaded using a sterile spatula into wells in a 1% low electroendoosmosis agarose gel made up in 0.5 x TBE(0.5). Air spaces were sealed with the same molten agarose solution. To resolve fragments of between 50 kb and 1200 kb, gels were electrophoresed in a Biorad, CHEF DR2, apparatus at 200 volts in 0.5 x TBE(0.5) buffer at 18.5°C. The initial switch time was 60 seconds for 15 hr followed by a switch time of 90 seconds for 9 hr. When the run was complete, the gel was stained for 40 minutes in 500 ml of 0.5 x TBE(0.5) buffer with 50μl ethidium bromide, and photographed on a UV transilluminator. The DNA was then partially depurinated by soaking
the gel in 500 ml of 0.25M HCl for 15 minutes and denatured in denaturing solution for 20 minutes. DNA was transferred onto a Hybond N+ nylon filter (Amersham, UK) by capillary blot for 24 hr in 0.4M NaOH. After blotting, the filter was rinsed briefly in 2 x SSC and allowed to air dry.

2.6.4 Hybridisation

PFG Hybridisation buffer 5 x Denhardt's solution, 0.5M Na₂HPO₄ (pH to 7 with orthophosphoric acid), 10% dextran sulphate, 100μg ml⁻¹ sonicated salmon sperm DNA, 1% sodium dodecyl sulphate (SDS)

Filters were prehybridised for 1 hr at 65°C in 20 ml of PF hybridisation buffer in rotating glass cylinders. Labelled probe was prepared using 60μCi of deoxycytidine[α-³²P], as described above. Filters were hybridised for 24 hr at 65°C. After hybridisation filters were washed for 20 minutes in 2 x SSC, 0.1% SDS at 65°C and then twice for ten minutes with 2 x SSC. If necessary further washes at 65°C with 0.1 x SSC, 0.1% SDS were made until the filter gave between 10 and 20 counts sec⁻¹ when monitored. Filters were exposed on X-ray film as described above.

2.7 Amplification of DNA by the polymerase chain reaction

2.7.1 Optimisation of reaction conditions for specific primer pairs

2.7.1.1 Annealing temperature

Several calculations exist which can be used to estimate the annealing temperature of a given oligonucleotide. The temperature at which 50% of an oligonucleotide and its complement are in duplex (Tₘ) will vary according to
the DNA sequence, the DNA length, and the concentration of each strand. Increased salt concentration and the presence of denaturants such as formamide or DMSO will tend to lower the $T_m$. Equations which estimate the $T_d$ are useful in the case of hybridisation of oligonucleotides to filter bound sequences, although the $T_d$ is approximately 7-8°C lower than the $T_m$ as a result of immobilisation of the target strand.

Wallace’s rule\textsuperscript{271} provides a simple way to estimate the $T_d$ of DNA oligonucleotides between 14 and 20 bases long in 0.9M NaCl:

$$T_d = 2(A+T) + 4(G+C)$$

$A$, $G$, $C$ and $T$ are the number of occurrences of each nucleotide. No compensation is made for variations in salt concentration.

Many primer design programs, for example Oligo 4, use a more complex calculation\textsuperscript{272}, to calculate the $T_m$.

$$T_m = 1000\Delta H/(A + \Delta S + R\ln(C_t/4) - 273.15 + 16.6 \log[Na^+]$$

$\Delta H$ (Kcal/mol) is the sum of the nearest neighbour enthalpy changes for hybrids, $A$ is a constant containing corrections for helix initiation, $\Delta S$ is the sum of nearest neighbour entropy changes, $R$ is the Gas Constant and $C_t$ is the total molar concentration of strands. This calculation gives values that are within 5°C of empirically determined values for the $T_m$.

Primer pairs were designed using the Oligo 4 program to have a $T_m$ of >60°C, to contain between 40 and 60% G+C, and to minimise the chance of secondary structure caused by self complementarity. Reaction conditions were optimised empirically using an annealing temperature of 5°C below the $T_m$ as a starting point.
2.7.1.2 Mg\(^{2+}\) concentration and pH

The magnesium ion concentration can affect the fidelity and activity of the Taq polymerase. It can also alter the specificity and efficiency of the PCR reaction by affecting the annealing and dissociation temperatures of primers, template and PCR product. DNA and dNTPs can chelate free magnesium ions and so disturb the apparent magnesium concentration. Most primer pairs work well with 1.5 mM magnesium ion concentration, but this may need to be increased by up to 2 mM in some cases. BSA (0.1 μg μl\(^{-1}\)) and 0.1% triton were included in reactions to help stabilise the enzyme and 50 mM KCl was included to facilitate primer annealing. Reactions were buffered with 10 mM Tris-HCl. The pH and magnesium ion concentration were optimised empirically but most primer pairs worked well at pH 9 with 1.5 mM MgCl\(_2\).

2.7.2 Amplification of genomic DNA

Care was taken to set up PCR reactions in a purpose made hood using dedicated pipettes with plugged pipette tips, in order to minimise cross contamination. As a further precaution, all surfaces were wiped with sodium hypochlorite before starting work. Genomic DNA was extracted from lymphocytes or cell lines as described above and diluted to a concentration of 100 ng μl\(^{-1}\). Two drops of light mineral oil (Sigma, UK) were added to each 0.5 ml eppendorf and 100 ng or 1μl of template DNA pipetted as a bubble within the oil. A master mix was made up containing all the reagents for PCR with the exception of the template DNA. DNA was denatured at 95°C for 3 minutes before addition of 49μl of PCR master mix. Typically thirty five cycles were
performed in a Biometra Trio thermoblock. Each cycle consisted of 1 minute at 95°C, 1 minute at the optimised annealing temperature and 1 minute per kb of expected product length at 72°C. The last cycle included a final step of 10 minutes at 72°C.

2.7.3 Amplification of cDNA

RT mix
50 mM KCl, 10 mM Tris-HCl, pH9 (at 25°C), 1% Triton X-100, 7.5 mM MgCl₂, 1 mM each dNTP, 1 unit µl⁻¹ Rnasin (Promega), 5 pmol µl⁻¹ random hexamers, 1.5 units µl⁻¹ AMV reverse transcriptase

Magnesium free PCR mix
50 mM KCl, 10 mM Tris-HCl, pH9 (at 25°C), 1% Triton X-100, 0.5 pmol µl⁻¹ each primer, 0.01 units ul⁻¹ Taq polymerase

RNA was first reverse transcribed from random hexamers using AMV reverse transcriptase. The reaction mix was then diluted so that magnesium ions and dNTPs were at suitable concentrations for PCR. The cDNA was then amplified under essentially the same conditions as described for genomic DNA.

RNA was extracted as described. Cytoplasmic RNA was denatured at 90°C for 5 minutes and then rapidly cooled on ice. In an RNase free eppendorf tube 1µg of denatured RNA (in a volume of 1µl or less) was mixed with RT mix to a final volume of 20µl and incubated at 37°C for 1hr. The reaction mix was then heat treated at 90°C for 5 minutes to inactivate the reverse transcriptase. To this was added 80µl of magnesium free PCR mix and the reaction covered with two drops of mineral oil. Thirty five cycles of PCR were performed in a Biometra Trio thermoblock. Each cycle consisted of
1 minute at 95°C, 1 minute at the optimised annealing temperature and 1 minute at 72°C. The last cycle included a final step of 10 minutes at 72°C.

2.8 Cloning and sequencing of PCR products

2.8.1 Purification of PCR product

Gel Loading Buffer  0.25% Bromophenol Blue, 40% Sucrose, 40µg ml⁻¹ Ethidium Bromide

TAE Buffer  0.04M Tris-acetate, 0.001M EDTA

PCR products were analysed directly by electrophoresis on 2% Nusieve™ agarose gels in TAE buffer. Between 10 and 20µl of the PCR reaction were removed from the PCR reaction tube and pipetted onto Sealon film (Camlab, UK) where it was mixed with 3µl of gel loading buffer. Any mineral oil transferred from the PCR overlay adhered to the Sealon film and the aqueous phase was pipetted into the gel sample wells. Gels were run at 50V for 1-2 hr and visualised on a long wave UV transilluminator in order to minimise DNA damage. The PCR product was excised in a minimum volume of agarose which was frozen at -20°C for 1hr and then thawed at 37°C for 5 minutes before being macerated with a sterile scalpel. PCR product was eluted from the gel slice by spinning through a 0.45µm Durapore membrane (Millipore) at 5,000g for 20 minutes.
2.8.2 Cloning of PCR product into pGEM-T and preparation of single stranded DNA

Ligase buffer 30 mM Tris-HCl pH 7.8 (at 25°C), 10 mM MgCl₂, 10 mM DTT, 5 mM ATP

PCR master mix 50 mM KCl, 10 mM Tris-HCl pH 9 (at 25°C), 1% Triton X-100, 1.5 mM MgCl₂, 200 μM each dNTP, 50 pmol each T7/SP6 primer, 0.2 units Taq polymerase, 10 ng μl⁻¹ BSA

Many thermostable polymerases add a non-template derived deoxyadenosine to the 3’ end of PCR products, which decreases the efficiency of blunt ended cloning protocols. Vectors that have single 3’-T overhangs at the insertion site have increased efficiency of ligation to PCR products. For this study PCR products were cloned into pGEMT (Promega, USA). Ligations were performed in a 10μl volume, using 1 Wiess unit of T4 DNA ligase (Promega, USA), 50 ng of pGEM-T DNA and an equimolar amount of purified PCR product and incubated at 15°C for 3 hr. After this time the enzyme was inactivated by incubating at 72 °C for 10 minutes, before transformation of the ligated product into competent XL1-blue cells (see above).

Blue/white screening using IPTG and XGAL to identify insertional inactivation of the pGEM-T lacZ gene, proved inefficient. Many colonies that contained insert appeared pale blue. Colonies were screened directly by PCR using T7 and SP6 primers. Each colony was picked into 200μl of H₂O using a sterile pipette tip and the bacterial cells were evenly resuspended. 1μl of cell resuspension was added to 24μl of PCR master mix in 0.5 ml eppendorf tubes.
and covered with 2 drops of light mineral oil. Twenty cycles of PCR were performed in a Biometra Trio thermoblock with an initial denaturing step of 3 minutes at 95°C. Each cycle consisted of 1 minute at 95°C, 1 minute at 56°C and 1 minute at 72°C.

PCR product was run on a 2% agarose gel against 1 kb ladder DNA markers to identify inserts of the expected size. These colonies were then infected with VCSM13 helper phage (Stratagene, USA) and grown up to isolate single stranded DNA for sequencing as described below. A single colony was inoculated into 10 ml of LB containing 75μg ml⁻¹ ampicillin and 10⁸ pfu ml⁻¹ VCSM13 helper phage. This was incubated at 37°C with vigorous aeration for 1-2 hr after which time kanomycin was added to 70μg ml⁻¹ to select for infected cells. Cultures were grown for a further 16-24 hr at 37°C with aeration, and then the bacterial cells were removed by centrifugation at 2500 rpm in a desk top centrifuge. Phage particles were precipitated from the supernatant by the addition of 1.5 ml of 20% PEG/2.5M NaCl and cooling on ice for 15 minutes. Phage particles were collected by spinning for 10 minutes at 3000 rpm in a desk top centrifuge and resuspended in 400μl of 0.3M sodium acetate pH6/1 mM EDTA by vortexing vigorously. This solution was extracted once with an equal volume of phenol/chloroform and spun briefly to separate the phases. The aqueous phase was transferred to a sterile 1.5 ml eppendorf tube with 1 ml of ethanol and then spun for 5 minutes in a microcentrifuge. The pellet of single stranded DNA was dried and resuspended in 20μl of H₂O and 2μl of this was run on a 1% agarose gel, against a known amount of lambda DNA digested.
with Hind III, to estimate the DNA concentration. Yields were typically between 10 and 20 µg DNA.

2.8.3 Sequencing by the chain termination method

2.8.3.1 Sequencing using Taq polymerase

Sequencing was performed using the chain termination method and the thermostable enzyme, Taq polymerase (Taqsequence, USB, USA). This allowed the reactions to be performed at higher temperatures allowing denaturation of secondary structure in the template DNA. The first exon of MYC is GC rich and attempts to sequence this region using lower reaction temperatures and the Klenow fragment produced regions of compression and bands in all four lanes on denaturing polyacrylamide gels, which were difficult to interpret.

Annealing Mix 40 mM Tris-HCl pH 8.8, 4 mM MgCl₂
0.5 pmol T7 primer, 1 µg (0.5 pmol) template DNA.

Labelling Mix 0.6 µM each dGTP, dCTP, dTTP (final concentration 0.176 µM each), 5 µCi [α-³⁵S] dATP, 2 units Taq polymerase

ddGTP Termination Mix 15 µM each dGTP, dATP, dTTP, 45 µM ddGTP
ddATP Termination Mix 15 µM each dGTP, dATP, dTTP, 600 µM ddATP
ddCTP Termination Mix 15 µM each dGTP, dATP, dTTP, 450 µM ddCTP
ddTTP Termination Mix 15 µM each dGTP, dATP, dCTP, 1200 µM ddTTP

Stop Solution 95% Formamide, 20 mM EDTA, 0.05% each Bromophenol Blue and Xylene Cyanol FF

The annealing of primer to template DNA was performed by heating 13 µl of annealing mix to 70°C for 2 minutes and cooling slowly to 21°C over a
30 minute period. On ice 4μl of labelling mix were added, and the reactions were incubated at 45°C for 5 minutes. Four microlitres from each labelling reaction were added to 4μl of each termination mix in a fresh tube and incubated at 70°C for 5 minutes. Reactions were stopped on ice with the addition of 4μl of stop solution to each tube.

2.8.3.2 Denaturing polyacrylamide electrophoresis

Gel Fix 10% Methanol, 12% Acetic Acid by volume

Sequencing reactions were resolved on 6% polyacrylamide gels containing 7M urea in 1x TBE buffer. Gels were preheated to 50°C and samples were heated to 75°C for 5 minutes before loading 3μl into each well. After running at 50W for between 2 and 8 hr gels were briefly rinsed with gel fix solution to remove urea, and dried onto Watman 3MM paper for 2 hr in a Biorad 583 gel drier. Dried gels were then placed in light tight cassettes and exposed to blue sensitive X-ray film at room temperature for 12-72 hr.

2.9 Analysis of polymorphic repeat markers

2.9.1 Amplification of CA repeats using fluorescent-dye labelled primers

PCR reactions were performed as described above using commercially prepared fluorescent labelled primers. Reactions were performed in a total volume of 10μl containing 25 ng template DNA, 0.2 mM dNTPs, 50 pmol each primer, 10 ng μl⁻¹ BSA, 2 mM MgCl₂, 50 mM KCl, 10 mM Tris HCl pH9, 1% Triton X-100, and 0.15 units Taq polymerase. After amplification using 28
cycles of touchdown PCR, products were diluted 20 fold in distilled H$_2$O and 2µl loaded into a single well on a 6% denaturing polyacrylamide gel on an ABI automatic sequencer. GeneScan 672 analysis software version 1.2.2-1 (Applied Biosystems) was used to determine the presence of zero, one or two peaks corresponding to allelic repeats. Peaks were sized by running marker DNA ladder labelled with a fluorescent dye (ABI) in each lane.
3. Chapter Three: Deletions of $p16^{\text{INK4A}}$ and $p15^{\text{INK4B}}$ in lymphoid malignancies

3.1 Introduction

DNA blot was performed to analyse $p16^{\text{INK4A}}$ configuration in a panel of lymphoid tumours and lymphoid cell lines of varying stages in development. The $p16^{\text{INK4A}}$ probe used was a 330 bp fragment containing exon I of the $p16^{\text{INK4A}}$ gene; this probe was obtained by PCR amplification from normal human genomic DNA using previously described primers. The PCR product was cloned into pBluescript vector and sequenced to confirm its identity. Using Bam HI digests, a region around exon 1 of over 20 kb could be examined by Southern blot. A genomic map of the $p16^{\text{INK4A}}$ gene is shown in Fig 3.1. All filters had been previously probed with immunoglobulin, T-cell receptor and various other oncogene probes that allowed an estimation of contaminating normal cells to be made.

PCR analysis was not used as contaminating normal cells may give false positive results. The sensitivity of the PCR reaction is such that even a tiny proportion of target DNA may successfully amplify to give a strong positive result. Most samples of leukaemic blood contain a proportion of normal cells which might mask detection of deletions of the $p16^{\text{INK4A}}$ gene within the leukaemic cells by PCR.
Figure 3.1 Genomic map of $p_{16}^{INK4A}$ locus $^{17,131}$

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Figure 3.1 Restriction map of $p_{16}^{INK4A}$ and $p_{15}^{INK4B}$. Note that Eco RI sites 3' of $p_{16}^{INK4A}$ exon 2 or 5' of $p_{15}^{INK4B}$ exon 2 are not shown. All samples were analysed using the $p_{16}^{INK4A}$ exon 1 probe shown here. UoCB4 was also analysed using a PCR derived $p_{16}^{INK4A}$ exon 2 probe that cross hybridises to $p_{15}^{INK4B}$ exon 2. $^{17}$
3.2 Results

A total of 231 fresh cases of lymphoid leukaemia or lymphoma and 66 cell lines representing malignancies of both B and T-cell lineages at various stages of differentiation were analysed. Overall, biallelic deletions of $p16^{INK4A}$ were observed in 17 (7.3%) and rearrangements of $p16^{INK4A}$ in 3 (1.3%) of fresh cases. Of the lymphoid cell lines, 9 (13.6%) exhibited biallelic deletion and 2 (3%) exhibited rearrangement. Results are summarised in Tables 3.2, 3.3 and 3.4 and are discussed according to the diagnostic subgroups below.

3.2.1 Acute lymphoblastic leukaemia

Fifty-three cases of BCP-ALL were examined, and of these, 11 cases (20%) exhibited biallelic deletions of $p16^{INK4A}$. Representative Southern blots are shown in Fig 3.2 and 3.4. Cases with biallelic deletions comprised a heterogeneous clinical group and were not associated with a consistent cytogenetic abnormality as shown in Table 3.4. In particular, of the 17 cases of BCP-ALL with the t(9;22)(q34;q11), only three (18%) exhibited $p16^{INK4A}$ deletions: two of these cases had cytogenetically detectable rearrangements of chromosome 9p (cases 6 and 11, Table 3.4). Two of the three cases with t(9;22)(q34;q11) and biallelic $p16^{INK4A}$ deletion were BCP-ALL arising as blast crisis of chronic myeloid leukaemia (cases 6 and 10 Table 3.4): whether loss of $p16^{INK4A}$ was associated with this transformation could not be determined, due to lack of suitable specimens in these two patients. One patient with t(4;11)(q21;q23) retained germline $p16^{INK4A}$. 
Figure 3.2 Deletions and rearrangements of $p16^{INK4A}$ in B-cell malignancy

Fig 3.2 Deletions and rearrangements of $p16^{INK4A}$ in B-cell malignancy. A) DNA digested with Eco RI and probed with both MYC and $p16^{INK4A}$ exon 1 probes. B) DNA digested with Bam HI and probed with $p16^{INK4A}$ exon 1. C) DNA digested with Pst I and probed with $p16^{INK4A}$ exon 1. Abbreviations: D = Daudi. 6, 16, 17, 18 refer to patient numbers in Table 3.4. Note 3 $p16^{INK4A}$ fragments of differing intensity in Daudi in both Bam HI and Pst I digests. Note biallelic deletion of $p16^{INK4A}$ in patient 6. Cross hybridising bands were seen with the $p16^{INK4A}$ exon 1 probe despite stringent washing in 0.1 x SSC 0.1% SDS.
Figure 3.3 Retention of germline $p^{16}_{\text{INK4A}}$ in BL-cell lines

*Figure 3.3 Retention of germline $p^{16}_{\text{INK4A}}$ in BL cell lines and lymphoblastic cell lines derived from the same patients. All BL cell lines with the exception of BL28 (arrowed) retained germline $p^{16}_{\text{INK4A}}$ as did all lymphoblastoid cell lines. Note cross-hybridising bands in Eco RI panel.*
Of the two BCP-ALL lines examined, one, UoCB1, showed biallelic \(^{p16^{\text{INK4A}}}_1\) deletion, whereas UoCB4 retained germline configuration in Eco RI digests. Use of an exon 2 probe that hybridises to both \(^{p16^{\text{INK4A}}}_1\) and \(^{p15^{\text{INK4B}}}_1\) revealed that \(^{p15^{\text{INK4B}}}_1\) was biallelically deleted in UoCB4, and also showed \(^{p16^{\text{INK4A}}}_1\) rearrangement in Bam HI digests. The availability of these cell lines permitted high resolution cytogenetics, but no structural abnormalities of the short arm of chromosome 9p were detected (E. Nacheva personal communication). These data, and those presented in Table 3.3 therefore indicated that \(^{p16^{\text{INK4A}}}_1\) deletions for the most part occurred in the absence of the cytogenetically detectable interstitial deletion 9p21-22.

Two cases (3.8% of the total) exhibited \(^{p16^{\text{INK4A}}}_1\) rearrangements (cases 13 and 14, Table 3.4 and Fig 3.4). These cases again lacked cytogenetic change at chromosome 9p. These rearrangements were seen in multiple restriction enzyme digests, were not seen in normal cases and were therefore neither simple polymorphisms nor point mutations. In case 13 the rearranged allele was associated with deletion of the other allele, whereas in case 14 both alleles of \(^{p16^{\text{INK4A}}}_1\) were rearranged, (Fig 3.4). In both of these cases and in all other cases with \(^{p16^{\text{INK4A}}}_1\) rearrangements (see below) a germline configuration was observed in Eco RI digests with the \(^{p16^{\text{INK4A}}}_1\) exon 1 probe.

Finally, leukaemic blasts from some cases of BCP-ALL have been shown to produce overt, transplantable disease when inoculated into SCID mice \(^{252, 275}\). Eleven such transplantable cell lines were established from a total of 27 different BCP-ALL samples: some of these lines have been shown
to maintain identical IGH rearrangements to those observed in patient material over several passages. Also, the immunophenotype and karyotype of transplanted cells remained the same with repeated passages (JM Heward, SE Height, GJ Swansbury, MJS Dyer, P Mitchell: unpublished observations, 1994). Seven out of ten BCP-ALL cases that grew in SCID mice and the original patient material showed biallelic p16^{INK4A} deletions (Fig 3.2 and Table 3.4). In contrast, of the eleven cases analysed that failed to grow in SCID mice, ten retained germline p16^{INK4A}. Only case 14 that showed biallelic p16^{INK4A} rearrangement failed to grow. Three cases of BCP-ALL with germline p16^{INK4A} did grow in SCID mice: details of these cases are given in Table 3.4, cases 16-18.

The difference in p16^{INK4A} configuration between the BCP-ALL population that grow in SCID mice and those that fail to grow in SCID mice was tested using the ARCUS program (Shareware) to calculate values for Fischers exact test. The probability that the null hypothesis (no difference in p16^{INK4A} configuration between the BCP-ALL population that grow in SCID mice and those that fail to grow in SCID mice) is true was calculated to be 0.007519 for the two tailed probability by summation. Therefore, p<0.01 and we reject the null hypothesis and accept that p16^{INK4A} is more frequently deleted in BCP-ALL that grow in SCID mice than in those that fail to grow in SCID mice.
Table 3.1 Configuration of $p16^{\text{NK4A}}$ in BCP-ALL inoculated into SCID mice

<table>
<thead>
<tr>
<th></th>
<th>Grow in SCID mice</th>
<th>Fail in SCID mice</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p16^{\text{NK4A}}$ D/D</td>
<td>7</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>$p16^{\text{NK4A}}$ G/G</td>
<td>3</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>11</td>
<td>21</td>
</tr>
</tbody>
</table>

Expectation of D/D + Grow = 3.809524
Two-tailed probability (by summation) = 0.007519

3.2.2 Burkitt’s lymphoma

A panel of 8 sporadic cases of Burkitt’s lymphoma and 26 cell lines derived from both sporadic and endemic cases was examined. One of the eight fresh cases and 1/26 cell lines exhibited biallelic $p16^{\text{NK4A}}$ deletions. One of the Burkitt’s cell lines (Daudi) exhibited two bands additional to the germline band in multiple enzyme digests, without any loss of the germline signal (Fig 3.2). All seventeen Epstein-Barr (EBV) transformed B-cell lines derived from patients with Burkitt’s lymphoma failed to demonstrate any $p16^{\text{NK4A}}$ abnormality (Fig 3.3).
Figure 3.4 Deletion and rearrangement of $p16^{INK4A}$ in B-cell malignancy

Figure 3.4A) DNA digested with Bam HI and probed with $p16^{INK4A}$ exon 1 probe. Abbreviations: N = normal peripheral blood DNA. 5, 6, 14, 16 and 17 correspond to cases in Table 3.3. Note rearranged fragment of 14 kb in case 14. B) DNA digested with Bgl II and probed with $p16^{INK4A}$ exon 1. Abbreviations: N = normal peripheral blood DNA; Do = DoHH2; P = Pleural effusion from which DoHH2 was derived; 14 corresponds to case 14 in Table 3.3. Note biallelic deletion of $p16^{INK4A}$ in DoHH2 and original tumour cells despite cytogenetically normal chromosome 9s. Note biallelic $p16^{INK4A}$ rearrangement in case 14.
Figure 3.5. DNA was digested with Eco RI and probed with \textit{p16}\textsuperscript{INK4A} exon 1. Rearrangements of cross hybridising bands are seen in cell lines and tumour DNA with germline \textit{p16}\textsuperscript{INK4A} and biallelic \textit{p16}\textsuperscript{INK4A} deletion. Karpas 1106, lane 2, shows rearrangement of the 6 kb cross hybridising band whereas DS, lane 12, and Namalwa IPN/45, lane 3, show deletion of this band. Abbreviations: 1= Wien 133; 2= Karpas 1106; 3= Namalwa IPN/45; 4= OCLY8; 5= Karpas 231; 6= SSK41; 7= Granta 452; 8= Granta 519; 9= DS; 10= UoCB4; 11= SuPT1.
3.2.3 De novo B-cell leukaemia with t(14;18)(q32.3;q21.3)

An aggressive subset of B-cell leukaemia has been recognised; these leukaemias have concurrent activation of \textit{BCL2} and \textit{MYC} oncogenes. Five cases consisting of 3 cell lines (Karpas 231, 353, and KMH-2B) were analysed along with 3 fresh cases. The primary tumours were the original sample from which the cell line Karpas 231 was derived, a case with both t(14;18)(q32.3;q21.3) and t(8;14)(q24.1;q32.3) and a case with t(8;9)(q24.1;p13.3). No cases exhibited biallelic deletion of \textit{p16\textsuperscript{INK4A}}. These data indicate that \textit{p16\textsuperscript{INK4A}} is not the target for 9p13.3 translocations in these leukaemias.

3.2.4 Non-Hodgkin’s lymphoma cell lines

Thirteen NHL, 10 B-NHL and 3 T-NHL cell lines were examined (Table 3.3): six cell lines (46%), namely DoHH2, Granta 452 and 519, Karpas 1106, SuPT1 and SKW3 exhibited biallelic \textit{p16\textsuperscript{INK4A}} deletions (see Fig 3.4 and 3.5). Of these, four represented transformed NHL. Cell line DoHH2 was derived from a patient with immunoblastic transformation of follicular B-NHL. Similarly Granta 452 was derived from a patient with B-cell acute leukaemia supravening follicular B-NHL. In both these cases it was possible to demonstrate that the biallelic deletions had occurred in the primary patient material and was therefore not an artefact of prolonged \textit{in vitro} culture (Fig 3.4). High resolution cytogenetic analysis of cell lines DoHH2 and Karpas 1106 failed to detect rearrangement of 9p21. However Karpas 1106 did possess isochromosome(9p) in addition to two normal chromosome 9. Cell
line Granta 519 was derived from a patient with B-cell acute leukaemia supravening mantle cell lymphoma: this cell line expresses the highest level of $CCND1$ in any of the six cell lines with t(11;14)(q31;q32) examined in our laboratory and yet also exhibited biallelic $p16^{INK4A}$ deletions. This indicates that in this case cyclin D1 overexpression was not in itself sufficient to overcome $p16^{INK4A}$ inhibition of cell cycle progression. However, this was the only case of six with t(11;14)(q13;q32.3) to exhibit any obvious $p16^{INK4A}$ abnormality.

3.2.5 Fresh cases of non-Hodgkin’s lymphoma

A total of 38 cases of various histological subtypes of NHL were examined: 7 cases were of T-cell lineage. No deletions were observed in 11 primary follicular B-NHL, 2 mantle-cell B-NHL or 16 cases of primary diffuse large-cell B-NHL. Biallelic rearrangement of $p16^{INK4A}$ with loss of all germline sequence was seen in one case of chemotherapy resistant monocytoid B-NHL. Also biallelic $p16^{INK4A}$ deletion was observed in an unusual and rapidly progressive B-NHL in leukaemic phase expressing both CD5 and CD10 and exhibiting translocation t(8;11)(q24.1;q13) with overexpression of $CCND1$ (D Jadayel, DG Oscier, MJS Dyer: unpublished observations).

Of the 7 cases of T-NHL, three of six cases of T-cell lymphoblastic lymphoma exhibited biallelic $p16^{INK4A}$ deletion. One case was of immunoblastic transformation of Sézary syndrome associated with HTLV-1 infection in a man of Caribbean origin. $P16^{INK4A}$ was present in a peripheral blood sample taken at diagnosis, but absent from the lymph node of immunoblastic transformation although analysis of the $TCR$ $\beta$ gene showed
the same T-cell clone was present in both samples. A similar loss of both alleles of $p16^{INK4A}$ was observed in a case of immunoblastic transformation of follicular B-NHL.

3.2.6 Leukaemias of mature lymphocytes

A series of 119 fresh cases comprising 34 cases of B-CLL, 22 cases of SMZL (four with t(11;14)(q13;q32), six cases of HCL, five cases of B-PLL, 46 cases of T-PLL and six cases of leukaemia of large granular lymphocytoma were investigated. No abnormalities of $p16^{INK4A}$ were detected. Similarly, no abnormalities were detected in three myeloma cell lines, U-266, Karpas 620 and Karpas 1272. Therefore biallelic $p16^{INK4A}$ deletion seems not to be associated with mature leukaemias, or lymphomas of either B-cell or T-cell lineage.
Table 3.2. Overall incidence of $p^{\text{NK4A}}$ deletions in lymphoid malignancies and lymphoid cell lines.

<table>
<thead>
<tr>
<th>Diagnostic Subgroup</th>
<th>n</th>
<th>Deletions</th>
<th>Rearrangements</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ALL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCP-ALL</td>
<td>53</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>BCP-ALL cell lines</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCP-ALL</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. Burkitt's lymphoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkitt's lymphoma</td>
<td>8</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Burkitt's lymphoma cell lines</td>
<td>26</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>EBV- transformed cell lines</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3. Leukaemia with t(14;18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 cell lines and 2 fresh cases</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4. Lymphoma cell lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-cell</td>
<td>10</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>T-cell</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>5. NHL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-cell</td>
<td>31</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>T-cell</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>6. Leukaemias of mature lymphocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-CLL</td>
<td>34</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B-PLL</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SMZL</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HCL</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T-PLL</td>
<td>46</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LGL</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7. Myeloma cell lines</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Table 3.2 Abbreviation: n= number of cases*
Table 3.3. **p16^{INK4A}** abnormalities in lymphoid cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Derivation</th>
<th>Chromosome 9 abnormalities</th>
<th>p16^{INK4A}</th>
</tr>
</thead>
<tbody>
<tr>
<td>UoCB1</td>
<td>BCP-ALL</td>
<td></td>
<td>D/D</td>
</tr>
<tr>
<td>UoCB4</td>
<td>BCP-ALL</td>
<td></td>
<td>R/R</td>
</tr>
<tr>
<td>BL 16-135 (n=20)</td>
<td>Burkitt's lymphoma</td>
<td>BL28=D/D 19=G/G</td>
<td></td>
</tr>
<tr>
<td>IARC (n=17)</td>
<td>Lymphoblastoid</td>
<td></td>
<td>All 17=G/G</td>
</tr>
<tr>
<td>Wien 133</td>
<td>Burkitt's lymphoma</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>Granta 56</td>
<td>Burkitt's lymphoma</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>Namalwa (plus Namalwa IPN/45)</td>
<td>Burkitt's lymphoma</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>Raji</td>
<td>Burkitt's lymphoma</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>Daudi</td>
<td>Burkitt's lymphoma</td>
<td></td>
<td>G/R/R/r</td>
</tr>
<tr>
<td>Karpass 231*</td>
<td>B-cell leukaemia with t(14;18)</td>
<td>t(8;9)(q24.1;p13.3)</td>
<td>G/G</td>
</tr>
<tr>
<td>Karpass 353</td>
<td>B-cell leukaemia with t(14;18)</td>
<td>t(8;9)(q24.1;p13.3)</td>
<td>G/G</td>
</tr>
<tr>
<td>KMH-2B</td>
<td>B-cell leukaemia with t(14;18)</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>SuDHL6</td>
<td>DLCL</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>Karpass 422</td>
<td>DLCL</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>OCLY8</td>
<td>DLCL</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>SSK41</td>
<td>DLCL</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>WSU-NHL</td>
<td>DLCL</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>NCEB1</td>
<td>MCL</td>
<td>t(9;?:13)(q32;?:q14)</td>
<td>G/G</td>
</tr>
<tr>
<td>Karpass 1106</td>
<td>Mediastinal B-NHL</td>
<td>iso(9p)</td>
<td>D/D</td>
</tr>
<tr>
<td>DoHH2*</td>
<td>Immunoblastic B-NHL</td>
<td></td>
<td>D/D</td>
</tr>
<tr>
<td>Granta 452*</td>
<td>Leukaemic transf. of B-NHL</td>
<td>t(2;9)(p11;p13)</td>
<td>D/D</td>
</tr>
<tr>
<td>Granta 519</td>
<td>Leukaemic transf. of MCL</td>
<td>t(9;14)(p13;q11)</td>
<td>D/D</td>
</tr>
<tr>
<td>JVM-2</td>
<td>B-PLL</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>Karpass 247*</td>
<td>B-PLL</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>Karpass 384</td>
<td>Leukaemic transformation of T-NHL</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>SKW-3</td>
<td>T-NHL</td>
<td></td>
<td>D/D</td>
</tr>
<tr>
<td>SuPT1</td>
<td>T-NHL</td>
<td></td>
<td>D/D</td>
</tr>
</tbody>
</table>

Table 3.3 All cell lines had cytogenetically normal chromosome 9 unless stated otherwise. For references for the derivation of cell lines see Chapter 2. Note DoHH2, Granta 452 and Granta 519 were all derived from cases of transformed B-NHL. Abbreviations: DLCL= diffuse large cell lymphoma; MCL = mantle cell B-NHL; B-PLL = B-cell prolymphocytic leukaemia; BCP-ALL = B-cell precursor ALL; G = germline p16^{INK4A}; R= rearrangement of p16^{INK4A}; r= faint rearrangement of p16^{INK4A}; *= identical configuration of patient material and derived cell line.
Table 3.4 Characteristics of BCP-ALL with $p16^{INK4A}$ abnormalities and engraftment in SCID mice

A) BCP-ALL with $p16^{INK4A}$ abnormalities

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/ Sex</th>
<th>WCC</th>
<th>Cytogenetic Abnormalities</th>
<th>Outcome</th>
<th>SCID</th>
<th>$p16^{INK4A}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/F</td>
<td>10.6</td>
<td>+21</td>
<td>A:2nd CR</td>
<td>+</td>
<td>D/D</td>
</tr>
<tr>
<td>2</td>
<td>5/F</td>
<td>17.9</td>
<td>No clone</td>
<td>A:1st CR</td>
<td>NT</td>
<td>D/D</td>
</tr>
<tr>
<td>3</td>
<td>10/F</td>
<td>470</td>
<td>t(7;9)(p15;q13)</td>
<td>A:1st CR</td>
<td>+</td>
<td>D/D</td>
</tr>
<tr>
<td>4</td>
<td>15/F</td>
<td>183</td>
<td>No clone</td>
<td>D</td>
<td>NT</td>
<td>D/D</td>
</tr>
<tr>
<td>5</td>
<td>15/M</td>
<td>9.3</td>
<td>No clone</td>
<td>A:1st CR</td>
<td>NT</td>
<td>D/D</td>
</tr>
<tr>
<td>6</td>
<td>16/F*</td>
<td>13.9</td>
<td>t(9;22)(q34;q11)</td>
<td>D</td>
<td>+</td>
<td>D/D</td>
</tr>
<tr>
<td>7</td>
<td>19/F</td>
<td>105</td>
<td>No clone</td>
<td>D</td>
<td>NT</td>
<td>D/D</td>
</tr>
<tr>
<td>8</td>
<td>29/M</td>
<td>97</td>
<td>t(9;22)(q34;q11)</td>
<td>D</td>
<td>+</td>
<td>D/D</td>
</tr>
<tr>
<td>9</td>
<td>36/M</td>
<td>21.9</td>
<td>Hyperdiploid</td>
<td>A:1st CR</td>
<td>+</td>
<td>D/D</td>
</tr>
<tr>
<td>10</td>
<td>40/F*</td>
<td>96</td>
<td>t(9;22)(q34;q11)</td>
<td>D</td>
<td>+</td>
<td>D/D</td>
</tr>
<tr>
<td>11</td>
<td>70/F</td>
<td>112</td>
<td>t(9;22)(q34;q11)</td>
<td>D</td>
<td>+</td>
<td>D/D</td>
</tr>
<tr>
<td>12</td>
<td>UoCB1</td>
<td>Complex</td>
<td></td>
<td>D</td>
<td>NT</td>
<td>D/D</td>
</tr>
<tr>
<td>13</td>
<td>19/F</td>
<td>19.6</td>
<td>No clone</td>
<td>A:2nd CR</td>
<td>NT</td>
<td>D/R</td>
</tr>
<tr>
<td>14</td>
<td>35/M</td>
<td>259</td>
<td>Hyperdiploid</td>
<td>D</td>
<td>-</td>
<td>R/R</td>
</tr>
<tr>
<td>15</td>
<td>UoCB4</td>
<td>Complex</td>
<td></td>
<td>NT</td>
<td>R/R</td>
<td></td>
</tr>
</tbody>
</table>

B) BCP-ALL with no $p16^{INK4A}$ abnormalities but SCID engraftment

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/ Sex</th>
<th>WCC</th>
<th>Cytogenetic Abnormalities</th>
<th>Outcome</th>
<th>SCID</th>
<th>$p16^{INK4A}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>2/F</td>
<td>86</td>
<td>t(9:15)(p21;q15)</td>
<td>A:1st CR</td>
<td>+</td>
<td>G/G</td>
</tr>
<tr>
<td>17</td>
<td>0.75/F</td>
<td>164</td>
<td>No clone</td>
<td>A:1st CR</td>
<td>+</td>
<td>G/G</td>
</tr>
<tr>
<td>18</td>
<td>17/M</td>
<td>26</td>
<td>Hyperdiploid</td>
<td>D</td>
<td>+</td>
<td>G/G</td>
</tr>
</tbody>
</table>

Table 3.4 Abbreviations: WCC = white cell count at presentation; A = alive; D = dead; CR = complete remission; + = engraftment in SCID mice; - = failure to engraft in SCID mice; NT = not tested. * = CML in BCP-ALL blast crisis.
4. Chapter Four: The Extent and Mechanism of Biallelic $p16^{INK4A}$ Deletions Identified In Lymphoid Cell Lines

4.1 Introduction

Deletions affecting the chromosomal arm 9p, range in size from the cytogenetically undetectable through to complete loss of chromosome 9. The number of genes affected by these deletions is potentially very large, but $p16^{INK4A}$ has been identified within the smallest common region of deletion and has been proposed as the critical gene affected in tumour cells. As reported in Chapter 3, $p16^{INK4A}$ was completely lost in a proportion of lymphoid tumours despite lack of cytogenetically visible 9p deletions. However this does not prove that $p16^{INK4A}$ is the target gene, since another closely linked gene might be the tumour suppressor gene on 9p in lymphoid malignancies, or $p16^{INK4A}$ may share the tumour suppressor role with one or more other genes at this locus. This chapter presents data from lymphoid cell lines with homozygous $p16^{INK4A}$ deletions, in which the extent of the deletion was mapped using amplification of sequence tagged sites (STS) in a region >70 kb around $p16^{INK4A}$ (see Fig 4.1).

Homozygous deletion of $p16^{INK4A}$ appears to be the major mechanism of inactivation of this gene in many haematological malignancies. Whilst other recognised tumour suppressor genes are frequently inactivated by point mutations, sequence changes in $p16^{INK4A}$ are relatively rare in lymphoid tumours.
There are several somatic mechanisms that could lead to homozygous deletion of $p_{16}^{INK4A}$ in tumour cells. Firstly, two independent deletions may occur on each chromosome 9. Alternatively, deletion of one chromosomal region may be followed by duplication of the deleted chromosome and subsequent loss of the normal chromosome 9 during mitosis. This is known as chromosomal non-dysjunction. Finally, a copy of the region containing the deleted gene may replace the homologous region on the normal chromosome by a process known as gene conversion. In order to investigate whether loss of one $p_{16}^{INK4A}$ allele had occurred in these cell lines via chromosomal non-dysjunction analysis of heterozygosity of polymorphic markers on chromosome 9 was performed. Data is
also included here of FISH analysis using a PAX5 probe to further pinpoint 9p breakpoints in cell lines Granta 452, Karpas 231, and Granta 519.

4.2 Results

4.2.1 STS Mapping

In order to investigate the size and extent of p16<sup>Ink4A</sup> deletions in lymphoid malignancies PCR analysis of STS spanning a region >70 kb around p16<sup>Ink4A</sup> was performed on 13 lymphoid cell lines and a BCP-ALL case grown in SCID mice. CC consisted of tumour cells from case 6 grown in SCID mice, 104 was a cell line derived from case 13 which failed to grow in SCID mice, see Table 3.4. Results are summarised in Table 4.1 and 4.2. Ten of these cell lines were shown to have p16<sup>Ink4A</sup> deletion by Southern blot (see Chapter 3). Three cell lines, Daudi, UoCB4 and 104, showed rearrangement of p16<sup>Ink4A</sup> exon 1. Karpas 231 possessed the translocation t(8;9)(q24.1;p13.3) but was germline for p16<sup>Ink4A</sup> exon 1 and p15<sup>Ink4B</sup>. JVM-2 showed no abnormalities of chromosome 9 by cytogenetics or Southern blot, and was included as a control. The possibility of contamination of PCR reactions was minimised by repeating each PCR reaction at least three times with the incorporation of several no DNA control tubes and three normal peripheral blood DNA positive controls.

Four cases UoCB1, BL28, Granta 519, and CC showed deletion of p16<sup>Ink4A</sup> or p15<sup>Ink4B</sup> by Southern blot, and gave no PCR product with any of the STS primers used (Table 4.2). This indicated that both p15<sup>Ink4B</sup> and p16<sup>Ink4A</sup>
were lost and that the deletions spanned more than 70 kb. In the remaining five cell lines with homozygous deletion of \( p16^{INK4A} \) by Southern blot, at least one STS was present. This was predominantly the most proximal or centromeric marker, c1.B (Fig 4.2). Five cell lines K1106, DoHH2, Granta 452, and SuPT1 still showed deletion of both \( p16^{INK4A} \) and \( p15^{INK4B} \), whilst SKW-3 retained \( p15^{INK4B} \), showing deletion of \( p16^{INK4A} \) only.

Two cell lines, Daudi and UoCB4, showed loss of \( p15^{INK4B} \) with retention of \( p16^{INK4A} \) coding sequences. Daudi and UoBC4 were included in the STS analysis as a result of rearrangements of \( p16^{INK4A} \) exon 1 observed by Southern blotting. Whether these deletions could functionally inactivate \( p16^{INK4A} \) by removing 5' regulatory sequences is unclear, but in both cases exon 1 \( \beta \) of \( p16^{INK4A} \) would be lost. This alternative transcription initiation site is located 10-20 kb upstream of \( p16^{INK4A} \) exon 1 and is transcribed in both normal and tumour cell lines. Although the exon 1 \( \beta \) transcript contains an open reading frame it is not thought to be transcribed in vivo and probably plays a role in transcriptional regulation of \( p16^{INK4A} \) throughout the cell cycle\(^{194}\).

Case 104, derived from a patient with BOP-ALL showed two separate regions of deletion within the area studied. The STS RN3.1 within exon 2 of \( p16^{INK4A} \) was lost as was \( p15^{INK4B} \) exon 2. By Southern blot this cell line was shown to have a deletion of one allele of \( p16^{INK4A} \) exon 1 and rearrangement of the second.
Figure 4.2 Amplification of STS at the $p16^{INK4A}$ locus in cell lines with abnormalities of $p16^{INK4A}$ by Southern blot. 50 ng genomic DNA was amplified using previously described primers.17 Abbreviations: 1= UoCB1; 2= BL28; 3= Granta 519; 4= CC; 5= DoHH2; 6= Granta 452; 7= SuPT1; 8= SKW3; 9= Karpas 1106; 10=UoCB4; 11= 104; 12= Daudi; 13= Karpas 231; 14= JVM-2; 15= Normal control. Expected size product arrowed, other bands were considered as non-specific amplification. Note 104 (Lane 11) shows loss of RN3.1 and MTS2 despite retention of c5.3 indicating the presence of two separate deletions at one allele in this cell line. Daudi (Lane 12) shows loss of MTS2 only.
Table 4.1. Comparison of Southern blot data and STS analysis of $p16^{INK4A}$ and $p15^{INK4B}$ deletions in lymphoid cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Derivation</th>
<th>Blot of $p16$ exon 1</th>
<th>$p16$ intact by PCR</th>
<th>$p15$ intact by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>UoCB1</td>
<td>BCP-ALL</td>
<td>D/D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BL28</td>
<td>Burkitt's lymphoma</td>
<td>D/D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G519</td>
<td>Leukaemic transformation of MCL</td>
<td>D/D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CC*</td>
<td>BCP-ALL secondary to CML</td>
<td>D/D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DoHH2</td>
<td>Immunoblastic B-NHL</td>
<td>D/D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Granta 452</td>
<td>B- ALL</td>
<td>D/D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SUPT1</td>
<td>T-NHL</td>
<td>D/D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SKW-3</td>
<td>T-NHL</td>
<td>D/D</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Karpas 1106</td>
<td>Mediastinal B-NHL</td>
<td>D/D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UoCB4</td>
<td>BCP-ALL</td>
<td>R/R</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>104</td>
<td>BCP-ALL</td>
<td>D/R</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Daudi</td>
<td>Burkitt's lymphoma</td>
<td>G/R/R/r</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Karpas 231</td>
<td>B-ALL with t(14;18)</td>
<td>G/G</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>JVM-2</td>
<td>BCP-ALL</td>
<td>G/G</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 4.1 Abbreviations: G = germ-line; R = rearranged; r = faint rearranged band; D = deleted; + = all STS within the gene were amplifiable by PCR; - = one or more STS sites within the gene failed to amplify; * = Tumour cells grown in SCID mice. Note although the exons 1-3 of $p16^{INK4A}$ were intact by STS PCR, exon 10 fell within the deleted region in UoCB4 and possibly also in Daudi.
Table 4.2 Extent of homozygous deletions of $p16^{INK4A}$ in lymphoid cell lines

<table>
<thead>
<tr>
<th>STS</th>
<th>1063.7</th>
<th>c18.B</th>
<th>c5.1</th>
<th>RN3.1</th>
<th>c5.3</th>
<th>R2.3</th>
<th>R2.7</th>
<th>$p15^{INK4B}$</th>
<th>c1.B</th>
<th>D9S171</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of PCR product (bp)</td>
<td>200</td>
<td>100</td>
<td>100</td>
<td>350</td>
<td>125</td>
<td>280</td>
<td>160</td>
<td>500</td>
<td>120</td>
<td>159-174</td>
</tr>
<tr>
<td>UoCB1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BL28</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td></td>
</tr>
<tr>
<td>G519</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CC*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>DoHH2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>G452</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>SuPT1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>SKW3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>K1106</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UoCB4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>Daudi</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>K231</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>JVM-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 4.2 DNA from cell lines was amplified using primers for STS or polymorphic markers as described in Chapter 2. Abbreviations: + = correct sized product amplified; - = no product of the correct size amplified. For the polymorphic marker D9S171, 2 = heterozygous at this locus; 1 = homozygous at this locus; 0 = nullizygous at this locus; NT = not tested; * = Tumour cells grown in SCID mice. See below for the STS map (Fig 4.1, see page 97 for legend).

![STS map](image-url)
4.2.2 Analysis of heterozygosity on chromosome 9

In order to investigate the mechanisms by which homozygous deletions of \( p16^{INK4A} \) arose in the lymphoid cell lines analysed, polymorphic dinucleotide repeats on chromosome 9 were amplified by PCR. Various possibilities can be considered. Homozygous deletions arising from non-dysjunction of a mutated chromosome 9 during mitosis would give rise to two identical copies of chromosome 9 within the tumour cell. Thus analysis of heterozygosity would show a single allele at all polymorphic markers along the chromosome 9. Other mechanisms, such as gene conversion or two separate deletion events may result in a small region of homozygosity around the deleted region including \( p16^{INK4A} \), with most of the remaining loci along chromosome 9 being heterozygous.

Nine lymphoid cell lines, shown to have deletion or rearrangement of \( p16^{INK4A} \) or \( p15^{INK4B} \) by Southern blot, were tested for heterozygosity of chromosome 9 loci by amplification of polymorphic repeat sequences. The cell lines JVM-2, K231 and K353 showed no deletion or rearrangement of \( p16^{INK4A} \) or \( p15^{INK4B} \) sequences and were included as controls. It was not possible to determine whether homozygosity reflected a tumour associated loss of heterozygosity without access to normal DNA from the patient from which each cell line was derived. However, nullizygous and heterozygous loci were informative for this study.
No cell line was homozygous or nullizygous for all markers tested on chromosome 9 (Table 4.3). Therefore duplication of the abnormal chromosome 9 and subsequent loss of the normal chromosome 9 was not the mechanism for homozygous deletion of \( p16^{INK4A} \) in these cell lines. Karpas 231 was homozygous for all loci tested on the 9q arm (Fig 4.4 and Table 4.3). All other cell lines were heterozygous for at least 2 loci tested (Table 4.3).

Several cell lines (5/12) were nullizygous for the marker D9S171 centromeric of \( p16^{INK4A} \) and only 2 cell lines were heterozygous at this locus. In cell lines BL28 and Granta 519 the presence of this nullizygous locus may reflect the proximal extent of the \( p16^{INK4A} \) deletion. However, cell lines K1106 and UoCB4 retained the STS marker c1.B which lies between \( p16^{INK4A} \) and D9S171 and which points to two separate deletion events occurring on at least one allele at 9p21 in these cell lines. Furthermore, the cell line JVM-2 which had no rearrangement or deletion of \( p16^{INK4A} \) showed complete biallelic loss of the D9S171 marker (Fig 4.6). These results may indicate that a further lymphoid tumour suppressor gene lies proximal to \( p16^{INK4A} \). YACs that contain both \( p16^{INK4A} \) and D9S171 have not been isolated. However D9S171 is thought to lie approximately 1.8 Mb centromeric \( p16^{INK4A} \).

4.2.3 Evidence for a lymphoid tumour suppressor on chromosome 9q

Several cell lines analysed here were homozygous for the D9S158 marker at 9q34 and two were nullizygous at this locus. This may reflect the presence of a tumour suppressor gene on the long arm of chromosome 9. Chaganti et al.
have reported a loss of heterozygosity at 9q31-34 in diffuse large cell lymphomas \(^9\) and Offit and colleagues have detected deletions in a diffuse B-cell subgroup of NHL centring on 9q31-32 \(^16\). Other researchers have found loss of heterozygosity at 9q34 in numerous tumours including bladder carcinomas \(^119\) and small lung carcinomas \(^282\) which further suggests the presence of a tumour suppressor gene in this region.

Figure 4.3 Markers are shown in linkage order with approximate physical location indicated by bars. Abbreviations: * = markers used for analysis of heterozygosity, see Chapter 2 for primer sequence and references.
Figure 4.4 Analysis of polymorphic markers on chromosome 9 in Karpas 231, a cell line with t(8;9)(q24.1;p13.3) but no abnormality of p16\textsuperscript{INK4A}.

Figure 4.4 Note that Karpas 231 is homozygous for all markers on 9q but heterozygous for markers on 9p (see also Table 4.3). Polymorphic repeats were amplified using dye labelled primers and separated on 6% polyacrylamide gels in an ABI automatic sequencer. Scale shows size in bp with respect to an internal size standard, and bars show expected size range for each marker. Peaks were identified using GeneScan 672 analysis software, version 1.2.2-1 (Applied Biosystems).
Figure 4.5 Analysis of polymorphic markers on chromosome 9 in SKW-3, a cell line with biallelic deletion of $p16^{INK4A}$.

Figure 4.5 Note that SKW-3 is homozygous for both markers on 9p (D9S171 and D9S147E). Polymorphic repeats were amplified using dye labelled primers and separated on 6% polyacrylamide gels in an ABI automatic sequencer. Scale shows size in bp with respect to an internal size standard, and bars show expected size range for each marker. Peaks were identified using GeneScan 672 analysis software, version 1.2.2-1 (Applied Biosystems).
Figure 4.6 Analysis of polymorphic markers on chromosome 9 in JVM-2, a cell line with no abnormality of $p16^{\text{NK4A}}$.

Figure 4.6 Note that JVM-2 is nullizygous for D9S171 at 9p21 despite having no deletion of $p16^{\text{NK4A}}$. When combined with data from other cell lines this indicates that a proportion of 9p deletions in lymphoid cells involve a region centromeric to $p16^{\text{NK4A}}$ that may contain a novel lymphoid tumour suppressor gene. Polymorphic repeats were amplified using dye labelled primers and separated on 6% polyacrylamide gels in an ABI automatic sequencer. Scale shows size in bp with respect to an internal size standard, and bars show expected size range for each marker. Peaks were identified using GeneScan 672 analysis software, version 1.2.2-1 (Applied Biosystems).
Table 4.3 Analysis of heterozygosity of polymorphic markers on chromosome 9 in lymphoid cell lines with deletion of \(p16^{INK4A}\)

<table>
<thead>
<tr>
<th>Locus</th>
<th>p16 Hz</th>
<th>p15</th>
<th>D9S171</th>
<th>D9S147E</th>
<th>D9S175</th>
<th>D9S118</th>
<th>D9S159</th>
<th>D9S158</th>
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<tbody>
<tr>
<td>Chromosome band</td>
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<td>0.78</td>
<td>0.86</td>
<td>0.83</td>
<td>0.78</td>
<td>0.70</td>
<td></td>
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<tr>
<td>pi 6</td>
<td>9p21</td>
<td>9p13</td>
<td>9q13-21</td>
<td>9q31-34</td>
<td>9q33-34</td>
<td>9q34-ter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UoCB1</td>
<td>D</td>
<td>D</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>BL28</td>
<td>D</td>
<td>D</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>G519</td>
<td>D</td>
<td>D</td>
<td>0</td>
<td>2</td>
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<td>2</td>
<td>1</td>
<td></td>
</tr>
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<td>G452</td>
<td>D</td>
<td>D</td>
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<td>2</td>
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<td>1</td>
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</tr>
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<td>D</td>
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<td>UoCB4</td>
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<td>2</td>
<td>0</td>
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</tr>
<tr>
<td>Daudi</td>
<td>G</td>
<td>D</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>K231</td>
<td>G</td>
<td>G</td>
<td>2</td>
<td>1</td>
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</tr>
<tr>
<td>JVM-2</td>
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<td>G</td>
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<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>K353</td>
<td>G</td>
<td>G</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3 Abbreviations: For \(p16^{INK4A}/p15^{INK4B}\), \(D\) = at least one STS within the gene failed to amplify; \(G\) = all STS within the gene amplified. Note although the exons 1-3 of \(p16^{INK4A}\) were intact by STS PCR, exon 1β fell within the deleted region in UoCB4 and possibly also in Daudi. For polymorphic markers; 2 = 2 distinct peaks within size range for that marker; 1 = only 1 distinct peak within size range for that marker; 0 := no distinct peaks within size range for that marker from two repeated samples. Hz = heterozygosity.
4.2.4 Molecular Analysis of PAX5 at 9p13

The PAX5 gene has been mapped to 9p13\(^{281}\) and codes for a transcription factor that is strongly expressed in developing B-cells\(^{121}\). It is therefore a good candidate for a gene that could deregulate expression of an oncogene juxtaposed by translocation. Two other genes from the PAX family have been implicated in translocations seen in human tumours. PAX3 and PAX7 are disrupted by the t(2;13)(q35;q14) and the t(1;13)(p36;q14) respectively in rhabdomyosarcoma. We therefore used probes for PAX5 to analyse the configuration and expression of this gene in cell lines derived from B-cell malignancies with translocations involving chromosome 9p.

Karpas 231 and Karpas 353 were both derived from patients with B-ALL and possessed, amongst other karyotypic abnormalities, the translocation t(8;9)(q24.1;p13.3). No deletion or rearrangement of p16\(^{INK4A}\) or p15\(^{INK4B}\) was observed in Karpas 231 or Karpas 353, by Southern blot or by amplification of STS markers in this region, indicating that neither p16\(^{INK4A}\) nor p15\(^{INK4B}\) were disrupted by the t(8;9)(q24.1;p13.3) translocation. No rearrangement of the PAX5 gene was found by Southern blot. By FISH analysis (Janet Shipley, data not shown) the PAX5 signal was not split between the der(8) and the der(9) t(8;9)(q24.1;p13.3) chromosomes. In cell line Karpas 231, PAX5 was translocated to the derivative chromosome 8 on both alleles, indicating that the target gene at 9p13 is centromeric to PAX5. Northern blots showed expression of a normal sized transcript in both cell lines (data not shown). PK1s is a
transcribed sequence isolated from the breakpoint of a t(9;14)(p13;q32.3) in a B-NHL cell line. Both Karpas 231 and Karpas 353 were germline for this probe by Southern blot.

Granta 452 was derived from a patient with an aggressive B-NHL and possessed, amongst other karyotypic abnormalities, two der(9) chromosomes. Southern blot and PCR of STS markers at the $p16^{INK4A}$ and $p15^{INK4B}$ locus showed biallelic deletion of both these genes and the surrounding STS. FISH analysis (Janet Shipley, data not shown) showed that one of the der(9) had a breakpoint at 9p23 distal to $PAX5$, and the other der(9) had a breakpoint at 9p13 proximal to $PAX5$. Again Southern blot showed no rearrangement of $PAX5$ and Northern blot showed expression of a normal sized transcript in this cell line.

Table 4.4 Expression of $PAX5$ in lymphoid cell lines with deletion of $p16^{INK4A}$ or 9p abnormalities

<table>
<thead>
<tr>
<th>Name</th>
<th>Disease</th>
<th>$p16^{INK4A}$</th>
<th>$PAX5$</th>
<th>GAPDH</th>
<th>9p abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>K231</td>
<td>B-ALL</td>
<td>G/G</td>
<td>+</td>
<td>+</td>
<td>t(8;9)(q24.1;p13.3)</td>
</tr>
<tr>
<td>K353</td>
<td>B-ALL</td>
<td>G/G</td>
<td>+</td>
<td>+</td>
<td>t(8;9)(q24.1;p13.3)</td>
</tr>
<tr>
<td>G452</td>
<td>B-ALL</td>
<td>D/D</td>
<td>+</td>
<td>+</td>
<td>9p+, 9p+</td>
</tr>
<tr>
<td>NCEB</td>
<td>B-NHL</td>
<td>G/G</td>
<td>+</td>
<td>+</td>
<td>t(9;?;13)(q32;?;q14)</td>
</tr>
<tr>
<td>SSK41</td>
<td>B-NHL</td>
<td>G/G</td>
<td>+</td>
<td>+</td>
<td>inv 9 (p24q22)</td>
</tr>
<tr>
<td>G519</td>
<td>B-NHL</td>
<td>D/D</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>DoHH2</td>
<td>B-NHL</td>
<td>D/D</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CC</td>
<td>BCP-ALL</td>
<td>D/D</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>104</td>
<td>BCP-ALL</td>
<td>D/D</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.4 Abbreviations: D/D = homozygous $p16^{INK4A}$ deletion by Southern blot. G/G = germline $p16^{INK4A}$ by Southern blot; + = distinct 10 kb band seen on Northern blot after 3-7 days exposure. - = 10 kb band not seen on Northern blot after 7 days exposure.
5. Chapter Five. MYC Abnormalities in Acute Leukaemias of Mature B-cells

5.1 Introduction

The MYC proto-oncogene is commonly deregulated in B-cell malignancies by translocations involving the immunoglobulin loci at 14q32.3, 22p11 and 2p11. In BL, these translocations are the primary chromosomal abnormality. However, in some cases of follicular lymphoma, translocation of MYC arises as a secondary event and is associated with progression to more aggressive disease. Follicular lymphoma is characterised by the t(14;18)(q32.3;q21.3) translocation, but rare cases of de novo acute leukaemia of mature B-cells with BCL2 deregulation via the t(14;18)(q32.3;q21.3) translocation have been described. The majority of these de novo leukaemias with BCL2 translocation also possess one of the MYC translocations typical of BL.

5.2 Molecular analysis of the t(8;9)(q24.1;p13.3) translocation

The recurrent translocation t(8;9)(q24.1;p13.3) has been reported by three different laboratories, in three cases of high grade B-NHL and three cases of de novo acute leukaemia of mature B-cells, in all cases in association with translocation of BCL2. This translocation is presumed to involve the MYC locus at 8q24.1 and an unknown gene at 9p13.3. Molecular analysis of two cell lines derived from de novo acute leukaemias...
with the t(8;9)(q24.1;p13.3) translocation was undertaken in order to map the breakpoint within the \textit{MYC} locus and determine the nature of the sequences at 9p13.3. A \textit{de novo} B-ALL patient sample with a t(8;9)(q24;p13) was also analysed.

Karpas 231 was derived from a TdT B-cell ALL (see Chapter 2). Two reciprocal translocations t(14;18)(q32.3;q21.3) and t(8;9)(q24.1;p13.3) were detectable, with a modal chromosomal number of 51 in both the major and minor clone. The major clone had several other additional structural abnormalities and some cells showed two copies of both derivatives of the t(8;9)(q24.1;p13.3) and duplication of the 18q- marker.

Karpas 353 was derived from a TdT B-cell ALL (see Chapter 2). Cells had a modal chromosomal number of 47 with trisomy 12 and occasional loss of chromosome 8. Three balanced translocations were seen in all cells; a t(8;9)(q24.1;p13.3), a t(14;18)(q32.3;q21.3) and a t(1;3;7)(p32.1;q21.1;q22.1).

The patient sample studied here was derived from the diagnostic bone marrow sample of a TdT B-cell ALL with expression of CD10, CD19 and CD20. The patient cells showed an extremely complex karyotype including a t(8;9)(q24;p13), a t(14;18)(q32;q21), a t(1;13)(q21;q1?) and a t(6;8)(q1?;q24)

5.2.1 Analysis of \textit{MYC} expression in Karpas 231 and 353

Northern blots of Karpas 231 and 353 RNA showed constitutive \textit{MYC} expression in Karpas 231 comparable to that seen in SSK41, a BL cell line with a \textit{MYC / Ig\lambda} translocation (Fig. 5.5 and Table 5.2). Karpas 353 showed a lower level of \textit{MYC} transcripts, but these were close to levels seen in
SSK41. However, analysis of Southern blots probed with *MYC* cDNA (cD1A) showed no rearrangement in a wide range of restriction digests. Hybridisation of Karpas 231 metaphase spreads with chromosome 8 centromere and *MYC* cos 72 \(^{212}\) probes also showed no rearrangement (Fig 5.1). However, hybridisation with a *BVR1* probe which was mapped 130kb telomeric of *MYC* exon 3 showed that *BVR1* did not remain on the der(8), but was translocated to another chromosome. The *BVR1* signal was not split indicating that the translocation breakpoint did not occur within *BVR1*, but centromeric to this probe (Fig 5.1). This indicated that the 8q24.1 breakpoint involved the *MYC* locus in Karpas 231 with deregulated expression of *MYC* being driven by unknown sequences on 9p13.3.

### 5.2.2 Mapping of the t(8;9)(q24.1;p13.3) MYC breakpoint

No rearrangement of the *MYC* locus could be detected by Southern blot, in either Karpas 231 or Karpas 353, using a *MYC* cDNA probe and a wide range of restriction enzyme digests. However analysis of the *MYC* locus in these cell lines by pulsed field gel electrophoresis revealed rearrangement in Karpas 231 of Not I, Sfi I and Bst ZI fragments spanning the gene (Fig 5.2). All rearranged bands were larger than germline bands, but partial digestion of the DNA could be eliminated by comparison with long range restriction maps of this locus (Fig 5.3). This mapped the breakpoint to within 50 kb telomeric of *MYC* exon 3. Further localisation of the breakpoint using probes derived from the region between 30 and 70 kb telomeric of *MYC* (Fig 5.3) was unsuccessful, all probes used gave germline bands (data not shown).
Figure 5.1 FISH analysis was performed by J. Shipley. Hybridisation of Karpas 231 metaphase spreads with chromosome 8 centromere and MYC (cos 72) or BVR1 probes showed that the translocation breakpoint on 8q occurred telomeric to MYC and centromeric to BVR1. Note both chromosome 8s hybridise to MYC, and centromere 8 probes. The der(8) chromosome does not hybridise to BVR1 but a BVR1 signal is seen on a chromosome with no 8 centromere. Therefore the 8q24.1 breakpoint occurs within 100kb 3' of MYC. For details of probes and protocols used see reference 356.
where is normal control for BSRZ1 digests
what about PFGE for BVR1
Figure 5.2 PFGE analysis of MYC rearrangements in K231

Figure 5.2 Abbreviations: Y = Yeast chromosome molecular weight marker (92kb band and 1050kb band arrowed). L = Lambda concatamer molecular weight marker (48.5kb band arrowed). K = Karpas 231; N = Normal human placental DNA; G = Granta 452. Agarose plugs containing 10μg DNA were digested with Not I, Bst ZI or Sfi I restriction enzymes and electrophoresed and blotted as described in Chapter 2. Blots were probed with MYC exon 2 probe. Note a germline and a rearranged band (arrowed) were seen in Karpas 231 in all digests, indicating that the t(8;9)(q24.1;p13.3) breakpoint probably fell within 50kb 3' of MYC. Rearranged bands were too small to be a result of partial digestion in the Not I digests.
Figure 5.3

A) Short range restriction map of the *MYC* locus

![Short range restriction map of the *MYC* locus](image)

*Figure 5.3A* Probes used in analysis of t(8;9)(q24.1;p13.3) breakpoint are shown in green. The three exons of *MYC* are indicated by red boxes numbered 1-3.

B) Long range restriction map of the *MYC* locus

![Long range restriction map of the *MYC* locus](image)

*Figure 5.3B* Bars indicate approximate size of rare cutting restriction enzyme fragments. Note that Not I and Bst ZI have several cutting sites within *MYC*.
5.3 Transcription of MYC in Granta 452: a cell line with both translocated and duplicated MYC alleles.

Amplification of MYC is rare in lymphoid cells, but has been reported in at least one T-NHL cell line \(^{238}\) and one B-NHL cell line \(^{239}\). A few cases have also been documented which show two copies of the translocated MYC allele formed by duplication of the entire translocated chromosome \(^{98,240}\).

A novel cell line has been derived in our laboratory from a case of leukaemic transformation of FL. This cell line, Granta 452, possesses both a duplicated MYC allele and a MYC translocation t(8;22)(q24.1;q11). In BL with MYC translocation, deregulated transcription occurs from the translocated allele and the normal allele remains silent or is expressed at low levels \(^{234,235}\). Analysis of the allele specific transcription of MYC was undertaken to determine the mechanisms affecting MYC transcription in transformed B-NHL.

Granta 452 was derived from a TdT \(^{+}\) B-cell ALL, arising subsequent to FL (Chapter 2). Immunophenotyping showed expression of CD19, and surface IgG2 with some cells also expressing CD10. The following cytogenetic abnormalities were observed: t(1;18)(q36;q21), t(6;20)(q11;p13), t(8;22)(q24.1;q11), 9p+, and 9p+. In addition a marker 8q+ was detected.

To characterise the genetic events occurring in this cell line genomic blots were analysed using probes for the oncogenes BCL2 and MYC and also the immunoglobulin loci probes J\(\gamma\) and C\(\kappa\) (Table 5.1). All oncogene probes used, MYC cD1A, PVT1, pFL1 and pFL2 gave a germline band only.
The *IGH* locus showed biallelic rearrangement with the *JH* probe and the *Cκ* locus was deleted.

Table 5.1. Summary of Southern blot analysis of Granta 452

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosomal Location</th>
<th>Probe</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MYC</em></td>
<td>8q24.1</td>
<td>cD1A</td>
<td>G/G</td>
</tr>
<tr>
<td><em>PVT1</em></td>
<td>8q24.1</td>
<td>PVT1</td>
<td>G/G</td>
</tr>
<tr>
<td><em>BCL2</em></td>
<td>18q21.3</td>
<td>pB16</td>
<td>G/G</td>
</tr>
<tr>
<td><em>BCL2</em></td>
<td>18q21.3</td>
<td>pFL1</td>
<td>G/G</td>
</tr>
<tr>
<td><em>BCL2</em></td>
<td>18q21.3</td>
<td>pFL2</td>
<td>G/G</td>
</tr>
<tr>
<td><em>IGH</em></td>
<td>14q32.3</td>
<td><em>JH</em></td>
<td>R/R</td>
</tr>
<tr>
<td><em>Cκ</em></td>
<td>2p11-12</td>
<td><em>Cκ</em></td>
<td>D/D</td>
</tr>
</tbody>
</table>

FISH analysis was performed by J. Shipley using paints for chromosome 8 and chromosome 22. This confirmed the presence of the t(8;22)(q24.1;q11) and showed that the other der(8q+) chromosome was composed solely of chromosome 8 material. No rearrangement of the *MYC* locus on the der(8)t(8;22)(q24.1;q11) chromosome could be detected using cosmids 400 kb 5' and 250 kb 3' of *MYC* coding sequences. However, duplication of the untranslocated *MYC* allele had occurred (Fig 5.4), and the other der(8q+) was in fact a chromosome 8 with duplication of part of band 8q24.1. PFGE analysis of the *MYC* locus in Granta 452 revealed rearranged bands in all digests (see Fig 5.2). This was unexpected, and may have been the result of abnormal methylation patterns, as FISH revealed that the 8q24.1 breakpoints were too distant to be detected by this method.
Figure 5.4 FISH analysis of the \textit{MYC} locus in Granta 452.

Figure 5.4 A) FISH analysis was performed by J.Shipley. Metaphase spreads of Granta 452 cells were hybridised to chromosome 8 centromere and \textit{PVT1} probes. Note that one chromosome 8 shows two copies of \textit{PVT1}, see Fig 5.3 for map. \textit{BVR1} and \textit{MYC} probes also showed duplication (data not shown). B) Hybridisation with a chromosome 8 centromere probe and chromosome 22 paint showed that chromosome 22 material was translocated to the long arm of chromosome 8. Chromosome 8 material was translocated to chromosome 22 (data not shown). For details of probes and protocols used see reference 356.
5.3.1 Northern analysis of MYC expression in Granta 452

Northern blots showed that MYC was expressed at high levels (5-6 times higher at the mRNA level) in Granta 452 when compared with a control B-NHL cell line, SSK41, with a t(8;22)(q24.1;q11). Quantitation of MYC expression and a GAPDH control in a selection of B-cell lines was performed by Imagequant™ analysis of an RNA blot hybridised sequentially with MYC and GAPDH labelled cDNA probes (Fig 5.5). A phosphorimager screen was exposed to the hybridised blot for 3 hrs and read by phosphorimager. Bands were selected manually, integrated volumes calculated, and values normalised to SSK41, see Table 5.2. To control for variations due to loading inaccuracies, a lane containing a double volume of Granta 452 was included.

Figure 5.5 MYC expression in Granta 452 and other B-cell lines.

![MYC expression in Granta 452 and other B-cell lines](image)

Figure 5.1 Lane 1= SSK41; 2= U698M; 3= Karpas 353; 4 = Karpas 231; 5 = Granta 452 x2; 6 = Granta 452; 10μg of total RNA were run in each lane, except lane 5 (20μg). The blot was probed with MYC cD1A and GAPDH. The blot was exposed to a phosphorimager screen for 3 hrs and the image quantified and analysed using ImageQuant™ software (see Table 5.2). Note that all cell lines show expression of a single transcript of approximately 2.5kb, whilst Karpas 353 also shows expression of a second smaller transcript. The smaller transcript probably results from transcription from P3.
Table 5.2 Relative MYC expression in Granta 452 and other lymphoid cell lines

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>GAPDH</th>
<th>MYC</th>
<th>RELATIVE RNA LOADING</th>
<th>RELATIVE MYC EXPRESSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granta 452</td>
<td>50863</td>
<td>81295</td>
<td>0.36</td>
<td>6.2</td>
</tr>
<tr>
<td>Granta 452 X2</td>
<td>112166</td>
<td>162311</td>
<td>0.79</td>
<td>5.6</td>
</tr>
<tr>
<td>K231</td>
<td>87545</td>
<td>39304</td>
<td>0.62</td>
<td>1.7</td>
</tr>
<tr>
<td>K353</td>
<td>50632</td>
<td>9937</td>
<td>0.36</td>
<td>0.8</td>
</tr>
<tr>
<td>U698M</td>
<td>68513</td>
<td>24150</td>
<td>0.48</td>
<td>1.4</td>
</tr>
<tr>
<td>SSK41</td>
<td>141837</td>
<td>36853</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5.2 A Northern blot with 10μg of total RNA per lane was probed with the MYC cDNA probe cD1A and GAPDH probe (Fig 5.5). For quantitation this blot was exposed to a phosphorimager screen for 3 hrs and the image quantified and analysed using ImageQuant ™ software. Abbreviations: Relative RNA Loading = GAPDH/SSK41 GAPDH; Relative MYC expression = MYC/ SSK41 MYC x relative RNA loading; Granta 452 X2 = double loading (20μg) of Granta 452 RNA.

5.3.2 Analysis of MYC allele specific transcription in Granta 452

In BL and other lymphomas with MYC translocations, the translocated MYC allele is frequently mutated within exon 1 220, 221, 290, 291, 292. Mutations have also been reported in exons 2 and 3 in some cases 224, 225, 226. To investigate whether one or more MYC alleles were transcribed in Granta 452, genomic DNA and cDNA was amplified using MYC exon I specific primers (Fig 5.6), and the 580 bp product cloned into pGEM-T. Single stranded DNA was prepared from individual clones and sequenced using Taq polymerase. If the translocated allele was mutated, or showed sequence polymorphisms, analysis of the genomic PCR product would show two different sequence groups. If both amplified alleles were identical then these groups should be represented by a 2:1 ratio of clones. If both copies of chromosome 8 were transcribed then both sequence groups would be detected in amplified cDNA.
clones. However if only one sequence was detected in the cDNA then only one chromosome 8 was transcribed at the MYC locus.

Figure 5.6 Map showing location of primers used for MYC exon I amplification

Figure 5.6 The MYC gene has four promoters P0- P3 (P3 not shown). In normal cells P2 and P1 are the major promoters with less than 5% of transcripts initiated at P0 which has no TATA box. Primers HC1-4 were each used in combination with HC0 to amplify genomic and cDNA.

Sequences of 16 MYC exon I PCR product clones were compared to seven MYC sequences from the GenBank database (Accession numbers: K01908, M14206, M13930, J03253, X00364, J00120, K03015). Bases were numbered from the first base of the 5' primer used for PCR amplification. Base 359 was polymorphic, with a C in four database sequences and a G in three database sequences and all Granta 452 sequences.
5.3.2.1 Sequence analysis of genomic MYC exon 1 PCR products

At least one alteration from the germline was observed in each clone, and in all 39 point mutations were detected in a total of 9,000 bp sequenced (Fig 5.7). The clones could be divided into two groups with respect to the presence of a C or a T at base 453 (Table 5.3 and Fig 5.8). Of the eleven clones which had a T at base 453, only one had any one of the mutations at bases 206, 274 or 422: namely mutation of base 274 (C to A). These clones were designated group A. A BLAST search of the Genbank database with a 40bp sequence including a T at base 453, revealed one identical sequence from a human foetal liver MYC gene (Accession number K00559). This base change may therefore be a rare polymorphism.

Four out of five subclones that had a C at base 453 also possessed point mutations at bases 274 (C to A), 206 (G to A) and 422 (C to G) (Fig 5.8): the fifth showed mutations at bases 274 and 206 but was germline at base 422. These were designated as group B.

All other 13 mutations occurring at positions separate from bases 206, 274, 422 and 453 were observed in one clone only with exception of a T to C mutation at base 76 which occurred in two clones from group B.

Table 5.3 Distribution of point mutations in Granta 452 subclones of MYC exon 1 genomic PCR.

<table>
<thead>
<tr>
<th>Genomic MYC exon 1</th>
<th>Group A (+453 polymorphism)</th>
<th>Group B (-453 polymorphism)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 clones</td>
<td>11 clones</td>
<td>5 clones</td>
</tr>
<tr>
<td>-206,-274,-422</td>
<td>-206,-274,-422</td>
<td>+422,+206,+274</td>
</tr>
<tr>
<td>10 clones</td>
<td>1 clone</td>
<td>4 clones</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-422,+206,+274</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 clone</td>
</tr>
</tbody>
</table>

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5.3.2.2 Sequence analysis of amplified reverse transcribed MYC exon 1 mRNA

Analysis of 10 clones of the amplified reverse transcribed MYC mRNA from Granta 452 revealed only sequences without mutations at position 453 and containing mutations at positions 206, 274, and 422 (Table 5.4 and Fig 5.8 and 5.9).

The mutation C to G observed at base 422 introduced a unique Mae III restriction site (GTNAC) into MYC exon 1. Incubation of uncloned Granta 452 MYC exon 1 RT PCR product with Mae III revealed complete digestion when products were electrophoresed on a 2% agarose gel.

Initial analysis of transcribed MYC exon 1 sequences in Granta 452 used a 5' PCR primer (HC1) that would only amplify transcripts initiating from P0. In order to amplify transcripts initiating from P1 and P2 an internal PCR primer HC4 was used to give a 333 bp product. The HC4 PCR product was digested with Mae III and electrophoresed on a 2% agarose gel. No uncut PCR product was seen (Fig 5.10).

Table 5.4 Distribution of point mutations in Granta 452 subclones of MYC exon 1 cDNA PCR.

<table>
<thead>
<tr>
<th>cDNA MYC exon 1</th>
<th>10 clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (+453 mutation)</td>
<td>0 clones</td>
</tr>
<tr>
<td>Group B (-453 mutation)</td>
<td>10 clones</td>
</tr>
<tr>
<td>-206, -274, -422</td>
<td>-206, +274, -422</td>
</tr>
<tr>
<td>0 clones</td>
<td>0 clone</td>
</tr>
<tr>
<td>+422, +206, +274</td>
<td>-422, +206, +274</td>
</tr>
<tr>
<td>0 clones</td>
<td>10 clones</td>
</tr>
<tr>
<td>-422, +206, +274</td>
<td>0 clones</td>
</tr>
</tbody>
</table>
Figure 5.7 Sequences of Granta 452 MYC exon I genomic PCR clones

---

Grants 452 sequences in black with mutations in red.

Germline sequence shown in blue.

Primer undefined.
Figure 5.8 Conserved MYC exon 1 mutations in Granta 452

Figure 5.8 MYC exon 1 was amplified from Granta 452 genomic DNA, cloned into pGEM-T and individual clones sequenced as described above. Mutations occurring in more than 2 clones are shown here arrowed. Note that clones that were germline at bases 206, 274 and 422 were mutated at 453 and vice versa. Germline sequences shown with mutated nucleotide in bold.
Figure 5.9 Sequence of Granta 452 MYC exon I mRNA PCR clones

- Germline sequence shown in blue
- Granta 452 sequences in black with mutations in red
- C polymorphic nucleotide
- Primer sequences underlined
Figure 5.10 Granta 452 cDNA was amplified using primers HC4 and HC0 to give a 333bp product, which was digested with Mae III restriction endonuclease and run on a 2% agarose gel. No uncut PCR product was seen, indicating that all MYC transcripts from PO, P1, and P2 were derived from the allele with a mutation at base 422. Lane 1= Granta 452 MYC exon 1 RT PCR product digested with Mae III; Lane 2,= Granta 452 MYC exon 1 RT PCR product uncut.

Errors made by the Taq polymerase enzyme fall within the range of 1/4000 to 1/11000 alterations per nucleotide per round of amplification which would be compatible with the level of mutation observed in this study. An average of 562.5 bases of each clone were sequenced which had undergone 30 rounds of amplification, giving a lower limit for the expected mean error rate due to Taq polymerase of 1.53 mutations per clone (1/11000 x 562.5 x 30 = 1.53) or 24.5 mutations overall and an upper limit of 4.22 (1/4000 x 562.5 x 30 = 4.22) mutations per clone or 67.5 mutations overall. The observed mutation rate was 39/(9000 x 30) = 1/6923. However the recurring mutations used to define each sequence group are unlikely to be the result of errors by Taq polymerase as they were observed in multiple clones which were derived from the products of four separate PCR reactions.
The conserved mutations at bases 206, 274, 422 were not typical of Taq polymerase errors, of which 70% are A to G or T to C \(^{295}\). All other mutations observed occurred in one clone only, with the exception of the alteration T to C at base 76 which was recorded twice. These mutations could be a result of Taq polymerase error. However only 5/13 of these errors were A to G or T to C mutations, compared to an expected frequency of around 9 (0.7 x 13 = 9.1). The high GC content of MYC exon 1 (65% GC) may have caused bias in the observed mutation frequencies and would reduce the target sites for A to G or T to C mutations. Alternatively a proportion of these mutations could be a result of sequence variations within the cell population. Other authors have reported ongoing mutations in BL cell lines \(^{229, 230}\), but no work has been done in other B-cell leukaemias with MYC translocations. However, none of the unconserved mutations seen in the sequences of amplified genomic DNA were repeated in the amplified cDNA sequences. Moreover, the frequency of isolated mutations was 1/20769 (13/(9000 x 30)) which is lower than predicted for Taq polymerase errors (1/11000). Therefore it seems likely that MYC mutation in Granta 452 is not ongoing.

5.3.3 Conclusions

Only one MYC allele was transcribed in Granta 452 from the major promoters P1 and P2, and also from P0. From the relative abundance of the two sequence groups in the genomic MYC exon 1 PCR, it seems probable that the transcribed allele is the translocated rather than the duplicated allele. MYC mutation does not appear to be ongoing in Granta 452, although due to PCR error this possibility cannot be excluded.
6. Chapter Six. Discussion

6.1 Introduction

The processes in human tumours by which dominant oncogenes are activated and recessive tumour suppressor genes are silenced are becoming more clearly understood. This is especially true of the genes involved in the pathogenesis of leukaemias and lymphomas.

In NHL, dominant oncogenes are frequently deregulated by translocation into the \textit{IGH} locus at 14q32.3, where lymphoid enhancers are thought to activate their transcription in B-cells. The \textit{MYC} oncogene was the first gene shown to be translocated to \textit{IGH}, and the t(8;14)(q24.1;q32.3) is the paradigm for this type of translocation. The coding sequences of the myc oncoprotein are often not directly disrupted by the translocation itself. However, removal or mutation of negative regulatory \textit{MYC} sequences may further enhance \textit{MYC} expression in tumours with the t(8;14)(q24.1;q32.3).

Loss of tumour suppressor gene function may arise from deletion of whole or part of the gene, inactivating point mutations or frameshift mutations, or alteration of transcriptional or translational regulatory factors. Recurring areas of chromosomal deletion or loss of heterozygosity (LOH) in tumour cells are considered as indicative of regions that contain tumour suppressor genes. However, the large extent of these deletions can frustrate the identification of the critical gene or genes.
Once a candidate tumour suppressor gene has been identified within a common region of chromosomal deletion, evidence of inactivating point mutations of this gene in tumour cells may confirm that it is the tumour suppressor gene. *P53* is the most widely inactivated tumour suppressor gene analysed to date, being inactivated in 50% of all human tumours. Point mutations are a common mechanism of *p53* inactivation in many tumours including some lymphoid malignancies, and in most cases where mutation of one allele has been observed the other allele is deleted. However, homozygous deletion of the gene has also been reported. *P53* is unusual in that some point mutations cause the mutant *p53* to act as a dominant oncogene.

Inactivation of known tumour suppressor genes, including *p53*, is uncommon in leukaemia and NHL. Nevertheless, in many haematological malignancies recurrent deletions of large chromosomal regions are frequently observed, and specific subsets of disease are associated with specific deletions. For example, the deletion of 5q in myeloid disorders, 9p21 in ALL, and 13q14 in B-CLL suggests distinct pathological mechanisms and consequences rather than a random loss of DNA.

This study examined both the activation of the *MYC* oncogene by translocation, and the inactivation of *p16<sup>INK4A</sup>* and *p15<sup>INK4B</sup>* in lymphoid malignancies.

### 6.2 Deletions of *p16<sup>INK4A</sup>* and *p15<sup>INK4B</sup>* in lymphoid tumours

Abnormalities of the human chromosome band 9p21 are associated with leukaemias, lymphomas, and melanomas.
gliomas, lung cancers and other tumours. These abnormalities include inversions, translocations and homozygous and heterozygous deletions. \( \text{p16}^{\text{INK4A}} \) was first proposed as a candidate tumour suppressor gene when it was shown to map within the smallest common region of deletion at 9p21. Subsequent studies showed frequent biallelic loss of this gene in a variety of malignant cell lines.

Because abnormalities of both 9p21 and the immediate centromeric chromosomal band 9p13.3 are frequent in both leukaemia and lymphoma, this study determined the frequency of alterations of \( \text{p16}^{\text{INK4A}} \) in a panel of 231 fresh malignancies and 66 lymphoid cell lines, with defined cytogenetic abnormalities, by DNA blot. Since completing this work, several reports of the extent of \( \text{p16}^{\text{INK4A}} \) and \( \text{p15}^{\text{INK4B}} \) deletions in lymphoid malignancies have been published. These data are reviewed here and summarised in Table 6.1 and Table 6.2 below.

6.2.1 Frequency of \( \text{p16}^{\text{INK4A}} \) deletions in leukaemias and lymphomas

The overall incidence of structural abnormalities of \( \text{p16}^{\text{INK4A}} \) in leukaemia and lymphoma was low, being observed in 20/231 (8.6%) of fresh cases and 10/66 (15%) of cell lines. Abnormalities of \( \text{p16}^{\text{INK4A}} \) were observed in two distinct subsets of lymphoid malignancy, both in a subset of BCP-ALL and also in some NHL of both T and B lineages. Overall, other investigators have found an incidence of between 14 and 40% of homozygous \( \text{p16}^{\text{INK4A}} \) deletions in ALL with only rare homozygous or hemizygous deletions in AML and NHL (1-2%) (see Table 6.1 and 6.2).
Biallelic deletions of $p16^{\text{INK4A}}$ were seen in 20% of cases of BCP-ALL, although no $p16^{\text{INK4A}}$ deletions were observed in eleven cases of TCP-ALL. This is in contrast to other reports of a high incidence of $p16^{\text{INK4A}}$ deletion in TCP-ALL, and may reflect the problems associated with analysis of small sample numbers. However, 3/6 cases reported here of the closely related disease, T-cell lymphoblastic NHL, did show $p16^{\text{INK4A}}$ deletion. Hebert and colleagues reported that 85% of cases of TCP-ALL had homozygous $p16^{\text{INK4A}}$ deletions as opposed to only 6.5% BCP-ALL (Table 6.1). Similarly, Okuda et al. reported an association of $p16^{\text{INK4A}}$ deletions with a T-cell phenotype in paediatric ALL. This T-cell bias was also observed in other studies, but the difference between the frequency of $p16^{\text{INK4A}}$ deletions in TCP-ALL and BCP-ALL was less marked. In the largest series of ALL, Ogawa et al. noted a higher percentage of $p16^{\text{INK4A}}$ deletions in BCP-ALL than in TCP-ALL, confirming the results reported here (Table 6.1). It is difficult to reconcile these differences. It may be that the varying frequencies of deletion reflect bias through case selection at tertiary referral centres.

In NHL, this study found deletion or rearrangement of $p16^{\text{INK4A}}$ in 3/31 (9.6%) cases with B-cell phenotype and 3/7 (42%) cases with T-cell phenotype and at higher frequencies in NHL cell lines (4/10 or 40% of B-NHL lines and 2/3 or 66.7% T-NHL lines). Deletion or rearrangement in 1/8 (12.5%) Burkitt’s lymphomas and 2/26 (7.5%) Burkitt’s lymphoma cell lines was also found. Gombart et al. reported $p16^{\text{INK4A}}$ loss in 2/52 B-NHL and 2/23 T-NHL, Ogawa et al. reported $p16^{\text{INK4A}}$ loss in 4/33 NHL, and Duro et al.
reported $p16^{\text{INK4A}}$ loss in 2/32 cases of NHL\textsuperscript{306}. Others have not observed deletions in NHL nor in NHL cell lines (see Table 6.2).

In contrast, no homozygous $p16^{\text{INK4A}}$ deletions or rearrangements were observed in 119 cases of leukaemia of mature T or B-cells, including 46 cases of T-PLL, 34 B-CLL, 22 SMZL, 6 HCL, 5 B-PLL, 6 LGL and 3 myeloma cell lines. Others have observed a very low frequency of $p16^{\text{INK4A}}$ deletion in tumours of mature lymphoid cells (see Table 6.2) with a mean of 2% of cases with deletions of $p16^{\text{INK4A}}$ in B-CLL\textsuperscript{277, 278, 310, 312, 313}, and no deletions in HCL\textsuperscript{307}.

This study, did not detect any hemizygous deletions of $p16^{\text{INK4A}}$, although Haidar et al. have reported an incidence of 8.6% (7/81) of hemizygous deletions in CLL in contrast with no hemizygous deletions in ALL (0/27)\textsuperscript{307}. Contamination of tumour cells with normal cells in some cases may make detection of hemizygous deletion difficult in fresh tumour samples. Moreover, as discussed below, deletion of $p16^{\text{INK4A}}$ appears to be a secondary event at least in some malignancies, and may in some cases be present in a subpopulation of tumour cells. As a result of these technical problems, hemizygous deletions are difficult to verify by Southern blot. Fluorescence in-situ hybridisation (FISH) may be a better method for the detection of hemizygous $p16^{\text{INK4A}}$ deletions.

Preliminary studies suggested that the frequency of biallelic loss of $p16^{\text{INK4A}}$ in fresh tumour material, was much lower than that observed in cell lines, and that the high incidence of $p16^{\text{INK4A}}$ loss in cell lines represented adaptation to in vitro culture\textsuperscript{131}. However, in the data presented in chapter 3 of this thesis, the absence of $p16^{\text{INK4A}}$ abnormalities in EBV transformed
lymphoblastoid cell lines suggests that prolonged in vitro culture per se does not necessarily lead to $p16^{INK4A}$ abnormalities. In this thesis and in other studies, a higher incidence of $p16^{INK4A}$ deletion has been reported in cell lines than in patient material from the same disease groups\textsuperscript{136, 196}. However, in this thesis where the patient material from which the cell line was derived was available for analysis, $p16^{INK4A}$ deletions were also noted in the original tumour (Chapter 3). Therefore it seems probable that biallelic deletion of $p16^{INK4A}$ provides an in vitro growth advantage to tumour cells, but that such deletions are not a result of in vitro culture.

6.2.2 Biological significance of $p16^{INK4A}$ deletions in lymphoid tumours

Cases of BCP-ALL with $p16^{INK4A}$ deletions observed in this thesis, did not constitute a distinct cytogenetic subgroup and did not appear to be associated with a particularly poor prognosis. In particular, 14 of the 17 Ph+ t(9;22)(q34;q11) and one case with t(4;11), disease subgroups associated with poor prognosis, retained germline $p16^{INK4A}$. However, both Fizzotti \textit{et al.} and Quesnel \textit{et al.} recently noted a correlation between $p16^{INK4A}$ deletions and an increased leukaemic mass or higher white blood cell counts\textsuperscript{277, 310} and concluded that deletions of $p16^{INK4A}$ characterise a subgroup of ALL with features of aggressive disease. Both studies also detected an association of $p16^{INK4A}$ deletions with translocations that carry poor prognosis in ALL, including 4 with t(9;22)(q34;q11), and 3 with t(4;11)(q21;q23), but only a subset of cases with each of these translocations exhibited $p16^{INK4A}$ deletions.
Therefore it may be that $p16^{INK4A}$ deletions in BCP-ALL are a secondary event, as in blast crisis of CML and NHL (see below).

Deletions of $p16^{INK4A}$ were shown in this thesis (Chapter 3), to correlate with the ability of BCP-ALL to grow in SCID mice ($p<0.01$). Seven of the ten cases of BCP-ALL that established overt transplantable leukaemia in SCID mice showed biallelic $p16^{INK4A}$ deletion, whereas none of the eleven cases that failed to engraft did so. The biological significance of these observations is not known. Within cytogenetically-defined subgroups of BCP-ALL, such as cases with t(4;11)(q21;q23), some cases will grow in SCID mice whereas others will not $^{314, 315, 316}$. The molecular basis for this heterogeneity is not known. Whilst the growth of human tumour cells in SCID mice clearly represents the result of many complex interactions, the data presented here indicate that loss of $p16^{INK4A}$ expression may be an important (although not essential) parameter in this process. Whether loss of $p16^{INK4A}$ expression is important for the growth of NHL in SCID mice has not been determined: however, both DoHH2 and Daudi cell lines grown in SCID mice for the evaluation of antibody and antisense therapeutic approaches exhibit abnormalities of $p16^{INK4A}$ $^{317, 318}$.

Despite the correlation of $p16^{INK4A}$ deletion with poor prognosis found by some groups, and the ability of tumour cells to grow in SCID mice, no correlation has been found between $p16^{INK4A}$ deletion and a specific karyotype. This may indicate that deletion of $p16^{INK4A}$ occurs as a secondary event in the development of lymphoid tumours. The demonstration that deletion of $p16^{INK4A}$ is concomitant with tumour progression in some cases of
NHL and CML also supports this hypothesis. In the series reported here, only 3 of 17 cases of BCP-ALL with t(9;22)(q34;q11) in chapter 3 showed $p16^{INK4A}$ loss, and two of these deleted cases were BCP-ALL arising as blast crisis of CML, which suggested a role for $p16^{INK4A}$ loss in progression of CML to lymphoid blast crisis. This point could not be demonstrated directly, due to lack of suitable patient samples. However, a study by Sill et al., showed that deletion of $p16^{INK4A}$ is associated with transformation to lymphoid but not myeloid leukaemic blast crisis of CML.

In NHL, $p16^{INK4A}$ abnormalities occurred in high grade malignancies including T-cell lymphoblastic NHL, Burkitt’s Lymphoma and NHL that had undergone progression from low to high grade disease in vivo. In two cases from which sequential samples were available, we were able to show that biallelic loss of $p16^{INK4A}$ was associated with immunoblastic transformation. In cell lines DoHH2 and Granta 452 that arose from immunoblastic and leukaemic transformation of follicular B-NHL respectively, biallelic deletion of $p16^{INK4A}$ was detected in patient material as well as the cell lines, indicating that deletions were not an artefact of in vitro culture. Another study on the progression of adult T-cell leukaemia, ATL, from chronic to acute phase, also indicated the involvement of $p16^{INK4A}$ and $p15^{INK4B}$ deletions in this process. Together, these data suggest that inactivation of $p16^{INK4A}$ may be a relatively late event in lymphoid malignancies, in contrast to bladder cancers, where loss of material from 9p is thought to occur early in tumour development.
6.2.3 Rearrangements of p16\(^{INK4A}\)

Use of Southern blots to detect p16\(^{INK4A}\) deletions in this thesis, also allowed detection of rearrangements of this locus. These have been reported in few other studies. However, many studies analysed deletions of p16\(^{INK4}\) and p15\(^{INK4B}\) by PCR alone, which would not detect these types of abnormalities. Rearrangements of p16\(^{INK4A}\) were seen in two cases of BCP-ALL, one Burkitt's cell line (Daudi) and in one case of monocytoid B-NHL. The significance of these rearrangements is not known. Since in all instances they occurred in the absence of cytogenetic change, it is likely that they represent smaller, internal deletions of the p16\(^{INK4A}\)/p15\(^{INK4B}\) locus. Use of STS mapping allowed further dissection of the molecular events in these rearranged cases (see below).

Ogawa et al. reported rearrangement of p16\(^{INK4}\) in 6 cases, 4 of BCP-ALL, 1 of AML and 1 of T-ALL. These were reported to be the result of recombination between exon 2 of p16\(^{INK4}\) and exon 2 of p15\(^{INK4B}\). These regions show considerable sequence homology and deletions could occur by homologous recombination between the two exons.

6.2.4 Abnormalities of p16\(^{INK4A}\) cross hybridising bands

Southern blots using a p16\(^{INK4A}\) exon 1 probe revealed several cross hybridising bands in all restriction enzyme digests of normal genomic DNA. Three lymphoid cell lines showed abnormalities of these cross hybridising bands including loss or rearrangement. These abnormalities occurred in both
malignant B-cell lines with germline $p16^{INK4A}$ and those with homozygous deletions of $p16^{INK4A}$.

The $p16^{INK4A}$ cross hybridising bands indicate the presence of genes homologous to $p16^{INK4A}$ within the human genome. Recently a cyclin dependant kinase inhibitor has been cloned, with 48% amino acid identity to $p16^{INK4A}$ within four ankyrin repeats. This protein is known as p19 and is expressed in all cells. p19 specifically inhibits cdk4 and cdk6 /cyclin D complexes, but may play a role in the cell cycle regulation of T-cells. Therefore p19 or other genes homologous to $p16^{INK4A}$ could possibly act as tumour suppressors in some lymphoid malignancies. Abnormalities of $p16^{INK4A}$ cross hybridising bands in this study may provide evidence for tumour suppressor activity of other cyclin dependant kinase inhibitors in lymphoid tumours.
Table 6.1a. Summary of published deletion data of $p16^{INK4A}$ and $p15^{INK4B}$ in acute leukaemias

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<th>Hemizygous deletion</th>
<th>Germline configuration</th>
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<td>11/53</td>
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<td>-</td>
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<tr>
<td></td>
<td>Okuda $^{308}$</td>
<td>7/32</td>
<td>7/32</td>
<td>6/32</td>
</tr>
<tr>
<td></td>
<td>Rassool $^{279}$</td>
<td>7/42</td>
<td>7/42</td>
<td>6/42</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
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</tr>
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<td>16/73</td>
<td>10/73</td>
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<td>Oshnishi $^{280}$</td>
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<td>20/56</td>
<td>-</td>
</tr>
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<td>2/20</td>
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<td>Duro $^{306}$</td>
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Table 6.1a Abbreviations: -, = not studied or not reported; ns, = disease subtype not specified.
Table 6.1b. Summary of published deletion data of $p16^{INK4A}$ and $p15^{INK4B}$ in acute myeloid leukaemias and leukaemic cell lines

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<td>Sill$^{311}$</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>Quesnel$^{277}$</td>
<td>0/35</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>Ogawa$^{278}$</td>
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Table 6.1b Abbreviations: -, = not studied or not reported; ns, = disease subtype not specified.

144
Table 6.2a Summary of published deletion data of $p16^{INK4A}$ and $p15^{INK4B}$ in chronic leukaemias

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<td>1/81</td>
<td>7/81</td>
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<td>1/11</td>
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Table 6.2a Abbreviations: -, = not studied or not reported; ns, = disease subtype not specified; CML LBC = lymphoid blast crisis of CML; CML OBC = non-lymphoid (usually myeloid) blast crisis of CML.
Table 6.2b Summary of published deletion data of $p_{16}^{INK4A}$ and $p_{15}^{INK4B}$ in lymphomas

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<td>$p_{15}$</td>
<td>$p_{16}$</td>
</tr>
<tr>
<td>B-NHL cell lines</td>
<td>Stranks$^{320}$</td>
<td>4/10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Otsuki$^{312}$</td>
<td>0/8</td>
<td>0/8</td>
<td>-</td>
</tr>
<tr>
<td>T-NHL cell lines</td>
<td>Stranks$^{320}$</td>
<td>2/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Otsuki$^{312}$</td>
<td>0/27</td>
<td>0/27</td>
<td>-</td>
</tr>
<tr>
<td>B-NHL</td>
<td>Stranks$^{320}$</td>
<td>2/31</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Gombart$^{309}$</td>
<td>1/37</td>
<td>1/37</td>
<td>1/37</td>
</tr>
<tr>
<td></td>
<td>Uchida$^{321}$</td>
<td>3/42</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T-NHL</td>
<td>Stranks$^{320}$</td>
<td>3/7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Gombart$^{309}$</td>
<td>1/16</td>
<td>0/16</td>
<td>1/16</td>
</tr>
<tr>
<td>NHL (ns)</td>
<td>Ogawa$^{278}$</td>
<td>4/33</td>
<td>4/33</td>
<td>0/33</td>
</tr>
<tr>
<td></td>
<td>Sill$^{311}$</td>
<td>0/43</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Duro$^{306}$</td>
<td>2/32</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.2b Abbreviations: -, = not studied or not reported; ns, = disease subtype not specified.
Table 6.3. Missense mutations of \( p16^{INK4A} \) reported in haematological malignancies.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Name</th>
<th>Reference</th>
<th>Exon</th>
<th>Codon</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-ALL*</td>
<td>KOPT-K1</td>
<td>Oshnishi²⁸⁰</td>
<td>2</td>
<td>72</td>
<td>CGA to TGA</td>
<td>Arg to stop</td>
</tr>
<tr>
<td>T-ALL*</td>
<td>DND-41</td>
<td>Oshnishi²⁸⁰</td>
<td>2</td>
<td>52-61</td>
<td>29bp deletion</td>
<td>Frameshift</td>
</tr>
<tr>
<td>T-ALL</td>
<td>Oshnishi²⁸⁰</td>
<td>2</td>
<td>58</td>
<td>CAC-CGC</td>
<td>His to Arg</td>
<td></td>
</tr>
<tr>
<td>T-ALL</td>
<td>Oshnishi²⁸⁰</td>
<td>2</td>
<td>63</td>
<td>AAC-AAGGTCG</td>
<td>Frameshift</td>
<td></td>
</tr>
<tr>
<td>T-ALL</td>
<td>Oshnishi²⁸⁰</td>
<td>2</td>
<td>50</td>
<td>CGA to CGGAGGGGA</td>
<td>Frameshift</td>
<td></td>
</tr>
<tr>
<td>AML*</td>
<td>HL-60</td>
<td>Nakamaki²¹⁵⁸</td>
<td>2</td>
<td>80</td>
<td>CGA to TGA</td>
<td>Arg to stop</td>
</tr>
<tr>
<td>BCP-ALL</td>
<td>Otsuki²¹²</td>
<td>2</td>
<td>115</td>
<td>CAT to CAA</td>
<td>His to Gln</td>
<td></td>
</tr>
<tr>
<td>BCP-ALL</td>
<td>Rasool²⁷⁹</td>
<td>1</td>
<td>20</td>
<td>TCCCGG to GCATGGA</td>
<td>Frameshift</td>
<td></td>
</tr>
<tr>
<td>BCP-ALL</td>
<td>Quesnel²⁷⁷</td>
<td>2</td>
<td>49</td>
<td>GCC to GTC</td>
<td>Ala to Val</td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td>Haidar²⁰⁷</td>
<td>intron 1</td>
<td>-</td>
<td>27bp del</td>
<td>Splice mutant</td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td>Haidar²⁰⁷</td>
<td>2</td>
<td>90</td>
<td>CAC to CGC</td>
<td>His to Arg</td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td>Haidar²⁰⁷</td>
<td>2</td>
<td>108</td>
<td>GAC to GTC</td>
<td>Asp to Val</td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td>Haidar²⁰⁷</td>
<td>2</td>
<td>45</td>
<td>ATG to ACG</td>
<td>Met to Thr</td>
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<tr>
<td>B-NHL</td>
<td>Uchida²¹¹</td>
<td>1</td>
<td>3-15</td>
<td>35 bp deletion</td>
<td>Frameshift</td>
<td></td>
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<tr>
<td>B-NHL</td>
<td>Uchida²¹¹</td>
<td>2</td>
<td>72</td>
<td>CGA to CAA</td>
<td>Arg to Gln</td>
<td></td>
</tr>
<tr>
<td>B-NHL</td>
<td>Uchida²¹¹</td>
<td>2</td>
<td>49-54</td>
<td>13bp deletion</td>
<td>Frameshift</td>
<td></td>
</tr>
</tbody>
</table>

* = cell line

6.2.5 Relationship between \( p16^{INK4A} \) deletions and cytogenetic abnormalities of chromosome 9p

Of the twenty lymphoid tumours reported here with deletion of \( p16^{INK4A} \) by Southern blot, only one had a cytogenetically visible deletion of 9p21. Therefore most \( p16^{INK4A} \) deletions occurred in the absence of detectable
cytogenetic abnormalities of this region. It was difficult to correlate
cytogenetic abnormalities with deletions of $p16^{INK4A}$ in other studies, as
karyotypes were not always reported. However, Okuda et al.\textsuperscript{308} and Ogawa et al.\textsuperscript{278} gave comprehensive data on 9p abnormalities observed in their studies.

Five out of 55 and 26 out of 39 patients with no cytogenetic abnormalities of
9p had lost both copies of $p16^{INK4A}$ indicating that the deletions on 9p are
frequently too small to be visible by cytogenetic techniques. Fizzoti et al. and
Sill et al. also reported 7 and 8 cases respectively with $p16^{INK4A}$ deletion and
no visible 9p loss\textsuperscript{310,311}.

In contrast, several studies noted cases with 9p deletion and retention
of both $p16^{INK4A}$ alleles. Of five patients with effective monosomy of 9p21,
Quesnel et al.\textsuperscript{277} stated that only three had biallelic deletion of $p16^{INK4A}$, whilst
Okuda et al.\textsuperscript{308} reported two cases with deletions of 9p22 that were telomeric
to, and did not include $p16^{INK4A}$. This suggests that a second lymphoid
tumour suppressor gene exists on 9p distal to $p16^{INK4A}/p15^{INK4B}$ (see below).

6.2.6 Does $p15^{INK4B}$ act as a tumour suppressor gene in lymphoid
malignancies?

Recent work provides biochemical evidence that $p16^{INK4A}$ acts as a
tumour suppressor gene in many malignancies. For example, ectopic $p16^{INK4A}$
expression in non small cell lung cancer cell lines with $p16^{INK4A}$ deletion can
inhibit \textit{in vitro} and \textit{in vivo} growth and entry into S-phase of these cells\textsuperscript{322}.

$P16^{INK4A}$ can also inhibit ras induced transformation of rat fibroblasts\textsuperscript{130}.

Introduction of $p16^{INK4A}$ into Granta 519, a B-NHL cell line with concurrent
abnormalities of $CCND1$, $p16^{INK4A}$ and $p15^{INK4B}$, prevented entry into S phase
However, work presented here and elsewhere, indicates that \( p15^{\text{INK4B}} \) is also important in the development of lymphoid malignancies. Both \( p16^{\text{INK4A}} \) and \( p15^{\text{INK4B}} \) are involved in the deletions seen in ALL in this thesis and in other studies, and the majority of homozygous deletions analysed spanned both genes in all types of lymphoid malignancy \(^{278, 304, 307, 308, 309, 320}\).

Kamb and colleagues suggested that \( p16^{\text{INK4A}} \) was the critical deleted gene involved in tumours with loss of 9p \(^7\). Analysis of several hundred tumour cell lines using PCR of sequence tagged sites (STS) spanning at least 70 kb surrounding \( p16^{\text{INK4A}} \) and \( p15^{\text{INK4B}} \) revealed 30 cell lines in which \( p16^{\text{INK4A}} \) was lost and \( p15^{\text{INK4B}} \) was retained, but only three cell lines in which \( p16^{\text{INK4A}} \) appeared to be germline whilst \( p15^{\text{INK4B}} \) was deleted.

However, \( p16^{\text{INK4A}} \) and \( p15^{\text{INK4B}} \) are highly homologous within their protein coding regions (93% sequence identity) and have a very similar function in control of the cell cycle. Both genes produce protein products that bind to and inhibit cdk4 and cdk6, preventing phosphorylation of the retinoblastoma gene product and causing cells to arrest in G1. This functional equivalence suggests that loss of function of both genes may play a similar role in tumour pathogenesis. P15 differs from p16 primarily in the regulation of its gene expression, which is induced by TGF-\( \beta \) \(^{195}\). Thus p15 may mediate the effect of external growth inhibitory signals. In contrast, \( p16^{\text{INK4A}} \) expression is suppressed by the presence of functional retinoblastoma gene product \(^{323}\). When this repression is lifted, as in small cell lung tumours and bladder cancer cell lines lacking retinoblastoma, \( p16^{\text{INK4A}} \) is strongly expressed \(^{324, 325}\).
Work presented here in Chapter 4, used primers for the STS sites described by Kamb et al.\textsuperscript{17} in 14 lymphoid cell lines, and supported the hypothesis that both $p_{15}^{\text{NK4B}}$ and $p_{16}^{\text{NK4A}}$ play a tumour suppressor role in lymphoid malignancies. Seven out of nine cases with abnormalities of $p_{16}^{\text{NK4A}}$ by Southern blot had lost both $p_{15}^{\text{NK4B}}$ and $p_{16}^{\text{NK4A}}$ and in primary lymphoid tumours this pattern of deletion appears to be similar, with loss of both genes the most common event at this locus. In three cell lines studied here, deletions spanned more than 70 kb. In the remaining six cell lines with homozygous deletion of $p_{16}^{\text{NK4A}}$ by Southern blot, one end of the region of homozygous deletion was within the region covered by the STS used. K1106 retained the most proximal and the most distal STS, whereas SKW-3, Granta 519, Granta 452, DoHH2, and SuPT1, retained the most proximal STS. These cell lines were all derived from NHL and may indicate that a hotspot for deletion occurs immediately centromeric of $p_{15}^{\text{NK4B}}$. One cell line, SKW-3, showed deletion of $p_{16}^{\text{NK4A}}$ with retention of $p_{15}^{\text{NK4B}}$.

However, two cell lines, Daudi and UoCB4, showed loss of $p_{15}^{\text{NK4B}}$ and retention of $p_{16}^{\text{NK4A}}$ coding sequences. Daudi was included in the STS analysis as a result of several rearrangements of $p_{16}^{\text{NK4A}}$ exon 1 observed by Southern blot. UoCB4 was apparently germline for $p_{16}^{\text{NK4A}}$ exon 1 in EcoRI digests by Southern blot, but showed rearrangements with an $p_{15}^{\text{NK4B}}$ exon 2 probe. Whether these deletions could functionally inactivate $p_{16}^{\text{NK4A}}$ by altering 5’ regulatory sequences is unclear, but in both cases exon 1 β of $p_{16}^{\text{NK4A}}$ may be lost. Transcripts from this alternative initiation site have been detected in both normal and tumour cell lines and probably play a role.
in transcriptional regulation of \( \text{p16}^{\text{INK4A}} \). However their influence on \( \text{p16}^{\text{INK4A}} \) expression has not yet been determined. Transcripts from \( \text{p16}^{\text{INK4A}} \) exon 1β continue to be expressed in cells that show methylation of the 5′CpG island that downregulates \( \text{p16}^{\text{INK4A}} \) expression.

Nevertheless the deletions in UoCB4 and Daudi do suggest a role for \( \text{p15}^{\text{INK4B}} \) as a putative tumour suppressor gene in lymphoid malignancies. Rasool and colleagues have recently reported two cases of BCP-ALL with deletion of \( \text{p15}^{\text{INK4B}} \) and retention of \( \text{p16}^{\text{INK4A}} \) sequences. These cases showed no intragenic mutations of \( \text{p16}^{\text{INK4A}} \) and suggest that either \( \text{p15}^{\text{INK4B}} \) or a separate gene at 9p21 plays a tumour suppressor role in these leukaemias.

A third cell line, 104, derived form a patient with BCP-ALL showed two separate regions of deletion within the area studied. The STS RN3.1 within exon 2 of \( \text{p16}^{\text{INK4A}} \) was lost as was \( \text{p15}^{\text{INK4B}} \) exon 2. Intervening sites were retained. By Southern blot, this cell line was shown to have a deletion of one allele of \( \text{p16}^{\text{INK4A}} \) exon 1 and rearrangement of the second. This complex pattern is difficult to interpret without separately cloning each allele, as STS analysis does not readily distinguish between hemizygous deletions and complete retention of markers. Also, the possibility that failure of one of the PCR reactions was due to a point mutation or small deletion within the primer binding sites was not eliminated. Nevertheless, this complex deletion pattern points to the importance of both \( \text{p16}^{\text{INK4A}} \) and \( \text{p15}^{\text{INK4B}} \) in this tumour, and in other lymphoid malignancies. Loss of \( \text{p16}^{\text{INK4A}} \) or \( \text{p15}^{\text{INK4B}} \) may be of different importance in different subsets of lymphoid disease. For example, three cases reported with \( \text{p15}^{\text{INK4B}} \) deletions only are BCP-ALL and the fourth is a
Burkitt's lymphoma cell line. Larger studies will be necessary to investigate this hypothesis.

6.3 Is \( p16^{\text{INK4A}} \) the only gene on 9p involved in lymphoid malignancies?

Abnormalities of 9p seen in lymphoid malignancies include both recurrent and sporadic translocations, and deletions. It seems probable that some translocations of 9p involve a gene different from \( p16^{\text{INK4A}} \) or \( p15^{\text{INK4B}} \). In this study, deletion of \( p16^{\text{INK4A}} \) was not observed in two B-ALL cell lines carrying the t(8;9)(q24.1;p13.3) or in one patient with BCP-ALL with a t(9;15)(p21;q15) translocation. This may indicate that other genes involved in lymphoid tumour development exist at 9p13.3 and 9p21. In contrast, Okuda et al. reported 11 cases of lymphoid malignancies with 9p translocations or inversions, of which three showed hemizygous \( p16^{\text{INK4A}} \) loss and eight showed bi-allelic \( p16^{\text{INK4A}} \) deletion. This implies that many of these translocations are not strictly reciprocal exchanges of DNA and that some do target the \( p16^{\text{INK4A}} \) locus.

6.3.1 Evidence of lymphoid tumour suppressor genes on chromosome 9p other than \( p16^{\text{INK4A}} \) and \( p15^{\text{INK4B}} \).

Studies of other tumours that show cytogenetic abnormalities of 9p21, for example melanoma and pancreatic adenocarcinoma, have revealed a significant rate of intragenic \( p16^{\text{INK4A}} \) mutation, consistent with \( p16^{\text{INK4A}} \) being a true tumour suppressor gene in these cancers. Sequence analysis of both \( p16^{\text{INK4A}} \) and \( p15^{\text{INK4B}} \) in lymphoid malignancies has revealed that inactivating point mutations of these genes are relatively rare in these
tumours (Table 6.3)\textsuperscript{277, 278, 279, 280, 321}. The relatively low number of point mutations found in $p16^{\text{INK4A}}$ in lymphoid malignancies may indicate that another gene at 9p21 is the major lymphoid tumour suppressor gene. However, this seems unlikely, given the importance of the role of $p16^{\text{INK4A}}$ in the control of the cell cycle and in the development of numerous other tumour types. Mutational analysis has concentrated on $p16^{\text{INK4A}}$ coding regions, and may have overlooked mutations in $p16^{\text{INK4A}}$ regulatory regions.

Alternatively, $p16^{\text{INK4A}}$ may be more frequently inactivated by other mechanisms in lymphoid tumours. The first exon of $p16^{\text{INK4A}}$ and an additional 139bp upstream is GC rich (67%) and has a high ratio of CpG to GpC dinucleotides (0.77)\textsuperscript{193}. De novo methylation of this 5'CpG island has been associated with transcriptional silencing of $p16^{\text{INK4A}}$ in human tumours, but is not detectable by sequence analysis or standard Southern blot techniques that use methylation insensitive restriction enzymes. Studies of the methylation status of $p16^{\text{INK4A}}$ in lymphoid tumours have not yet been undertaken.

However, the possibility that another unidentified tumour suppressor gene adjacent to $p16^{\text{INK4A}}$ and $p15^{\text{INK4B}}$ was affected by these deletions cannot yet be excluded. To begin to investigate this, PCR analysis of both polymorphic and STS markers on chromosome 9 was performed in both lymphoid cell lines with $p16^{\text{INK4A}}$ and $p15^{\text{INK4B}}$ abnormalities and controls with no evidence of alteration of these genes.

In three cell lines, UoCB1, BL28 and G519, the endpoints of the deletion were outside the region covered by the STS primers used. Of the
nine cell lines with abnormalities of $p^{16^{\text{INK4A}}}$, none were heterozygous at the locus immediately centromeric of $p^{16^{\text{INK4A}}}$ at 9p21, D9S171. Five cell lines were nullizygous at this locus, including JVM-2 which has no deletion of $p^{16^{\text{INK4A}}}$ or $p^{15^{\text{INK4B}}}$ by Southern blot or STS mapping. This may indicate that in these cell lines with loss of all STS markers at the $p^{16^{\text{INK4A}}}$ locus and D9S171, the deletions extended centromeric of $p^{16^{\text{INK4A}}}$ (BL28 and G519). For cell lines that retained the most centromeric STS marker c1.B (K1106 and UoCB4), a second separate deletion can be postulated at one allele. JVM-2 was included as a negative control with no detectable deletion of $p^{16^{\text{INK4A}}}$ or $p^{15^{\text{INK4B}}}$, however it appears to have a homozygous deletion on 9p, centromeric of these genes. This may indicate that another unidentified lymphoid tumour suppressor is located at 9p21. However, without normal DNA from the patient from whom JVM-2 was derived, it cannot be ascertained whether this deletion was tumour associated. Takeuchi et al. have reported loss of heterozygosity at D9S171 in 7/24 informative cases of childhood ALL with germline $p^{16^{\text{INK4A}}}$ and $p^{15^{\text{INK4B}}}$ genes, which also suggests the presence of a tumour suppressor gene on 9p, centromeric to $p^{16^{\text{INK4A}}}$ and $p^{15^{\text{INK4B}}}$.

Evidence from other tumour cell lines supports the hypothesis that more than one tumour suppressor locus is situated on 9p. Some melanomas have two non-contiguous deletions of 9p, one involving the region containing $p^{16^{\text{INK4A}}}$ and the other a more centromeric region on 9p. Furthermore a malignant mesothelioma cell line and two melanoma cell lines have been isolated that have 9p deletions with a distal boundary proximal to the MTAP.
However Holland et al. have reported that in melanoma the two regions of deletion fall either side of the IFNA cluster, and that the most centromeric, which includes the p16 locus, is linked with familial melanoma. Closely linked tumour suppressor genes may not be uncommon in the human genome, and have previously been implicated at the RB locus on 13q in CLL and breast cancer and at the p53 locus on 17p. However cloning and detailed mapping of one 9p deletion in glioma has revealed a complex rearrangement including both an inversion and a deletion, which would have the appearance of two separate deletions by PCR analysis of polymorphic markers. Thus the presence of non-contiguous deletions of 9p21 could reflect the instability of this region in tumour cells.

6.4 Mechanisms of p16\textsuperscript{INK4A} and p15\textsuperscript{INK4B} inactivation in lymphoid tumours

In order to inactivate two unlinked tumour suppressor genes by point mutation in one cell, four mutational events are required. However, if the tumour suppressor genes are closely linked physically, a single deletion event can inactivate both genes on one chromosome. Therefore the rarity of observed point mutations of p16\textsuperscript{INK4A} and p15\textsuperscript{INK4B} in lymphoid tumours, and the high frequency of homozygous deletions affecting both genes, may indicate that both genes act as lymphoid tumour suppressors.
6.4.1 Analysis of polymorphic repeats on chromosome 9: are bi allelic deletions at 9p21 caused by non-dysjunction of chromosome 9?

Many human cancers have been shown to have functional loss of both alleles of various tumour suppressor genes, and this can occur in several ways. Typically, one allele is inactivated by mutation or methylation, for example by genomic imprinting. The second is lost by deletion, resulting in a loss of heterozygosity for DNA markers adjacent to the tumour suppressor gene.

There are three somatic mechanisms identified that could lead to loss of heterozygosity.\(^334, 335, 336\):

1) Deletion of a chromosomal region.

2) Loss of a whole chromosome.

3) Replacement of a chromosomal region by a copy of the homologous sequence from the partner chromosome at mitosis. This occurs by homologous recombination.

In tumours with homozygous gene deletions two independent deletions may occur at each allele or the mechanisms number 2 or 3 above may inactivate the second allele. Loss of whole or part of one chromosome can be detected by the presence of a single allele only, for all polymorphic loci within the deleted region. In contrast two independent subchromosomal deletion events give rise to nullizygous loci within the deletion overlap and loss of heterozygosity in regions where the deletions do not coincide. Gene conversion would appear to be the same as two independant deletions by analysis of heterozygosity.
All the lymphoid cell lines analysed here, showed at least two heterozygous loci and so could not have undergone loss or duplication of a whole copy of chromosome 9.

Of the nine cell lines with p16INK4A abnormalities that were homozygous or nullizygous for D9S171, five were also homozygous at D9S147E. More detailed loss of heterozygosity studies using non-tumour DNA controls from each case would be necessary to further investigate the mechanisms of deletion in these cell lines. In particular, analysis of more loci on chromosome 9p would be necessary to pinpoint areas of loss of heterozygosity and deletion.

Karpas 231, whilst heterozygous for both loci on 9p was homozygous for all loci tested on 9q. This is difficult to interpret in the context of the cytogenetic data which shows that Karpas 231 has two copies of both the der(8) and der(9) of the t(8;9)(q24.1;p13.3). No cytogenetically normal chromosome 9 was observed in this cell line. Karpas 1106, which can be seen cytogenetically to have four copies of the short arm of chromosome 9 (two normal 9 chromosomes, and iso(9p)) was nullizygous at D9S171 probably implying that the +iso(9p) was generated after the deletion of p16INK4A. Alternatively, 4 separate deletions at 9p21 may have occurred in this cell line.

6.5 ALLELIC EXCLUSION OF MYC IN GRANTA 452

Translocation of MYC is a common event in some types of lymphoma, and may be associated with the progression of follicular lymphoma to higher
grade disease. Whilst amplification of the MYC locus is frequently seen in solid tumours, only a few cases of leukaemia or lymphoma have been reported with extra copies of the MYC oncogene and these are commonly of myeloid lineage. The cell line Granta 452 is unusual in that it was derived from a lymphoid leukaemia possessing three MYC alleles, one of which was translocated to the immunoglobulin lambda locus on chromosome 22. The untranslocated chromosome 8 showed duplication of the MYC locus.

In most BL studied to date the translocated MYC allele was expressed whilst the remaining MYC allele remained silent, or was expressed at low levels. Differentiation between the two alleles may be made by the accumulation of point mutations within the first exon and intron of translocated MYC genes, since in BL and other lymphomas the translocated MYC allele frequently accumulates point mutations within this region. It has been suggested that this may be the result of the t(8;14)(q24.1;q32.3) translocation being aberrantly recognised as a functional VDJ recombination by the IGH hypermutation mechanism. However, evidence in support of this hypothesis is inconclusive and MYC hypermutation and IGH hypermutation may occur via unrelated mechanisms. Furthermore, Burkitt like MYC mutations have been reported in an untranslocated MYC allele in a case of prolymphocytic leukaemia.

In Granta 452, amplification and sequencing of the first exon of MYC from genomic and cDNA was undertaken in order to determine whether the duplicated or translocated allele was mutated, and also to investigate whether one or more MYC alleles were transcribed.
6.5.1 Structural Analysis

Sequencing of the Granta 452 genomic MYC exon 1 amplification product revealed essentially two groups of sequences and no normal germline clones were isolated. The ratio of 11:5 of the frequency of the two sequence groups (here called A and B) suggested that group A clones were derived from the duplicated allele and that group B clones were derived from the translocated allele. This would also indicate that any sequence difference in the duplicated allele occurred before duplication.

The high level of MYC expression in this cell line was not the result of transcription of all three MYC alleles. From the sequence analysis of the cDNA MYC exon 1 amplification product, it was determined that only one MYC allele in Granta 452, sequence B, was expressed from promoter P0. Sequence A was either transcriptionally silent, transcribed at low levels, or transcribed only from P3. This last possibility was not examined, but this promoter is not usually active in translocated MYC alleles that retain MYC exon 1 sequences. However, Mae III restriction endonuclease digestion of RT PCR product confirmed that transcription of sequence A was negligible from P0, P1 and P2. The relative abundance of the two sequences from the genomic PCR (11:5/silent:expressed) suggests that the translocated MYC allele and not the duplicated allele was transcribed.

In small cell lung carcinoma and breast cancer, amplification of MYC is associated with increased levels of MYC expression \(^{337}\). However in lymphoma, MYC amplification is rare and it is translocation of MYC that is associated with deregulation of MYC expression. Therefore, the lack of
transcripts detected from the duplicated MYC allele in Granta 452 may reflect the transcriptional repression of the untranslocated MYC allele in lymphoma cells with a MYC translocation. Moreover, in many tumours that exhibit MYC amplification, copy numbers of 40-150 per cell are typical, and in bladder cancer no association has been found between a low level MYC copy number increases and myc protein expression \(^{337}\). The duplicated region of 8q24.1 in Granta 452 may have targeted another lymphoma associated oncogene. For example, the PVT1 gene located 3’ of MYC which has been shown to be expressed in mouse plasmacytomas was also duplicated.

6.5.2 Mechanisms of mutation

In Granta 452 the untranslocated duplicated allele was mutated as no germline sequences were cloned. However, the sequence of the putative duplicated allele differed at only one base (453) from most germline sequences. Searching the Genbank database with a 40 bp sequence including the mutation at base 453 using the BLAST program to identify sequence homologies revealed one identical sequence (GenBank accession no. K00559) derived from a human foetal liver MYC gene \(^{342}\). Consequently, the “mutation” at base 453 may in fact be a rare polymorphism. Whatever the interpretation of the sequence analysis this mutation must have been present before duplication of this locus and may be a germline event.

6.5.3 Consequences of mutation

In BL and other B-cell malignancies with t(8;14)(q24.1;q32.3), mutations of the MYC gene are common throughout the first exon and intron
and have been seen to cluster at the 3’ end of the first exon, often altering a 
Pvu II site 40 bp upstream of the exon I /intron I boundary. This restriction 
enzyme site marks the location of sequences that are important in the 
regulation of transcriptional attenuation, and mutations in this region have 
been shown to increase transcriptional readthrough. This attenuation site 
has been shown to be more effective at blocking transcripts from P2 than P1. 
Mutations in Granta 452 that were seen in clones derived from both genomic 
and cDNA amplification did not affect this Pvu II site. However, one genomic 
clone and 3 cDNA clones showed mutations in this 6bp region.

In BL cells a switch in promoter usage to favour P1 has been reported 
by some authors which may increase the number of full length transcripts 
produced by overcoming attenuation at the exon 1/intron 1 boundary. What 
causes this shift in promoter usage is not fully understood and the relative 
promoter usage in Granta 452 has not been investigated. However, the point 
mutation at base 206 is within 8 bp of the beginning of the P2 promoter, and 
could conceivably reduce the efficiency of this promoter in favour of P1. The 
cell line Raji also shows a mutation at base 206 (G to C) in addition to two C 
to T mutations at bases 203 and 204. Raji is unusual in that 30% of its 
MYC expression is from the normal MYC allele, and originates from the P3 
promoter. Transcripts from P3 in Granta 452 would not possess any of the 
exon 1 sequences analysed here and so could not be amplified or assigned 
to a specific allele. Further work using RNase protection assays would 
determine whether P3 transcripts exist in this cell line.
Comparison of ten translocated MYC sequences, aligned using the pileup program (GCG: Wisconsin Package) did not show a consensus between the sites of mutation in Granta 452 and other cell lines. This may indicate that these mutations are merely a consequence of MYC translocation and do not confer a selective growth advantage on Granta 452. In support of this hypothesis is evidence that human MYC cDNA in a retroviral vector is equally potent when transforming chicken haematopoietic cells as v-myc which is has no exon I \(^{357}\). However, nearly all BL that have been examined in detail possess abnormalities of the translocated MYC exon I and these appear to cluster in transcriptional regulatory regions \(^{220}\). Therefore it seems likely that they play some role in tumour development.

7. CONCLUSIONS

Abnormalities of chromosome 9p are common in lymphoid malignancies and fall into two categories, translocations and deletions. This study has determined that homozygous deletion of \(p16^{INK4A}\), located at 9p21, occurred in a subset of ALL and transformed NHL, but was not observed in malignancies of mature lymphocytes or myeloid lineages. \(P16^{INK4A}\) is likely to be the target for 9p deletions or is closely linked to the target gene. \(P16^{INK4A}\) deletion was most frequently observed in the absence of cytogenetically detectable loss of 9p. Progression of B-NHL to high grade disease was associated with homozygous loss of \(p16^{INK4A}\) in two cases. Both cases also showed translocation of the MYC oncogene concurrent with disease progression.
Loss of $p16^{INK4A}$ was more frequent in cell lines than in primary tumours. Furthermore, the ability of B-ALL to grow in SCID mice was closely associated with deletion of $p16^{INK4A}$. Analysis of paired fresh tumour and cell line samples shows that all $p16^{INK4A}$ deletions in cell lines were also present in the original tumour and lymphoblastoid cell lines established from normal lymphoid cells showed no $p16^{INK4A}$ deletions despite prolonged in vitro culture. This indicates that deletion of $p16^{INK4A}$ facilitates in vitro growth of tumour cells, but is not a direct result of long term in vitro culture.

Analysis of the extent of these deletions in lymphoid cell lines, has revealed that $p15^{INK4B}$, a homologous gene closely linked to $p16^{INK4A}$, was also a target for deletion. The presence of two putative tumour suppressor genes at the same locus may account for the high frequency of homozygous deletion reported here, and the lack of inactivating point mutations of these genes observed in other studies of leukaemias and lymphomas. There is some evidence, from analysis of polymorphic markers on chromosome 9, to suggest that a third tumour suppressor gene may exist centromeric to $p15^{INK4B}$.

Duplication of an abnormal chromosome 9 and loss of the normal copy of chromosome 9 was not a mechanism for the production of homozygous deletion in the cell lines studied here.

The translocation t(8;9)(q24.1;p13.3) did not target $p16^{INK4A}$ or $p15^{INK4B}$ but did involve the MYC locus at 8q24.1, at least in Karpas 231, and appeared to drive expression of the MYC oncogene. Cell line Karpas 231 which possessed this translocation showed strong expression of the MYC
oncogene. Karpas 231 showed duplication of the der(9) chromosome, and exhibited approximately double the MYC mRNA expression by Northern blot, than Karpas 353.

Studies of the MYC gene in Granta 452 showed the presence of three MYC alleles. Translocation of MYC drove expression from P0, P1 and P2, rather than amplification of MYC alleles in this cell line. This is fundamentally different to events in lung carcinomas and breast cancers where MYC amplification is associated with MYC overexpression.
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Deletions and Rearrangement of CDKN2 in Lymphoid Malignancy


Recurrent abnormalities of the short arm of chromosome 9, including translocations and interstitial deletions, have been reported in both leukemia and lymphoma. The pathologic consequences of these abnormalities remain unknown. The cyclin-dependent kinase 4 inhibitor (CDKN2) gene, which maps to 9p21, has been implicated by the finding of a high frequency of biallelic deletions in leukemic cell lines. We have determined the incidence of structural abnormalities affecting CDKN2 by DNA blot in a panel of 231 cases of leukemia and lymphoma and 66 cell lines derived from patients with lymphoid malignancies with defined cytogenetic abnormalities. Structural alterations of CDKN2 were seen in 20 (8.3%) of all fresh cases and 10 (15.1%) of all cell lines. Biallelic CDKN2 deletions were seen in 11 of 53 (21%) cases of B-cell precursor acute lymphoblastic leukemia (BCP-ALL). There was no association with any particular cytogenetic abnormality. Biallelic deletions were also found in high-grade and transformed non-Hodgkin's lymphoma (NHL) of both B- and T-cell lineages. In two cases of transformed NHL, analysis of sequential samples showed loss of CDKN2 with transformation. Neither deletions nor rearrangements of the CDKN2 gene were seen in any of the 119 leukemias of mature B or T cells analyzed. Biallelic deletions of CDKN2 were observed in 6 of 13 NHL cell lines. Three of the 6 cases had undergone transformation from low- to high-grade disease; in 2 of these cases it was possible to show that the CDKN2 deletions were present in fresh material from the patient and were therefore not an artifact of in vitre culture. Rearrangements of CDKN2 were seen in 2 cases (4%) of BCP-ALL, in 1 case of B-NHL, and in 1 Burkitt's lymphoma cell line and suggest the presence of a "hot spot" for recombination in the vicinity of the CDKN2 gene. These data indicate that the loss of CDKN2 expression may be involved in the pathogenesis of a subset of BCP-ALL, some high-grade NHL, and in the transformation of NHL from low- to high-grade disease. CDKN2 deletions and rearrangements occurred in the absence of detectable cytogenetic changes of chromosome 9p in 25 of 30 (83%) cases. Finally, of 10 cases of BCP-ALL that produced overt, transplantable leukemia in mice with severe combined immunodeficiency (SCID), seven showed biallelic CDKN2 deletions. In contrast, none of 11 cases that failed to engraft showed biallelic CDKN2 deletions. BCP-ALL cases that lack CDKN2 expression may have a particular propensity to grow in SCID mice.

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control of the cell cycle. However, preliminary studies in primary bladder tumors have shown that CDKN2 deletions occur at a much lower frequency in primary tumors than in cell lines.\textsuperscript{23} This raised the possibility that the high frequency of deletions observed in cell lines may represent an artifact of in vitro culture.

We have determined the incidence of CDKN2 abnormalities by DNA blot analysis. Results are summarized in Table 1 and are discussed further. CDKN2 may confound analysis by polymerase chain reaction (PCR). DNA was isolated from a mononuclear fraction of peripheral blood, bone marrow, biopsied lymph node, or splenectomy specimens after informed patient consent was obtained.

**Materials and Methods**

**Patient population.** Diagnosis was made on the basis of combined morphologic, immunophenotypic, cytogenic, and genotypic analyses, using previously defined criteria.\textsuperscript{22,23} High molecular weight DNA was isolated from a mononuclear fraction of peripheral blood, bone marrow, biopsied lymph node, or splenectomy specimens after informed patient consent was obtained.

**Cell lines.** All cell lines were grown in RPMI 10% fetal calf serum (FCS) under standard conditions. Cell lines were kindly derived by the following: (1) Burkitt’s lymphoma-derived cell lines and Epstein-Barr virus (EBV)-transformed normal B-cell lines were derived by Prof G.M. Lenoir; (2) cell lines Karpas 231, 247, 353, and 519 by Dr A. Karpas; (3) cell lines Granta 56, 452, and Epstein-Barr virus (EBV)-transformed normal B-cell lines were derived by Prof G.M. Lenoir; (4) cell lines UoCB 1 and UoCB4 by Dr A. Karpas; (3) cell lines Granta 56, 452, and Epstein-Barr virus (EBV)-transformed normal B-cell lines were derived by Prof G.M. Lenoir; (4) cell lines UoCB 1 and UoCB4 by Dr A. Karpas; (5) cell lines derived from patients with lymphoid malignancies with biallelic CDKN2 deletions may have increased ability to proliferate when transplanted into mice with severe combined immunodeficiency (SCID).

**CDKN2**

\[ \text{Coding region of exon 1 of CDKN2 (130 bp) plus 200 bp of flanking sequence. This probe should cross hybridize with the related p15.} \]

\[ \text{Note that EcoRI sites 3' of exon 2 are not shown; B, BamH1. The position of the exon 1 probe is shown.} \]

**RESULTS**

A total of 231 cases of lymphoid leukemia or lymphoma and 66 cell lines representing malignancies of both B- and T-cell lineages at various stages of differentiation were analyzed. Overall, biallelic deletions of CDKN2 were observed in 17 (7.3%) and rearrangements of CDKN2 in 3 (1.3%) fresh cases. Of the lymphoid cell lines, 9 (13.6%) exhibited biallelic deletion and 1 (1.5%) exhibited rearrangement. Results are summarized in Tables 1 and 2 and are discussed according to the diagnostic subgroups below.
Table 2. CDKN2 Abnormalities in Lymphoid Cell Lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Derivation</th>
<th>Chromosome 9 Abnormalities</th>
<th>CDKN2</th>
</tr>
</thead>
<tbody>
<tr>
<td>UoCB1</td>
<td>BCP-ALL</td>
<td></td>
<td>D/D</td>
</tr>
<tr>
<td>UoCB4</td>
<td>BCP-ALL</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>BL 15-135 (n = 20)</td>
<td>Burkitt’s lymphoma</td>
<td>BL28 = D/D 19 = G/G</td>
<td></td>
</tr>
<tr>
<td>JARC (n = 17)</td>
<td>Lymphoblastoid</td>
<td>All 17 = G/G</td>
<td></td>
</tr>
<tr>
<td>Wien 133</td>
<td>Burkitt’s lymphoma</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>Granta 56</td>
<td>Burkitt’s lymphoma</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>Namalwa (plus Namalwa IPN/45)</td>
<td>Burkitt’s lymphoma</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>Raji</td>
<td>Burkitt’s lymphoma</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>Daudi</td>
<td>Burkitt’s lymphoma</td>
<td>G/R/R/r</td>
<td></td>
</tr>
<tr>
<td>Karpas 231*</td>
<td>B-cell leukemia with t(14;18)</td>
<td>t(6;9)(q24.1;p13.3)</td>
<td>G/G</td>
</tr>
<tr>
<td>Karpas 353</td>
<td>B-cell leukemia with t(14;18)</td>
<td>t(6;9)(q24.1;p13.3)</td>
<td>D/D</td>
</tr>
<tr>
<td>KHM-2B</td>
<td>B-cell leukemia with t(14;18)</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>SuDHL6</td>
<td>DLCL</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>Karas 422</td>
<td>DLCL</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>OCLY8</td>
<td>DLCL</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>SSK41</td>
<td>DLCL</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>WSU-NHL</td>
<td>DLCL</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>NCEB1</td>
<td>MZ-NHL</td>
<td></td>
<td>G/G</td>
</tr>
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<td>Karpas 1106</td>
<td>Mediastinal B-NHL</td>
<td>iso(9p)</td>
<td>D/D</td>
</tr>
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<td>DoHH2*</td>
<td>Immunoblastic B-NHL</td>
<td></td>
<td>D/D</td>
</tr>
<tr>
<td>Granta 452*</td>
<td>B-cell ALL</td>
<td>t(2;9)(p11;p13)</td>
<td>D/D</td>
</tr>
<tr>
<td>Granta 519</td>
<td>Leukemic transformation MZ-NHL</td>
<td>t(9;14)(p13;q11)</td>
<td>D/D</td>
</tr>
<tr>
<td>JVM-2</td>
<td>B-PLL</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>Karpas 247*</td>
<td>B-PLL</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>Karpas 384</td>
<td>Leukemic transformation T-NHL</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>SKV-3</td>
<td>T-NHL</td>
<td>D/D</td>
<td></td>
</tr>
<tr>
<td>SuPT1</td>
<td>T-NHL</td>
<td>D/D</td>
<td></td>
</tr>
</tbody>
</table>

All cell lines had cytogenetically normal chromosome 9s unless otherwise stated. References for the derivation of all cell lines will be provided on request to M.J.S.D. Note that DoHH2, Granta 452 (G.S., E.N., and M.J.S.D., manuscript in preparation) and Granta 519 were all derived from cases of transformed B-NHL.

Abbreviations: DLCL, diffuse large-cell lymphoma; MZ-NHL, mantle cell B-cell NHL; G, germline configuration of CDKN2; D, deletion of CDKN2; R, rearrangement of CDKN2; r, faint rearrangement of CDKN2.

* Identical configuration of patient material and derived cell line.

ALL. Fifty-three cases of BCP-ALL were examined and, of these, 11 cases exhibited biallelic deletions of CDKN2 (Figs 2 and 3). Cases with biallelic deletions comprised a heterogeneous clinical group and were not associated with any consistent cytogenetic abnormality, as shown in Table 3. In particular, of the 17 cases of BCP-ALL with the t(9;22)(q34;q11), only 4 exhibited CDKN2 deletions; 2 of these cases had cytogenetically detectable rearrangements of chromosome 9p (cases no. 6 and 11, Table 3). Two of the four cases with t(9;22)(q34;q11) were BCP-ALL arising as blast crisis of chronic myeloid leukemia (cases no. 6 and 10, Table 3); whether loss of CDKN2 was associated with this transformation could not be determined because of the lack of suitable specimens. One patient with t(4;11)(q21;q23) retained germline CDKN2. Of the two BCP-ALL lines examined, one (UoCB1) showed biallelic CDKN2 deletion, whereas UoCB4 retained germline configuration. The availability of these cell lines permitted high-resolution cytogenetics, but no structural abnormalities of the short arm of chromosome 9p were detected. These data therefore confirm that CDKN2 deletions for the most part occurred in the absence of the interstitial deletion 9p21-22.

Two cases (3.8% of the total) exhibited CDKN2 rearrangements (cases no. 13 and 14, Table 3 and Fig 3). These cases again lacked cytogenetic change at chromosome 9p. Rearrangements were seen in multiple restriction enzyme digests, were not seen in normal cases, and were therefore neither simple polymorphisms nor point mutations. In case no. 13, the rearranged allele was associated with deletion of the other allele, whereas, in case no. 14, both alleles of CDKN2 were rearranged (Fig 3). In both of these cases and in all other cases with CDKN2 rearrangements (see below), a germline configuration was observed in EcoRI digests with the CDKN2 exon I probe. Molecular cloning experiments in case no. 14 are being undertaken to define further the nature of these rearrangements.

Finally, leukemic blasts from some cases of BCP-ALL have been shown to produce overt, transplantable disease when inoculated into SCID mice. We have established 11 such transplantable cell lines from a total of 27 different BCP-ALL samples. Some of these lines have been shown to maintain identical IGH rearrangements to those observed in patient material over several passages (J.M. Heward, M.J.S.D., and P.M., unpublished observations, 1994). Seven of 10 BCP-ALL lines that grow in SCID mice and the original patient material showed biallelic CDKN2 deletions (Ta-
Fig 2. Deletions and rearrangements of CDKN2 in B-cell malignancy. (A) DNAs digested with EcoRI and probed with both MYC and CDKN2 exon 1 probes. (B) DNAs digested with BamHI and probed with CDKN2 exon 1. (C) DNAs digested with PstI and probed with CDKN2 exon 1. D, Daudi DNA. Lanes 6, 15, 16, and 17 refer to patient numbers in Table 3. Note 3 CDKN2 hybridizing fragments of differing intensity in Daudi in both BamHI and PstI digests. Note also biallelic CDKN2 deletions in patient no. 6. Cross-hybridizing bands of varying intensity were seen with the CDKN2 exon 1 probe despite stringent washing in 0.1 x SSC 0.1% sodium dodecyl sulfate; the significance of these bands is unknown.

Note that there is little homology between the 5' regions of CDKN2 and the related p15 gene that is also located at 9p21.27

ble 3). In contrast, 10 of the 11 cases analyzed that failed to grow in SCID mice retained germline CDKN2, whereas case no. 14, which showed biallelic CDKN2 rearrangement, also failed to grow. The differences between these groups are statistically significant (P = .001, Fisher's exact test).

Three cases of BCP-ALL with germline CDKN2 did grow in SCID mice; details of these cases are given in Table 3 as cases no. 15 through 17. Case no. 15 exhibited a t(9;15)(p21;ql5) translocation, but no rearrangement of CDKN2 on DNA blot (Fig 2). None of the 11 cases of TCP-ALL showed deletion or rearrangement of CDKN2.

Burkitt's lymphoma. A panel of 8 sporadic cases of Burkitt's lymphoma and 26 cell lines derived from both sporadic and endemic cases was examined. One of the eight fresh cases and 1 of 26 cell lines exhibited biallelic CDKN2 deletions. One of the Burkitt's cell lines (Daudi) exhibited three apparently rearranged fragments of differing intensity in multiple enzyme digests, without any loss of the germline signal (Fig 2). All EBV-transformed normal B-cell lines derived from patients with Burkitt's lymphoma failed to show any CDKN2 abnormality (Fig 4).

De novo B-cell leukemias with t(14;18)(q32.3;q21.3). An aggressive subset of B-cell leukemia has been recognized; these leukemias have concurrent activation of BCL2 and MYC oncogenes. Three cell lines (Karpas 231, Karpas 353,13 and KMH-2B29) and three fresh cases were analyzed. Karpas 231 was derived from one of the fresh cases. Of the other two fresh cases, one possessed both t(14;18)(q32.3;q21.3) and t(8;14)(q24.1;q32.3) and the other had t(8;9)(q24.1;p13.3).14 Only one cell line, Karpas 353, exhibited biallelic deletion of CDKN2. These data indicate that CDKN2 is not likely to be the target for 9p13.3 translocations in leukemias with t(8;9)(q24.1;p13.3).

NHL cell lines. Thirteen NHL, 10 B-NHL, and 3 T-NHL cell lines were examined (Table 2). Six cell lines, namely DoHH2, Karpas 1106, Granta 452 and 519, SUPT1, and
SKW3, exhibited biallelic CDKN2 deletions. Of these, three represented transformed NHL. Cell line DoHH2 was derived from a patient with immunoblastic transformation of follicular B-NHL. Similarly, Granta 452 was derived from a patient with B-cell acute leukemia supervening follicular B-NHL (G.S., M.J.S.D., and E.N., manuscript in preparation). In both these cases it was possible to show that these biallelic deletions had occurred in the original patient material from which the cell line was derived and was therefore not an artifact of prolonged in vitro culture (Fig 3). High-resolution cytogenetic analysis of cell lines DoHH2 and Karpas 1106 failed to detect rearrangement of 9p21. Cell line Granta 519 was derived from a patient with B-cell acute leukemia supervening mantle cell lymphoma; this cell line expresses the highest level of cyclin D1 in any of the six cell lines with t(11; 14)(q13; q32) examined in our laboratory and yet also exhibited biallelic CDKN2 deletions. However, this was the only case, of seven with t(11; 14)(q13; q32) examined, to exhibit any CDKN2 abnormality.

**Fresh cases of NHL.** A total of 38 cases of various histologic subtypes of NHL were examined; 7 cases were of T-cell lineage. No deletions were observed in 11 primary follicular B-NHL, 2 mantle cell B-NHL, or 16 cases of primary diffuse large-cell B-NHL. Biallelic rearrangement of CDKN2 with loss of all germline sequence was seen in one case of chemotherapy-resistant monocytoid B-NHL. Also, biallelic CDKN2 deletion was observed in an unusual and rapidly progressive B-NHL in leukemic phase expressing both CD5 and CD10 and exhibiting translocation t(8; 11)(q24.1; q13) with overexpression of cyclin D1 (D.J., D.G.O., and M.J.S.D., unpublished observations).

Of the 7 cases of T-NHL, two of six cases of T-cell lymphoblastic lymphoma exhibited biallelic CDKN2 deletion. One case was of immunoblastic transformation of Sézary syndrome associated with human T-lymphotropic virus type 1 (HTLV-1) infection in a man of Caribbean origin. CDKN2 was present in a peripheral blood sample taken at diagnosis, but absent from the lymph node of immunoblastic transformation, although analysis of the TCR β gene showed that the same T-cell clone was present in both samples (E. Matutes, M.J.S.D., et al, manuscript submitted). A similar loss of both alleles of CDKN2 was observed in immunoblastic transformation of follicular B-NHL (data not shown). However, two cases of B-PLL arising after B-CLL and one case of Richter’s syndrome retained germline CDKN2.

**Leukemias of mature lymphocytes.** A series of 119 fresh cases comprising 34 cases of B-CLL, 22 cases of splenic lymphoma with villous lymphocytes (SLVL) [4 with t(11; 14)(q13; q32)⁵], 6 cases of hairy cell leukemia (HCL), 5 cases of B-cell prolymphocytic leukemia (B-PLL), 46 cases of T-cell prolymphocytic leukemia (T-PLL), and 6 cases of leukemia of large granular lymphocytes were investigated. No abnormalities of CDKN2 were detected. Similarly, no abnormalities were detected in 3 myeloma cell lines, U-266, Karpas 620, and 1272.
Table 3. Characteristics of BCP-ALL With CDKN2 Abnormalities and Engraftment in SCID Mice

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/Sex</th>
<th>WCC (x10^9/L)</th>
<th>Cytogenetic Abnormalities</th>
<th>Outcome</th>
<th>SCID</th>
<th>CDKN2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) BCP-ALL with CDKN2 abnormalities</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4/F</td>
<td>10.6</td>
<td>+21</td>
<td>A: 2nd CR</td>
<td>+</td>
<td>D/D</td>
</tr>
<tr>
<td>2</td>
<td>5/F</td>
<td>17.9</td>
<td>No clone</td>
<td>A: 1st CR</td>
<td>NT</td>
<td>D/D</td>
</tr>
<tr>
<td>3</td>
<td>10/F</td>
<td>470</td>
<td>t(7;9)(p15;q13)</td>
<td>A: 1st CR</td>
<td>+</td>
<td>D/D</td>
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<td>4</td>
<td>15/F</td>
<td>183</td>
<td>No clone</td>
<td>D</td>
<td>NT</td>
<td>D/D</td>
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<tr>
<td>5</td>
<td>15/M</td>
<td>9.3</td>
<td>No clone</td>
<td>A: 1st CR</td>
<td>NT</td>
<td>D/D</td>
</tr>
<tr>
<td>6</td>
<td>16/F*</td>
<td>13.9</td>
<td>t(9;22)(q34;q11), add9p</td>
<td>D</td>
<td>+</td>
<td>D/D</td>
</tr>
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<td>7</td>
<td>19/F</td>
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<td>D/D</td>
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<td>8</td>
<td>29/M</td>
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<td>10</td>
<td>40/F*</td>
<td>96.1</td>
<td>t(9;22)(q34;q11)</td>
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<td>+</td>
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<td>D/D</td>
</tr>
<tr>
<td>12</td>
<td>UoCB1</td>
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<tr>
<td>(B) BCP-ALL with no CDKN2 abnormalities/ SCID engraftment</td>
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<tr>
<td>15</td>
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<td>85.5</td>
<td>t(9;15)(p21;q15)</td>
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<td>17</td>
<td>17/M</td>
<td>26</td>
<td>Hyperdiploid</td>
<td>D</td>
<td>+</td>
<td>G/G</td>
</tr>
<tr>
<td>18</td>
<td>UoCB4</td>
<td>Complex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: WCC, white cell count at presentation; A, alive; D, dead; CR, complete remission; +, engraftment in SCID mice; −, failure of engraftment in SCID mice; NT, not tested.

* CML in BCP-ALL blast crisis.

DISCUSSION

Deletion of segments of DNA ranging from single base-pairs to the megabase deletions visible cytogenetically are a common mechanism of human disease. In many hematologic malignancies, recurrent deletions of large chromosomal regions are frequently observed. The association of specific deletions with specific subsets of disease, eg, deletion of 5q in myeloid disorders, 13q in B-CLL, 9p in BCP-ALL, etc, suggests distinct pathologic mechanisms and consequences rather than a random loss of DNA. However, identification of the crucial genes is hampered by the large size of the deletions.

The mapping of the CDKN2 gene to 9p21 and the observation of frequent biallelic loss of this gene in a variety of malignant cell lines raised the possibility that CDKN2 is a tumor-suppressor gene, the loss of which is important in the development of several types of malignancy. However, preliminary studies on fresh tumor material suggested that the frequency of biallelic loss of CDKN2 was much lower than that in cell lines and that the high incidence of loss in cell lines represented adaptation to in vitro culture. 21

Because abnormalities of both 9p21 and the immediately centromeric chromosomal band 9p13.3 are frequent in both leukemia and lymphoma, we determined the frequency of structural alterations of the CDKN2 gene in a panel of 231 fresh malignancies and 66 lymphoid cell lines, with defined cytogenetic abnormalities, by DNA blot. We chose this method of analysis because PCR, although adequate for cell lines, can be confounded by contaminating normal cells in patient samples. A number of points emerged from this analysis. Firstly, overall the incidence of structural abnormalities of CDKN2 was low, being observed in 20 of 231 (8.6%) fresh cases and 10 of 66 (17%) cell lines. No CDKN2 deletions or rearrangements were observed in 119 cases of leukemia of mature T or B cells, including 46 cases of T-PLL, a highly aggressive T-cell malignancy associated with a median survival of only 7 months. 22 Second, the absence of CDKN2 abnormalities in EBV-transformed lymphoblastoid cell lines suggests that prolonged in vitro culture does not necessarily lead to CDKN2 abnormalities.

Abnormalities of CDKN2 were observed in two distinct subsets of lymphoid malignancy, both in a subset of BCP-ALL and also in some NHL of both T and B lineages. In NHL, CDKN2 abnormalities occurred in high-grade malignancies, including T-cell lymphoblastic NHL, Burkitt's lymphoma, and NHL, that had undergone progression from low- to high-grade disease in vivo. In two cases in which sequential samples were available, biallelic loss of CDKN2 was associated with immunoblastic transformation. In cell lines DoHH2 and Granta 452, which arose from immunoblastic and leukemic transformation of follicular B-NHL, respectively, biallelic deletion of CDKN2 was detected in patient material as well as the cell lines, indicating that deletions were not an artifact of in vitro culture. These data suggest that inactivation of CDKN2 may be a relatively "late" event in NHL, in contrast to other tumors, in which loss of material from 9p is thought to occur "early" in tumor development. 4

In BCP-ALL, biallelic deletions of CDKN2 were seen in 20% of cases. These cases did not have a common cyto-
Genetic abnormality and did not appear to be associated with a particularly poor prognosis, although this point requires confirmation from larger studies. Four of the 17 cases analyzed with t(9;22)(q34;q11) exhibited biallelic CDKN2 deletions; of these 4, 2 were lymphoid blast crisis of chronic myelogenous leukemia (CML). Whether loss of CDKN2 accompanied the acute transformation could not be determined.

Of the 30 cases with either biallelic deletion or rearrangement of CDKN2, 25 (83%) retained cytogenetically normal copies of chromosome 9p; CDKN2 deletions mostly occurred in the absence of detectable cytogenetic abnormalities of 9p. Only 1 case of BCP-ALL with interstitial deletion of 9p21-p22 was analyzed (Table 3, case no. 11), and this case exhibited biallelic CDKN2 deletion. However, the lack of CDKN2 abnormalities in cases with other abnormalities of 9p13/21, e.g., cell line K231 with t(8;9)(q24.1;p13.3) and case no. 15 in Table 3 with t(9;15)(p21;q15), may indicate the presence of other genes in this region of importance in the pathogenesis of lymphoid malignancies.

Seven of the 10 cases of BCP-ALL that established overt transplantable leukemia in SCID mice showed biallelic CDKN2 deletions, whereas none of the 11 cases that failed to engraft did so. The biologic significance of these observations is not known. Within cytogenetically defined subgroups of BCP-ALL, such as cases with t(4;11)(q21;q23), some cases will grow in SCID mice, whereas others will not. The molecular basis for this heterogeneity is not known. Whereas the growth of human tumor cells in SCID mice clearly represents the result of many complex interactions, the data presented here indicate that loss of CDKN2 expression may be an important (although perhaps not essential) parameter in this process. Whether the loss of CDKN2 expression is important for the growth of NHL in SCID mice has not been determined; however, both DoHH2 and Daudi cell lines, grown in SCID mice for the evaluation of novel therapeutic approaches, exhibit abnormalities of CDKN2.

Finally, rearrangements of CDKN2 were seen in two cases of BCP-ALL, in one Burkitt’s cell line (Daudi), and in one
case of monocytoid B-NHL. Because in all instances they occurred in the absence of cytogenetic change, it is likely that they represent smaller, internal deletions of chromosome 9p21, and might therefore indicate a "hot spot" for recombination in the vicinity of the CDKN2 gene. Molecular cloning of these rearrangements is being undertaken.

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