ANALYSIS OF GENE REARRANGEMENTS AND PROTEIN EXPRESSION OF THE TUMOUR SUPPRESSOR GENES, \textit{RB} and \textit{p16}, IN ACUTE MYELOID LEUKAEMIA: POSSIBLE ROLES IN LEUKAEMOGENESIS

by

A.RAHMAN A.JAMAL

A thesis submitted for the degree of Doctor of Philosophy at the University of London

supervised by
Professor David C.Linch
Department of Haematology
University College London Medical School
London

1995
ACKNOWLEDGEMENTS

I would like to mention my co-sponsors, the Universiti Kebangsaan Malaysia (National University of Malaysia) and the Public Services Department of Malaysia, for the scholarship award.

I am indebted to Professor David C. Linch for giving me the opportunity to undertake this Ph.D project and for his excellent supervision and moral support despite his hectic schedule.

I would also like to convey many special thanks to Dr.Rosemary E. Gale and Dr.Shaun Thomas for their wholehearted tutelage and for sharing their vast experience and scientific knowledge.

My appreciation as well to everyone in the department who have made my life as a scientist in Chenies Mews an unforgettable experience. Thank you for the top tips!

Last but not least, I am immensely grateful to my wife, Rashidah Karim, and my three daughters, Afifah, Fatin and Sara, for encouraging and motivating me through their expression of pride and appreciation, and for their seemingly limitless patience throughout my personal endevour. The gratitude I owe my wife is far too deep to be adequately expressed in words and I shall eternally be indebted to her.
ABSTRACT

Carcinogenesis is a multi-step process involving the activation of oncogenes and/or inactivation of tumour suppressor genes. One of these tumour suppressor genes is the human retinoblastoma susceptibility gene, \( RB1 \), which encodes for a 110 kilodaltons (kDa) nuclear phosphoprotein, pRB. The hypophosphorylated form of pRB inhibits the progression of a cell from the G\(_1\) to the S phase of the cell cycle. Phosphorylation of pRB is mediated by the cyclin dependent kinase-4 (CDK4) and the latter is inhibited by a 16kDa protein, the product of another tumour suppressor gene, \( p16 \). It has been shown that a negative feedback loop exists between pRB and p16 protein in cell lines. The roles of \( RB1 \) and \( p16 \) in the pathogenesis of acute myeloid leukaemia (AML) were investigated. Using Southern blotting analysis, none of the 106 patients analysed had gross rearrangements in the \( RB1 \) gene. Fourteen patients showed the presence of polymorphic bands. The protein analysis revealed that 31% of 86 cases studied had abnormal pRB expression. Although, AML patients with normal pRB showed a higher complete remission rate and a longer median survival compared to those with abnormal pRB, the differences were not statistically significant. Using quantitative Southern blotting analysis, none of the 70 samples analysed were found to have \( p16 \) gene deletions. No point mutations were found in 25 cases analysed using single strand conformational polymorphism technique. p16 protein expression was investigated by western blotting in 60 cases. Six (10%) were found to have a reduced expression. There was no methylation of the 5' CpG island found in these 6 cases. Another six cases (10%) had overexpression of the p16 protein and four of these had abnormal expression of pRB providing further evidence that the reciprocal relationship between p16 and pRB also exists in primary tumours. However this interaction does not seem to occur universally as in cell lines.
TABLE OF CONTENTS

Thesis title .................................................................................................. 1
Acknowledgements ................................................................................... 2
Abstract ...................................................................................................... 3
Table of contents ........................................................................................ 4
List of figures .............................................................................................. 9
List of tables ............................................................................................... 11

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Carcinogenesis ................................................................................ 12
1.2 Myelopoiesis and the molecular basis of leukaemia ...................... 13
1.3 Tumour suppressor genes ................................................................ 20
1.4 The human retinoblastoma susceptibility gene (RB1) ................... 22
   1.4.1 The RB protein (pRB) and cell cycle control .......................... 23
   1.4.2 The role of pRB in differentiation ...................................... 31
   1.4.3 The role of pRB in apoptosis ........................................... 32
   1.4.4 The role of RB1 in tumour formation and progression ...... 33
1.5 The role of cyclin dependent kinase inhibitors (CDKIs) in cell cycle regulation ........................................................................ 35
1.6 p16 and p15 genes ........................................................................... 37
   1.6.1 p16 protein function in the cell cycle ................................. 38
1.7 Oncogenic potential of G1 checkpoint control .............................. 40
1.8 Aims of study ................................................................................... 41

CHAPTER TWO

MATERIALS AND METHODS

2.1 Patients and normal controls .......................................................... 43
2.2 DNA extraction ............................................................................... 43
2.3 RNA extraction ............................................................................... 44
2.4 Preparation of protein lysates ......................................................... 45
2.5 Southern blotting analysis .............................................................. 45
2.6 SDS-PAGE and western blotting ................................................... 46
2.7 Immunoprecipitation............................................................... 48
2.8 Cell culture.........................................................................................49
2.9 Polymerase Chain Reaction........................................................... 50
2.10 Reverse Transcription-PCR............................................................ 51
2.11 Sequencing of PCR and RT-PCR products..................................... 51

CHAPTER THREE

ANALYSIS OF REARRANGEMENTS IN THE RB1 GENE IN ACUTE
MYELOID LEUKAEMIA PATIENTS

3.1 Introduction......................................................................................52
3.2 Materials and Methods.................................................................. 56
  3.2.1 AML patients and normal controls..................................... 56
  3.2.2 Southern blotting analysis and Polymorphisms............... 57
  3.2.3 Optimisation of conditions for RB1 Southerns............. 59
    3.2.2.1 Testing different RB1 cDNA fragments as
           probes and establishing the germline bands....... 59
    3.2.2.2 Determining the amount of genomic DNA
           to be used in digest.......................................... 60
    3.2.2.3 Establishing positive and negative controls...... 60
    3.2.2.4 Comparing 2 different membranes....................... 62
    3.2.2.5 [α-32P]dATP versus [α-32P]dCTP for
           labelling probes.............................................. 62
    3.2.2.6 Comparing 3 different hybridisation
           protocols................................................................ 62
  3.2.4 Testing the sensitivity of the Southern blotting
       technique.............................................................................. 64
  3.2.5 Southern blot analysis of AML patients.............................. 64
3.3 Results.............................................................................................. 64
  3.3.1 Germline configuration in normal controls..................... 64
  3.3.2 Polymorphisms in the RB1 gene........................................ 67
  3.3.3 Sensitivity of detection of bands by Southern blotting..... 69
  3.3.4 Southern blotting results of 106 AML patients.............. 69
  3.3.5 Further investigative analysis of the 9 samples with
       possible common mutation/polymorphisms at
       the 5' end of the RB1 gene............................................. 75
    3.3.5.1 PCR amplification of the promoter region........... 75
3.3.5.2 Southern blotting of samples using the RB1 promoter region as a probe ................................ 75
3.3.5.3 RT-PCR of exons 1-9 ............................................ 76
3.3.5.4 Southern blotting analysis using 3 different PCR probes ........................................ 76

3.4 Discussion ............................................................................................ 81

CHAPTER FOUR

ANALYSIS OF PROTEIN EXPRESSION OF THE RETINOBLASTOMA GENE IN PATIENTS WITH ACUTE MYELOID LEUKAEMIA

4.1 Introduction ..................................................................................... 89
4.2 Materials and Methods .................................................................. 92
  4.2.1 The western blotting technique ...................................................... 93
  4.2.2 Showing the different phosphorylation states of pRB during the cell cycle ........................................ 93
4.3 Results ............................................................................................ 93
  4.3.1 pRB expression in cell lines ......................................................... 93
  4.3.2 Protein loading and integrity in the AML samples ....................... 95
  4.3.3 pRB expression in normal controls and AML patients .......... 97
  4.3.4 Complete remission and survival data of AML patients .... 100
4.4 Discussion ....................................................................................... 100

CHAPTER FIVE

THE ANALYSIS OF DELETIONS AND REARRANGEMENTS IN THE p16 GENE USING MULTIPLEX POLYMERASE CHAIN REACTION AND QUANTITATIVE SOUTHERN BLOTting TECHNIQUE

5.1 Introduction ..................................................................................... 111
5.2 Materials and Methods .................................................................. 117
  5.2.1 Setting up the multiplex PCR ....................................................... 117
     5.2.1.1 PCR of exon 1 or 2 of the p16 gene .................................... 117
     5.2.1.2 Multiplex PCR of exon 1 or 2 with an internal control ........ 118
5.2.1.3 Multiplex PCR of both exons 1 and 2 with an internal control ....................................................... 120
5.2.1.4 Evaluating the contribution of non-leukaemic cells in the PCR analysis of leukaemic samples ................................................................. 122

5.2.2 Quantitative Southern blotting analysis ...................................................................................... 124
5.2.2.1 Introduction ......................................................................................................................... 124
5.2.2.2 Preparing the p16 gene probe ......................................................................................... 126
5.2.2.3 Southern blot analysis using different enzymes digests and the incorporation of an internal control ........................................................................ 126
5.2.2.4 Quantification of bands .................................................................................................. 127
5.2.2.5 Optimisation of quantitative Southern blotting analysis .................................................. 127
5.2.2.6 Testing the sensitivity of quantitative Southern blotting ................................................... 131
5.2.2.7 Quantitative Southern blotting analysis of 18 normal controls ........................................ 131
5.2.2.8 Quantitative Southern blotting analysis of 76 AML patients ............................................. 133

5.3 Results ........................................................................................................................................... 133
5.4 Discussion ...................................................................................................................................... 133

CHAPTER SIX

ANALYSIS OF THE p16 GENE FOR POINT MUTATIONS AND POLYMORPHISMS USING RT-PCR-SSCP TECHNIQUE

6.1 Introduction ..................................................................................................................................... 139
6.2 Materials and Methods .................................................................................................................. 142
6.2.1 RT-PCR ................................................................................................................................. 142
6.2.2 SSCP analysis .......................................................................................................................... 143
6.2.3 Sequencing of RT-PCR products ............................................................................................. 143
6.3 Results ............................................................................................................................................. 143
6.3.1 Results of SSCP analysis using 7 different RT-PCRs ............................................................. 143
6.3.2 Results of sequencing of samples showing at least two abnormal SSCP patterns ................... 146
6.4 Discussion ....................................................................................................................................... 146
CHAPTER SEVEN

THE STATUS OF p16 PROTEIN EXPRESSION IN AML PATIENTS AND THE CORRELATION BETWEEN pRB AND p16 DATA

7.1 Introduction ..................................................................................... 151

7.2 Materials and Methods .................................................................. 153

7.2.1 p16 western blotting ............................................................ 153

7.2.2 CDK4 western blotting ........................................................ 153

7.2.3 Analysis of p16 protein expression in different cell lines and during differentiation .................................................... 154

7.2.4 The specificity of p16 by western blotting .................. 154

7.2.4.1 Peptide inhibition experiment ...................................... 154

7.2.4.2 In-vitro translation of p16 cDNA followed by immunoprecipitation .............................................. 156

7.2.4.3 Analysing the methylation status of the 5' CpG island of p16 gene in samples with reduced p16 protein expression ................................................. 156

7.3 Results ............................................................................................. 157

7.3.1 p16 protein expression in AML patients ..................... 157

7.3.2 p16 protein expression in cell lines .................................... 157

7.3.3 pRB levels in AML patients with altered p16 expression ............................................................................. 159

7.3.4 CDK4 protein levels in AML patients with altered p16 expression ............................................................................. 159

7.3.5 Methylation status of the 5' CpG island in cases with reduced p16 expression .................................................... 163

7.4 Discussion ............................................................................... 163

CHAPTER EIGHT

CONCLUSIONS ......................................................................................... 168

REFERENCES ........................................................................................... 174

ABSTRACTS AND SUBMISSIONS ........................................................ 208
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>No.</th>
<th>Figure title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic diagram of myelopoiesis</td>
<td>14</td>
</tr>
<tr>
<td>1.2</td>
<td>Oncogenes known to be activated in leukaemia and their roles in signal transduction pathways</td>
<td>16</td>
</tr>
<tr>
<td>1.3</td>
<td>Two-hit mutation of the $RB1$ gene in retinoblastoma</td>
<td>24</td>
</tr>
<tr>
<td>1.4</td>
<td>Cell cycle progression and the phosphorylation of pRB by cyclins and cyclin dependent kinases</td>
<td>25</td>
</tr>
<tr>
<td>1.5</td>
<td>The binding of pRB to E2F represses the transcription of genes involved in cell proliferation</td>
<td>30</td>
</tr>
<tr>
<td>1.6</td>
<td>p16 inhibits CDK4/6 to prevent the phosphorylation of pRB and progression of cells from G$_1$ into the S phase</td>
<td>39</td>
</tr>
<tr>
<td>3.1</td>
<td>Genomic map of $RB1$ and restriction sites for $EcoRl$ and $HindWl$</td>
<td>53 &amp; 54</td>
</tr>
<tr>
<td>3.2</td>
<td>cDNA probes used for $RB1$ Southern blotting</td>
<td>61</td>
</tr>
<tr>
<td>3.3</td>
<td>Germline bands of $RB1$ in normal controls detected by Southern blotting analysis (5' probe and 3' probe)</td>
<td>65 &amp; 66</td>
</tr>
<tr>
<td>3.4</td>
<td>$BamHl$ polymorphism in the $RB1$ gene</td>
<td>68</td>
</tr>
<tr>
<td>3.5</td>
<td>Polymorphisms in the $RB1$ gene</td>
<td>70</td>
</tr>
<tr>
<td>3.6</td>
<td>Sensitivity of Southern blotting using $BamHl$ polymorphic site as marker</td>
<td>71</td>
</tr>
<tr>
<td>3.7</td>
<td>Representative Southern blots showing rearranged bands consistently detected in 5 AML patients using digestion with both $EcoRl$ and $HindWl$</td>
<td>72</td>
</tr>
<tr>
<td>3.8</td>
<td>Novel bands detected in single enzyme digest</td>
<td>74</td>
</tr>
<tr>
<td>3.9</td>
<td>PCR probes used in Southern blotting analysis of AML patients with the 'common' rearrangements</td>
<td>77</td>
</tr>
<tr>
<td>3.10</td>
<td>Southern blotting using the $RB1$ promoter as a probe</td>
<td>78</td>
</tr>
<tr>
<td>3.11</td>
<td>Southern blotting analysis using 4 different probes of 2 AML patients with common rearrangements</td>
<td>79 &amp; 80</td>
</tr>
<tr>
<td>4.1</td>
<td>RB protein structure showing phosphorylation sites and the binding domains</td>
<td>90</td>
</tr>
<tr>
<td>4.2A</td>
<td>pRB expression in Daudi and WERI-1 cell lines</td>
<td>94</td>
</tr>
<tr>
<td>4.2B</td>
<td>Different phosphorylation states of pRB in Daudi cells</td>
<td>94</td>
</tr>
<tr>
<td>4.3A</td>
<td>Actin western of AML samples showing multiple bands</td>
<td>96</td>
</tr>
<tr>
<td>4.3B</td>
<td>Evaluation of multiple actin bands for possible degradation</td>
<td>96</td>
</tr>
</tbody>
</table>
4.4 Spectrum of pRB expression in AML patients........................... 98
4.5 Western blot of 8 patients using Pab-2 anti-RB antibody...... 99
4.6 Kaplan-Meier survival curves of patients with normal
and abnormal pRB............................................................. 101
4.7 Possible role of RB1 mutation in leukaemia progression...... 108

5.1 Genomic organisation of p16 gene................................. 113
5.2A&B PCR of exon 1 or 2 of the p16 gene.............................. 119
5.3A&B Multiplex PCR of exon 1 or exon 2 using globin as
internal control.................................................................. 121
5.4 Multiplex PCR of exons 1 and 2 using globin as
an internal control............................................................ 123
5.5 Amplification of non-leukaemic cells in leukaemic samples.. 125
5.6 Germline p16 bands in 4 different enzymes using p16
and c-fms probes............................................................... 128
5.7 Quantitative Southern blotting: Optimisation of p16
and c-fms probes............................................................... 129
5.8 Southern blot and graph showing the sensitivity of p16
detection and linearity of signal intensity........................... 132
5.9 Representative Southern blot of 3 AML patients using
EcoRI digests and probed with p16 and c-fms probes........... 134

6.1 Basis of SSCP analysis technique................................. 141
6.2 RT-PCR-SSCP analysis of exon 2 p16 gene using
3 different gel running conditions....................................... 144
6.3A&B SSCP analysis of 16 AML patients showing variability
in the mobility shifts using 6 different RT-PCR reactions...... 147

7.1 Specificity of p16 western blotting............................... 155
7.2 Variable p16 expression in AML patients......................... 158
7.3 p16 expression in cell lines with and without
differentiating agents....................................................... 160
7.4 Absence of p16 expression in J6 and HL60 cell lines........ 161
7.5 CDK expression in 9 samples with abnormal
p16 expression.................................................................. 162
7.6 Southern blot showing methylation status of CpG island
in 6 cases with reduced p16 expression.............................. 164
7.7 Feedback loop between pRB and p16............................. 166
<table>
<thead>
<tr>
<th>Table no.</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>The different cyclins, their preferential CDK partners and site of action in the cell cycle</td>
<td>28</td>
</tr>
<tr>
<td>3.1</td>
<td>Frequency of <em>RB1</em> gene rearrangements in various primary solid tumours</td>
<td>55</td>
</tr>
<tr>
<td>3.2</td>
<td>Frequency of <em>RB1</em> gene rearrangements in AML</td>
<td>56</td>
</tr>
<tr>
<td>3.3</td>
<td>Breakdown of AML patients according their FAB types</td>
<td>57</td>
</tr>
<tr>
<td>3.4</td>
<td><em>RB1</em> Germline bands detected by Southern blotting</td>
<td>67</td>
</tr>
<tr>
<td>3.5</td>
<td>AML patients with their respective additional bands seen on different enzyme digests</td>
<td>73</td>
</tr>
<tr>
<td>4.1</td>
<td>Frequency of pRB inactivation in solid tumours</td>
<td>92</td>
</tr>
<tr>
<td>4.2</td>
<td>Studies of pRB expression in AML</td>
<td>92</td>
</tr>
<tr>
<td>4.3</td>
<td>Spectrum of abnormal protein expression in AML patients</td>
<td>97</td>
</tr>
<tr>
<td>4.4</td>
<td>Comparison of complete remission rate and median survival in patients with normal and abnormal pRB</td>
<td>100</td>
</tr>
<tr>
<td>4.5</td>
<td>Frequency of pRB abnormalities in AML cases according to their FAB types</td>
<td>105</td>
</tr>
<tr>
<td>5.1</td>
<td>Frequency of <em>p16</em> gene deletions in primary solid tumours</td>
<td>112</td>
</tr>
<tr>
<td>5.2</td>
<td><em>p16</em> gene deletions in haemopoietic malignancies</td>
<td>114</td>
</tr>
<tr>
<td>5.3</td>
<td>Predicted range of intensity of <em>p16</em> band in homozygous and heterozygous deletions</td>
<td>137</td>
</tr>
<tr>
<td>6.1</td>
<td>Results of SSCP analysis of exon 2-3 of the <em>p16</em> gene using 7 different RT-PCR reactions</td>
<td>145</td>
</tr>
<tr>
<td>7.1</td>
<td>Correlation between <em>p16</em> protein overexpression and pRB expression in 60 AML cases</td>
<td>159</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

1.1 Carcinogenesis

Genetic alterations play a central role in the development of cancer (Klein, 1987; Bishop, 1987) and tumour formation is believed to be a multi-step process (Knudson, 1971; Pitot et al., 1991) involving the accumulation of many genetic mutations, whereby critical molecular targets are deregulated culminating in the malignant transformation and the clonal expansion of a cell. Central to this theory are the roles of proto-oncogenes and tumour suppressor genes whose activation and inactivation respectively cause disruption of critical events in cell division, cell differentiation and cell death or apoptosis. The oncogenes and the tumour suppressor genes may well be likened to the accelerator and the brake pedal of a fast car cruising on a busy motorway. A stuck accelerator or failed brakes could lead to loss of control of the vehicle, a potential disaster for the occupants. Within the cellular context, this would mean continued and dysregulated cell proliferation leading to malignancy.

Any attempt to understand the process of carcinogenesis requires the identification of these genes and knowledge of their fundamental roles and behaviour both in health and disease. The advances in cytogenetics and molecular biology have allowed identification, detailed characterisation and analysis of many of these genes. Developments in protein biochemistry have also enabled the nature and cellular functions of the products of these genes to be elucidated. Within this framework, it is equally crucial to analyse the interaction between genes as well as protein-protein interaction with regard to their specific and accumulative contribution to tumour initiation and/or progression so as to enable us to understand further the biology of disease and to design novel approaches to cancer therapy in the future.

The multi-step model of carcinogenesis was put forward based on both cancer epidemiology data and experimental observations. There is an increase in cancer incidence with age, suggesting that multiple genetic alterations accumulate with time (Cook et al., 1969). Studies in transgenic mice have shown that overexpression of the oncogene c-myc alone does not cause the development of breast tumours until late in life. However when H-ras, another proto-oncogene, is co-expressed the mice develop tumours at a much earlier
age (Stewart et al., 1984), confirming the prediction that supplying multiple activated or unregulated oncogenes should shorten the lag period to initiate tumours.

The overall process of carcinogenesis can be divided into at least 3 phases: initiation, promotion and progression. The initiation phase is the result of an irreversible genetic alteration(s) in the DNA. It is believed that the reversible stage of tumour promotion does not involve structural changes in DNA, but rather the inappropriate expression of downstream genes mediated through promoter-receptor interactions. Finally, the irreversible phase of tumour progression is characterised by karyotypic instability and malignant growth of cells (Pitot, 1993).

1.2 Haemopoiesis and the molecular basis of leukaemia

Haemopoiesis is a process of self renewal and differentiation of pluripotential stem cells. It is believed that a common pluripotential stem cell gives rise to a series of progenitor cells for each of the main lineages. A representative schematic diagram of myelopoiesis is depicted in Figure 1.1 (Metcalf and Nicola, 1995). The clonal selection and malignant proliferation of a haemopoietic progenitor cell is the basic process which gives rise to leukaemia. For example, acute myeloid leukaemia (AML) probably arises from a single abnormal myeloid progenitor cell. This disease is characterised by (1) the accumulation of abnormal (leukaemic) blast cells, principally in the bone marrow, and (2) impaired production of normal blood cells. In line with the multi-step model proposed by Knudson, leukaemogenesis, which is considered to be a special type of carcinogenesis, is thought to be at least a two-step phenomenon (Knudson, 1973; Farber and Cameron, 1980). First, a critical mutation of a proto-oncogene in a single cell leads to clonal haemopoiesis, and progression to AML is accompanied by further genetic changes, perhaps involving one or more oncogenes or tumour suppressor genes, often reflected by chromosomal abnormalities or DNA rearrangements. The activation of oncogenes and/or inactivation of tumour suppressor genes is believed to endow the leukaemic cell clone with a proliferative advantage or inhibit its normal differentiation and subsequent death (Williams et al., 1995).

Structural aberrations in specific gene families have been shown to be consistently associated with specific phenotypes of leukaemia and a selection of these is discussed in the following paragraphs. Most of these
Figure 1.1: Schematic diagram of myelopoiesis
(Adapted from Metcalf and Nicola, 1995)

MULTIPOTENTIAL STEM CELLS

LYMPHOID STEM CELL

ERYTHROID

GRANULOCYTE-MACROPHAGE

EOSINOPHIL PROGENITOR

COMMITTED PROGENITOR CELLS

IMMATURE PROLIFERATING CELLS

Myeloblast

Monoblast

Promyelocyte

Myelocyte

Metamyelocyte

Neutrophil

Monocyte

MATURING POSTMITOTIC CELLS

Release to blood

MACROPHAGE

Exit to tissues

OSTEOCLAST

Marginating pools
leukaemogenic genes are normal genes which have become oncogenic as a result of a genetic change which can either be a point mutation, gene rearrangement, deletion, amplification or fusion to other genes giving rise to deregulation or abnormal expression. Some of these genetic changes are gross, hence they can be seen at the chromosomal level by cytogenetical analysis while others require detection by molecular methods such as Southern blotting. Yet a further subset of microscopic mutations will only be detected by sequencing the involved gene.

Many of the proto-oncogene products which are potentially leukaemogenic provide the vital elements in the signal transduction pathway (reviewed by Butturini and Gale, 1990; Cantley et al., 1991; Sawyers et al., 1991; Cline, 1994). A diagrammatic representation of some of the proto-oncogenes involved in signal transduction is shown in Figure 1.2. Some encode growth factors, like sis and hst which are genes for the platelet derived growth factor beta (PDGF-β) and the fibroblast growth factor respectively. Others like fms and erb-b1 encode for receptors. The binding of growth factors to their receptors triggers a cascade of events involving protein kinases some of which are encoded by oncogenes such as abl, pim, src, ras and ret. The ultimate event is the transduction of the signals to the nucleus where the genes controlling transcription, cell proliferation and cell differentiation reside, such as myc, myb, fos, and jun. Hence, within the signal transduction pathway itself each level of control may be disrupted which can be potentially disastrous for the cell.

The t(8;14)(q24;q32) translocation provides the paradigm for oncogene activation arising from a genetic event resulting in a specific leukaemia phenotype. This translocation which is characteristic of Burkitt’s leukaemia/lymphoma, brings into juxtaposition the myc proto-oncogene at 8q24 with the immunoglobulin heavy chain (IgH) locus at chromosome 14 (Dalla-Favera et al., 1982). The myc gene which normally functions as a transcription factor, is overexpressed as a result of this translocation event resulting in the excessive transcription of downstream target genes, leading to oncogenesis. Another well known example of translocation resulting in a phenotype-specific leukaemia is the t(9;22)(q34;q11) translocation present in about 95% of chronic myeloid leukaemia (CML) patients. This event translocates the abl proto-oncogene to a specific site at 22q11 known as the major breakpoint cluster region (M-BCR) (Heisterkampe et al., 1983, Shtivelman et al., 1985). The resultant chimeric gene, named bcr-abl, is
Figure 1.2: Oncogenes known to be activated in leukaemia and their roles in signal transduction pathways
(Adapted from Whittaker, 1993)
transcribed as a chimeric mRNA which in turn translates into a fusion protein p210 which has an inappropriately high level of tyrosine kinase activity as compared with the product of the normal abl gene (Lugo et al., 1990). Hence, the change in the protein expression of the leukaemogenic genes can provide a specific marker for analysing primary leukaemias.

Another unique leukaemogenic event is the t(15;17)(q22;q21) translocation which is found in almost all cases of acute promyelocytic leukaemia (APML) (de The et al., 1990). The gene at the breakpoint on chromosome 17 encodes the retinoic acid receptor α chain while the gene on chromosome 15 has been called PML, for promyelocytic leukaemia. One of the postulations for the resultant effect of the translocation is that dysfunctional PML proteins are produced leading to an arrest of differentiation of promyelocytes (Mu et al., 1994). Interestingly, in APML, treatment with all-trans retinoic acid results in remission in the majority of patients not only at the phenotypic level but also in the disappearance of the rearrangement at the gene level (Chomienne et al., 1989). The t(15;17) translocation provides further evidence for the specific causal relationship between genetic events and certain leukaemias and highlights the potential of harnessing this knowledge in the management of the patient.

Another proto-oncogene which has been reported to be mutated in certain leukaemias is the fms gene. This gene encodes the receptor for the macrophage and monocyte specific growth factor CSF1 (Sherr et al., 1985). The binding of CSF1 to the receptor activates its tyrosine kinase resulting in autophosphorylation of the receptor as well as phosphorylation of other proteins. This cascade of events ultimately triggers events which leads to mitogenesis. Point mutations and deletions of fms have been described in myelodysplasia and AML and in some cases overexpression of fms has also been reported (Ridge et al., 1990; Tobal et al., 1990).

The involvement of ras oncogenes in haemopoietic malignancies has also been extensively studied. These genes encode the G-proteins, a class of transcription factors involved in signal transduction. Mutations of the ras gene family (H, K and N-ras) result in abnormal protein products which have transforming activities in certain cells. In leukaemias, ras mutations have been found in up to 30% of AML cases, 40% of patients with myelodysplastic syndromes (MDS), 30% of multiple myeloma patients, 20% of ALL and has
also been reported in the acute phase of CML (Bos et al., 1987; Lyons et al., 1988; Neri et al., 1989; Janssen et al., 1987).

Genes which control for programmed cell death (apoptosis) also play a major part in haemopoietic malignancies. The \textit{bcl-2} gene is the prototype repressor of cell death (Hockenberry et al., 1990). The gene is characteristically involved in human follicular lymphoma through the translocation t(14:18), which juxtaposes the \textit{bcl-2} gene and the immunoglobulin heavy chain gene locus on chromosome 14 (Tsujimoto et al., 1985). This results in deregulated overexpression of \textit{bcl-2} mRNA and its protein. Studies in transgenic mice models have shown that the presence of this chimeric \textit{bcl-2-IgH} resulted in follicular hyperplasia in the lymphoid system (McDonnell et al., 1989; Korsmeyer, 1992). \textit{bcl-2} has the unique role of promoting cell survival by repressing apoptosis, rather than promoting cell proliferation.

Loss of tumour suppressor genes in leukaemia has also been widely reported. For example, a diverse variety of mutations of the \textit{p53} gene have been reported in many different leukaemias although none of the studies has been conclusive. The main evidence for \textit{p53} involvement in leukaemia is provided by CML where 30\% of cases in blast crisis has been reported to have \textit{p53} mutations (Ahuja et al., 1989), suggesting a role during progression of the disease. For AML, some groups have failed to detect \textit{p53} protein expression whilst others have found overexpression up to 10-100 fold greater than normal marrow cells (Prokocimer et al., 1986; Smith et al., 1986; Fenaux et al., 1992; Koeffler et al., 1986). Point mutations and deletions have also been described in many leukaemic cell lines. The role of another tumour suppressor gene, the retinoblastoma gene, in leukaemias will be discussed later.

There are also several constitutional abnormalities that predispose to leukaemia. These include Down's syndrome (Trisomy 21) and Klinefelter's syndrome, both of which are associated with an increased risk of AML. Patients who have DNA repair disorders or fragile chromosomes also have an increased susceptibility to acute leukaemia, such as Fanconi's anaemia, Bloom's syndrome and ataxia telangiectasia. The putative gene for ataxia telangiectasia has recently been cloned and sequenced (Savitsky et al., 1995).

Accumulative genetic events do not only contribute to the initiation of leukaemia. It is believed that further molecular lesions occur even after a cell
clone has been transformed or specifically during progression. Hence, a new clone of even more aggressive leukaemic cells may arise as a consequence of this sub-clonal selection. This could probably explain the progression of many leukaemias and lymphomas from a relatively indolent phase to a more aggressive phase. Examples include the acute blastic transformation in CML, for which the role of \( p53 \) was described earlier, and the evolution of chronic lymphocytic leukaemia into an aggressive lymphoma. It is crucial that the genetic events responsible for this change in tumour behaviour are defined so that novel leukaemia therapies may be designed to combat leukaemia progression.

The preceding simplistic review of the involvement of oncogenes and tumour suppressor genes in haemopoietic malignancies highlights the critical role of genetic events in leukaemogenesis. Some of these events are phenotype specific whilst others appear to occur in a diverse group of leukaemias. Analysis of these events can be carried out at the DNA, RNA or the protein level of the genes concerned. An important inference is the fact that these various oncogenes and tumour suppressor genes encode for products which are the major players in signal transduction, cell proliferation and cell death.

It must be borne in mind that the concept of accumulative genetic alterations in carcinogenesis as a whole, or leukaemogenesis specifically, makes it difficult to assign the exact role for each oncogene and tumour suppressor gene in leukaemia initiation or progression. For example, although mutation of the \( ras \) gene is the most commonly detected abnormality in AML (Bos et al., 1987; Farr et al., 1988), it may not be the initial fundamental abnormality in those 30% of cases since it may be present only in a subset of the leukaemic cells (Bashey et al., 1992). Furthermore, AML patients with N-ras mutations have no characteristic phenotypic features, clinical patterns, or response to therapy (Radich et al., 1990). Hence, unless it is possible to show a direct causal relationship between the alteration of an oncogene or a tumour suppressor gene with a particular leukaemia phenotype, assigning specific roles for these genes in leukaemia initiation or progression must be exercised with caution. It is possible that all of them are capable of playing both roles in a pleiotropic manner although some may show specificity for the development of specific leukaemia phenotypes.
1.3 Tumour suppressor genes

In the mid 1980s, several lines of evidence were put forward to support the idea that loss of genetic information is a critical step in tumorigenesis. These genes were postulated to function as negative regulators of cell proliferation and the loss of these genes resulted in removal of the existing brakes to cell growth, thereby endowing cells with a growth advantage.

Somatic cell genetics provided the first firm evidence indicating the importance of loss of genetic information from tumour cell genomes. It was shown that fusion of tumour cells with normal non-tumorigenic cells resulted in hybrids which were generally non-tumorigenic (Harris et al., 1969). This elegant experiment suggested that the normal partners in the fusion were supplying genes that reimposed normal growth control on the malignant cell. A more conclusive experiment was conducted by Weissman et al. (1987), where it was shown that the introduction of a normal chromosome 11 into a Wilm's tumor cell line resulted in the loss of tumorigenicity.

The second line of evidence pointing towards genetic loss during tumorigenesis derived from karyotypic analysis of specific human tumours. It was nearly 17 years ago that cytogeneticists observed that retinoblastoma tumour cells had interstitial deletions affecting the q14 band of chromosome 13 (Yunis and Ramsay, 1978). Similar analysis on Wilm's tumour cells revealed a specific loss of genetic material associated with the p13 band of chromosome 11 (Riccardi et al., 1980). These hereditary cancers have revealed a new class of gene that is important in the pathogenesis of cancer. These genes, which are clearly different from oncogenes, have been called tumour suppressor genes (TSG) or anti-oncogenes, because of their potential to induce cancer in a recessive mode i.e. one normal allele being sufficient to protect against a particular cancer. TSGs have been shown to be highly conserved genes that are thought to be involved in the regulation of normal cell growth. The genes are considered to function as housekeeping genes which are ubiquitously expressed. As opposed to oncogenes, loss of TSGs through deletions or mutations will result in loss of the gene function resulting in an increased risk of developing cancer (Marshall, 1991).

*p53* and *RB1* are the two best characterised tumour suppressor genes, their inactivation lead to deregulated cell proliferation and is a key factor in human tumorigenesis. Both are frequently inactivated in many naturally occurring
human malignancies including sarcomas, small cell lung carcinomas, oesophageal carcinomas and cervical carcinomas amongst others. The inactivation of both of these TSGs can be the result of mutations at the gene level and what is most fascinating is that both are also inactivated through being targeted by viral oncoproteins (Lane, 1992 and 1994; Bargonetti et al., 1991). Studies in transgenic mice using single or double knockout systems have also shown that these two tumour suppressors may be involved in the same cell cycle regulatory mechanism and that each of them may compensate for the loss of the other's tumour suppressor function, at least in vitro (Picksley and Lane, 1994; Williams et al., 1994, Harvey et al., 1995).

p53 is a sequence specific DNA-binding protein which functions as a transcription factor. It was first discovered by virtue of its association with SV40 T-antigen (Lane and Crawford, 1979), a viral oncoprotein which binds to pRB as well. p53 is also the commonest target for genetic alterations across all types of human cancer (Hollstein et al., 1991). Allelic losses or mutations of p53 gene occur either early, as in the Li-Fraumeni syndrome (Malkin et al., 1990), or late, as in colorectal cancer, during the process of carcinogenesis. It has been nicknamed the guardian of the genome by virtue of its property of blocking the division of cells that have sustained DNA damage and in some cases triggering cell death by apoptosis (Lane, 1992). The potent tumour suppressor properties of p53 are shown by the fact that introduction and expression of wild-type p53 protein inhibits the growth of tumour cells whatever their other molecular genetic changes may be (Baker et al., 1990).

A number of genes involved in the control of cell growth are now known to be transcribed in a p53-dependent manner. Following DNA damage, p53 protein expression is induced, it binds to specific DNA sequences and activates the expression of these target genes (Vogelstein and Kinzler et al., 1992). Two of the well characterised target genes include MDM2, an oncogene from murine double minute chromosomes (Fakharzadeh et al., 1991), and p21, which encodes for a recently discovered cyclin dependent kinase inhibitor protein. In fact, p21 has been suggested to mediate p53-induced growth arrest and apoptosis following DNA damage (El-Deiry et al., 1994; Dulic et al., 1994).

The characterisation of p53 and RB1, both in terms of their genetic configuration and biological activities, led to the inevitable convergence of cell cycle and cancer research. It is easy to understand that the disruption of cell cycle control is the result of the breakdown in the function of the molecules
which control the regulatory pathways of cell growth. It is thus crucial to define
the events in the cell cycle and elucidate the function of the genes which
control cell proliferation to understand fully the impact of deregulation in
disease states.

1.4 The human retinoblastoma susceptibility gene (RB1)

The human retinoblastoma susceptibility gene has become the paradigm for
the class of recessively acting tumour suppressor genes, whereby the
functional loss of both alleles is critical for tumour formation. It was identified
as the gene responsible for the childhood cancer retinoblastoma (Cavanee et
al., 1983; Godbout et al., 1983) as well as giving a predisposition to
osteosarcoma (Abramson et al., 1984; Draper et al., 1986; Hansen et al.,
1985). Retinoblastoma is a malignant tumour affecting the developing retina
usually recognised before the age of 5 years and has had a reasonably high
cure rate for much of this century. It affects between 1 in 15,000 and 1 in
34,000 live births. 30-40% of patients with retinoblastoma have a heritable
predisposition to the tumour as well as to a number of other cancers,
especially osteosarcoma.

The location of the retinoblastoma gene has been defined in three ways: (a)
the existence, in about 5% of patients, of cytologically visible deletions of
chromosome 13 with a minimum region of overlap in the q14 region (Yunis
and Ramsay, 1978), (b) genetic linkage analysis in affected families, between
the RB1 phenotype and a protein polymorphism of the enzyme esterase D
(ESD), the gene for which also lies in chromosome 13q14 (Sparkes et al.,
1980), (c) the development of loss of heterozygosity for loci on chromosome
13, again with a minimum region of overlap in the q14 region, in sporadic
tumours (Dryja et al., 1986). Based on these data, a search was made for a
gene expressed in fetal retinal cells but inactivated in retinoblastoma tumours,
culminating in the cloning of a candidate gene called 4.7R (Friend et al., 1986;
Fung et al., 1987; Lee et al., 1987a). Subsequent work by many groups has
authenticated this candidate gene, now called RB1. The genomic structure
and organisation of the RB1 gene will be discussed in chapter 3.

Two forms of retinoblastoma are distinguished on a genetic basis, the
hereditary form (comprising 30-40% of all cases) and the non-hereditary form.
The hereditary form is an autosomal dominant trait and 90% of carriers
eventually develop retinoblastoma. These individuals also have a high risk of
developing additional primary neoplasms later in life. In contrast, patients with non-hereditary retinoblastoma have no increased risk of second cancers. Because of this clear-cut heritability, retinoblastoma has been a prototype for the study of genetic determination in cancer. It also provided the best model for a tissue specific neoplasm arising as a consequence of deletion or mutation of both copies of the gene.

It is likely that all retinoblastoma tumours have both RB1 alleles mutated, as originally postulated by Knudson (1971) and proven experimentally later by Cavanee et al (1983). In fact, no retinoblastoma tumours or cell lines analysed to date have been shown to have an intact RB protein. The classic 'two-hit hypothesis' proposed by Knudson states that two mutational events are necessary for tumorigenesis not only for the hereditary cases but also for the non-hereditary cases (Figure 1.3). This hypothesis was based primarily upon statistical data indicating that individuals with the hereditary form of retinoblastoma developed tumours earlier than those with non-hereditary disease (Knudson, 1971). In the inherited form of the disease, one mutation is acquired from the parent and the other occurs during somatic development. In the sporadic form, both mutations occur in the same cell clone during somatic development. The two events were regarded as involving the same gene for both the hereditary and non-hereditary forms. Structural analysis of the RB1 locus in retinoblastoma and many types of adult tumours has shown that both copies of the wild type RB1 alleles are deleted in these tumours.

Once the retinoblastoma gene was defined, intense research was dedicated to characterise the function(s) of the RB protein.

1.4.1 The RB protein (pRB) and cell cycle control

In order to proliferate, a cell must traverse through the cell cycle (Figure 1.4). The cell cycle is made up of a series of processes controlled by an intricate network of extracellular factors and intracellular signalling pathways. These pathways in turn are regulated by both positive and negative growth signals from the environment. From the resting state (Go) a cell goes through four phases to complete the cycle: G1, a phase during which the cell prepares to synthesise DNA; S, where DNA synthesis takes place; G2, a period in which preparations are made for cell division; and eventually M phase where mitosis and subsequent cell division occurs. After completion of mitosis, cells can either enter a new cycle or return to a quiescent state. Cells that are not
FIGURE 1.3: Two-hit mutation of the RB1 gene in retinoblastoma

Germline mutation of an RB1 allele

Somatic mutation of 2nd RB1 allele

HEREDITARY RETINOBLASTOMA

No germline mutation

1st somatic mutation

2nd somatic mutation

NON-HEREDITARY RETINOBLASTOMA
Figure 1.4: Cell cycle progression and the phosphorylation of pRB by cyclins and cyclin dependent kinases.
proliferating may be either terminally differentiated, and thus unable to re-enter the cell cycle, or remain in G₀. To maintain a controlled proliferation rate and to coordinate the timing and order of cell cycle events, there are in-built mechanisms which function as checkpoints. These checkpoints are also critical for the fidelity in the transmission of genetic information. The restriction point, R, is a crucial control checkpoint in late G₁. Cells which pass through this point are committed to complete the cycle, even if growth factors are subsequently withdrawn (Pardee et al., 1974). One of the characteristics of a transformed cell is the partial or complete loss of this control checkpoint (Pardee et al., 1978). A key cellular protein which regulates progression through the G₁ phase of the cell cycle is the retinoblastoma protein (pRB). By sitting and functioning at a most crucial time window during which the cell makes most of its decisions about growth versus quiescence, pRB is likely to function as a regulator in cell proliferation (Weinberg, 1989).

The RB protein (pRB) was identified by antibodies which had been raised against three peptides based on the predicted amino acid sequence from the RB1 cDNA. These polyclonal antibodies immunoprecipitated a protein of about 110 kDa from normal cells. The pRB was localised primarily to the nucleus by cell fractionation and immunostaining analysis and later shown to be constitutively expressed in most cultured cells and normal tissues (Lee et al., 1987b; Bernards et al., 1989; Mancini et al., 1994). In populations of asynchronously proliferating cells, SDS-containing polyacrylamide gel electrophoresis and western blotting analysis detect pRB bands ranging in size from approximately 110 to 116 kilodaltons. This heterogeneity in the molecular sizes detected reflects the multiple phosphorylation states of the protein (Burke et al., 1992). Specifically, in G₀ or in early G₁, the hypophosphorylated form of pRB is found exclusively. In late G₁, pRB becomes highly phosphorylated and maintains this configuration throughout the remainder of the cell cycle, losing its multiple phosphates only upon emergence from mitosis via the actions of phosphoprotein phosphatases.

Several lines of evidence indicate that pRB plays a pivotal role as a growth suppressor. It has been shown through gene transfer experiments using malignant cell lines lacking in RB1 that some of the neoplastic properties are reverted by introduction of the RB1 gene either by retroviral infection (Huang et al., 1988), microinjection (Goodrich et al., 1991) or transfection (Qin et al., 1992). The common observation for all these experiments is that the tumour cells demonstrate a reduced growth rate which is attributable to G₁ arrest.
Clues as to how pRB carries out this inhibitory function stem from observations that pRB is phosphorylated in a cell cycle dependent manner (Chen et al., 1989; Mihara et al., 1989; De Caprio et al., 1989). This reversible phosphorylation event is believed to be the mechanism for affecting the inactivation of the growth inhibitory functions of pRB as suggested by three further experimental findings. Firstly, transforming viral oncoproteins such as the E1A proteins of adenovirus, the SV40 T antigen and the E7 protein of papillomavirus, bind only to the hypophosphorylated pRB to eliminate its function (Ludlow et al., 1989). The binding of pRB to this set of oncoproteins occurs at a specific domain called the 'pocket' region of the pRB. Two other members of the pRB family, p107 and p130, have similar pocket regions, form complexes with E1A (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993) and have been shown to have growth suppressive properties similar to pRB (Claudio et al., 1994). Secondly, hypophosphorylated pRB binds and seemingly controls several other cellular proteins (see below). In fact, no cellular protein is known to bind to the hyperphosphorylated form of pRB which is considered to be inactive. Thirdly, in vitro studies have shown that conditions that cause phosphorylation of pRB favour cell proliferation (Cobrinik et al., 1992).

More than a dozen potential phosphorylation sites are present within pRB either on serine or threonine residues, suggesting that either multiple kinases are involved in the phosphorylating event or a kinase working sequentially. It is now known that a kinase complex consists of a regulatory subunit called cyclin and a catalytic subunit called cyclin dependent kinase (CDK). To date at least 8 different cyclins and 7 different CDKs are known to exist (reviewed by Pines, 1995). Table 1 provides a list of the cyclins and CDKs as well as the preferential pairing and the observed site of action for each heterodimer in the cell cycle.

The first kinase demonstrated to phosphorylate pRB in vitro is cdc2, also called CDK1 (Lin et al., 1991). Many different CDKs have since been shown to phosphorylate pRB in vitro at the same sites as those phosphorylated in vivo. It is not clear though as to which kinase(s) phosphorylate pRB in vivo. One possible scenario is that there is a sequential wave of phosphorylation affected by different cyclin-CDK complexes as the cell traverses through the G1 phase of the cell cycle (Peters, 1994). The CDK4 and CDK6 however have been implicated as the major kinases in the phosphorylation of pRB, working in concert with the G1 cyclins, namely cyclins D1, D2 and D3 (Kato et al.,
1993; Ewen et al., 1993). Cyclin E-CDK2 also contributes to pRB phosphorylation possibly in late G1 as evidenced by observations that the levels of cyclin E mRNA and protein increase in mid-to-late G1 (Lew et al., 1991; Koff et al., 1991). Further evidence came from experiments using cell lines transfected with either cyclin D1, cyclin E or both. The ectopic expression of each cyclin independently causes pRB phosphorylation. Furthermore, expression of both cyclins shortens the G1 phase significantly greater than when only cyclin D1 is expressed (Resnitzky and Reed, 1995).

Table 1.1: The different cyclins, their preferential CDK partners and site of action in the cell cycle (Pines, 1995)

<table>
<thead>
<tr>
<th>Cyclin</th>
<th>CDK</th>
<th>Site of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CDK1, CDK2</td>
<td>S/G2</td>
</tr>
<tr>
<td>B</td>
<td>CDK1</td>
<td>Mitosis</td>
</tr>
<tr>
<td>C</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>D</td>
<td>CDK2, 4, 5, 6</td>
<td>G1</td>
</tr>
<tr>
<td>E</td>
<td>CDK2</td>
<td>G1/S</td>
</tr>
<tr>
<td>F</td>
<td>?</td>
<td>G2?</td>
</tr>
<tr>
<td>G</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>H</td>
<td>CDK7</td>
<td>All</td>
</tr>
</tbody>
</table>

The classical cyclins such as cyclins A and B undergo periodic accumulation and destruction in phase with the cell cycle, suggesting this as the mechanism for regulating the activities of various cyclin-CDK complexes. In contrast, in continuously proliferating cells the level of D-type cyclins remains less variable during the cell cycle. The presence of three types of cyclin D suggests that there is redundancy in their individual actions but studies have shown that the expression of each particular member is tissue specific (Inaba et al., 1992). For example, T-lymphocytes express predominantly cyclin D3 and to a lesser degree cyclin D2 but lack cyclin D1 (Sutherland et al., 1993; Tam et al., 1994).

Evidence pointing to cyclin D1 as the cyclin responsible for inducing the phosphorylation of pRB came from observation that the levels of cyclin D1 peak in mid-to-late G1, which approximates the time phase for phosphorylation of pRB (Motokura et al., 1991). Direct interaction of pRB with cyclin D1 has been shown in vitro (Dowdy et al., 1993) and experiments
using the insect cell system have demonstrated that cyclin D1-CDK4 complexes phosphorylate pRB (Kato et al., 1993). Cyclin D1 has a rapid turnover time with a half life of about 15-30 minutes and its steady state levels decline rapidly upon removal of extracellular mitogen (Matsushime et al., 1991). This suggests that there is a narrow window within the G₁ phase at the time of anticipation of pRB phosphorylation, whereby the cell increases the level of cyclin D1 until it reaches a certain threshold level. The D-type cyclins have also been shown to bind directly to pRB in vitro via a conserved LXXE motif, where X is any amino acid, and this is thought to account for the ability of D-cyclins to reverse the pRB-mediated growth arrest of SAOS-2 cells (Hinds et al., 1992; Ewen et al., 1993). The interaction between cyclin D1 and pRB has been further explored by analysing tumour cell lines lacking pRB. The level of cyclin D1 is dramatically reduced in these cell lines but upon transfection of a functional RB1 gene expression of cyclin D1 is induced (Muller et al., 1994). This observation suggests that pRB stimulates the expression of cyclin D1, a key component in the cell cycle itself, and that a regulatory loop exists between pRB and cyclin D1 (Bates et al., 1994).

Another mechanism for regulating the effector functions of pRB is through its modulation of gene transcription. Many cellular genes have been identified as targets of transcriptional regulation by pRB. These include c-fos, c-myc, transforming growth factor beta-1 (TGF-β1), and the neu genes (Chen et al., 1994). What is most fascinating is that pRB can both positively and negatively regulate the promoters of TGF-β1, c-fos, c-myc through a common motif called the retinoblastoma control element (RCE) (Kim et al., 1991).

Recent studies have also shown that pRB directly interacts with many transcription factors. The best characterised of these is E2F, which actually denotes a group of 5 distinct transcription factors, namely E2F1-5, which are all targeted to variants of the consensus nucleotide sequence TTTCGCGC (Nevins, 1992; La Thangue, 1994). The site is found in the promoter region of many genes important for cell proliferation such as c-myc, N-myc, B-myb, DNA polymerase-α, dihydrofolate reductase (DHFR), thymidine kinase, RB1 and the promoter of the E2F-1 gene itself. Complexes of pRB-E2F were initially detected in various cell extracts by several independent approaches (Chellapan et al., 1991; Chittenden et al., 1991; Bandara and La Thangue, 1991). The binding of hypophosphorylated pRB to E2F is believed to cause a repression in the transcription of these genes. Phosphorylation of pRB causes it to lose its grip on E2F hence allowing transcription to occur (Figure 1.5). Of
Figure 1.5: The binding of pRB to E2F represses the transcription of genes involved in cell proliferation.

Hypophosphorylated pRB

Hyperphosphorylated pRB

Mutated pRB
the five known E2Fs, only three, E2F-1, E2F-2, and E2F-3, are believed to be controlled directly by pRB whilst E2F-4 and E2F-5 are believed to be under the control of the pRB related proteins p107 and p130 (Ginsburg et al., 1994, Cobrinik et al., 1993; Dyson et al., 1993; Shirodkar et al., 1992).

So, if pRB is inactivated in a cell by its phosphorylation or rendered defective through deletion/mutation of the gene, the E2F transcription factors could be constantly turned on, causing derepression of the genes required for S-phase entry and eventually leading to deregulated cell growth. This could endow the cell with a proliferative advantage, contributing to a neoplastic state.

Apart from E2F, pRB also binds to other transcription factors. These include Elf-1, Myo-D, PU-1, ATF2 and c-abl. Some of these have roles in differentiation. Elf-1 and PU-1 belong to the ets family of transcription factors and they are specific for T- and B-lymphocytes respectively (Wang et al., 1993; Hagemeier et al., 1993). The transactivating potential of both of these factors is suppressed by pRB. By contrast, the transcriptional activation of ATF2 is enhanced by pRB (Kim et al., 1992). The most intriguing of these interactions is shown by the modulation of c-abl by pRB. c-abl is a tyrosine kinase with oncogenic potential and pRB is reported to bind directly to the active catalytic domain of c-abl, thus blocking its kinase activity which is activated upon phosphorylation of pRB (Welch and Wang, 1993).

The preceding discussion underscores the diversity of pRB functions as a positive and negative regulator of cellular effectors which regulate cell proliferation.

1.4.2 The role of pRB in differentiation

There is accumulating evidence that pRB also has an important role in cell differentiation. The most compelling evidence comes from RB1 gene knockout studies (Lee et al., 1992; Jacks et al., 1992; Clarke et al., 1992). These studies also suggested that pRB is not required for the function of many tissues. Homozygous RB1 deficient mice develop normally until day 12 of gestation. The apparently normal development of all tissue types up to this time clearly implies that much of normal differentiation does not require pRB. On day 13, when the homozygous RB1 negative embryos begin to show abnormalities, only two tissues, part of the central nervous system and the haemopoietic system, show macroscopic defects. The RB1 +/- mice dies with
a failure of erythropoietic and neuronal differentiation, manifesting increased mitotic index and cell death in these tissues. It is possible therefore that the target for pRB might be terminal differentiation of certain tissues as further suggested by observations that RB1 mRNA levels increases when erythroleukaemia, myoblast or embryonic carcinoma cells undergo differentiation (Coppola et al., 1990; Richon et al., 1992; Slack et al., 1993).

Other indirect evidence for the role of pRB in differentiation is provided by studies of the interaction of pRB with cellular proteins involved in differentiation. For example, muscle development involves the direct interaction of pRB with muscle-specific basic helix-loop-helix (bHLH) factors of the Myo-D family (Gu et al., 1993). It seems that the interaction of pRB and bHLH factors promotes the permanent withdrawal of muscle cells from the cell cycle and the activation of the myogenic differentiation pathway. A recent study by Kreider et al (1995) showed that another helix-loop-helix protein known as Id was able to inhibit the differentiation of a murine myeloid precursor cell line, 32DC13(G). It will be interesting to see whether bHLH, which interacts with pRB, can inhibit myeloid differentiation in human cells.

Hence, the role of pRB may not be solely related to the control of cell cycle progression but also the control of differentiation of specific tissues. In terms of tumour biology, the inactivation of pRB could potentially block one of the differentiation mechanisms in the malignant cell clone. For certain malignancies such as acute leukaemia where there is a maturational arrest, this blockade may be a key event in leukaemia progression.

1.4.3 The role of pRB in apoptosis

The preservation of tissue homeostasis and the prevention of carcinogenesis require the maintainence of an equilibrium between cellular proliferation and cell death or apoptosis. Apoptosis is a genetically controlled process, and amongst the genes important for the regulation of apoptosis are p53 (Lowe et al., 1993) and c-myc (Evan et al., 1992). It has also been shown in vitro that cellular proteins such as c-myc (Eilers et al., 1991) and E2F have dual functions as positive regulators of apoptosis and cellular proliferation. Interestingly, pRB binds to both these proteins and the resulting effect of this interaction is growth suppression. It is possible that pRB can also act as a negative regulator of apoptosis as well. Evidence for this was recently put forward by Hass-Kogan et al (1995) in a study analysing ionising radiation-
induced apoptosis occurring in SAOS-2 cells, which have defective pRB. It was shown that transfection of wild-type RB1 into these cells conferred a significant degree of protection against apoptosis when compared to untransfected cells. Since SAOS-2 cells lack p53 as well, this result also suggests that the ability of pRB to protect cells from apoptosis does not require wild-type p53 expression. Further evidence which suggests that pRB may inhibit apoptosis comes from studies of RB1-deficient mice. These mice exhibit widespread apoptosis not seen in normal mice (Clarke et al., 1992).

1.4.4 The role of RB1 in tumour formation and progression

It can be predicted from its biochemical and biological properties that inactivation or absence of pRB, either through deletions and mutations or via excessive phosphorylation, can predispose a cell to malignant transformation. A cell which has a defective pRB will not encounter the control normally imposed by pRB and hence will have a growth advantage, may fail to differentiate or undergo apoptosis and may continue to divide inappropriately which may eventually lead to malignant transformation. Likewise, a malignant cell which acquires RB1 mutation will have a further growth advantage, leading to a more progressive disease.

The first indication that the RB1 gene may contribute to cancers other than retinoblastoma was the development of secondary non-ocular malignancies, which include both sarcomas and gliomas, in individuals who have inherited a germline RB1 mutation (Abramson et al., 1984; Meadows et al., 1985). Osteosarcoma, the most common second malignancy, occurs at a several hundred-fold increased incidence in retinoblastoma survivors (Draper et al., 1986).

The first study of RB1 changes in tumours not usually associated with the development of retinoblastoma was that of small cell lung cancer (SCLC). Structural changes in the RB1 gene were seen in a primary SCLC and four SCLC cell lines as well as a pulmonary carcinoid cell line. Moreover, the absence of RB1 transcripts was noted in 60% of the SCLC lines as well as a pulmonary carcinoid cell lines (Harbour et al., 1988).

Further direct evidence for the oncogenic properties of pRB is provided by gene knockout studies. Homozygous RB1 -/- mice die at 12-16 days. However, heterozygous RB1 +/- mice have been successfully bred (Clarke et
al., 1992; Jacks et al., 1992; Lee et al., 1992) and they develop pituitary tumours with almost 100% penetrance. Although it is not retinoblastoma which develops in these mice, the resultant evolution of a tumour is consistent with the proposed role of \( RB1 \) as a tumour suppressor gene.

Inactivation of pRB has been shown to be a key factor in the initiation and/or progression of several other common human malignancies including small cell lung carcinoma (Harbour et al., 1988; Horowitz et al., 1990), non-small cell lung carcinoma (Reissmann et al., 1990; Xu et al., 1991a), breast carcinoma (Lee et al., 1988; T’Ang et al., 1988), bladder carcinoma (Horowitz et al., 1989), osteogenic sarcomas (Toguchida et al., 1988), sarcomas (Cance et al., 1990), prostate carcinoma (Bookstein et al., 1990), renal cell carcinoma (Ishikawa et al., 1991), oesophageal carcinoma (Boynton et al., 1991) and parathyroid carcinoma (Cryns et al., 1994).

The broad range of malignancies in which pRB inactivation occurs suggests a pleiotropic role rather than a specific one. It is important to remember that most of these malignancies develop in individuals without a history of retinoblastoma. Why hereditary mutations in \( RB1 \) predispose so strongly to retinoblastoma and not to other tumour types is unclear. The key issue is whether loss of \( RB1 \) gene function is related to initiation of the tumour or with progression of disease. Assigning the exact role of \( RB1 \) in the genesis of these tumours is not easy. It is probably adequate to say that \( RB1 \) mutations contributes to the accumulative genetic events leading to cancer. The fact that these mutations are only seen in a subset of these malignancies suggests that pRB inactivation is not the rate limiting step in the development of these cancers nor is it the initiating event (Benedict et al., 1990).

The availability of purified high-affinity polyclonal antibodies has provided a more sensitive method for analysing inactivation of pRB. It has also enabled researchers to examine the status of pRB at the single cell level in each type of malignancy using techniques such as immunostaining and flowcytometry. This has allowed the screening of subpopulations of tumour cells with different pRB expression. For example, histochemical staining of soft tissue sarcomas showed that there was a sub-population of tumour cells with normal pRB and another with non-detectable pRB (Cance et al., 1990). This heterogeneity in pRB expression could be explained by a further genetic alteration occurring in a sub-clone of tumour cells, allowing this particular sub-clone a further growth advantage.
It has been shown in high grade human sarcomas that the absence of pRB predicts for a poorer prognosis both in therapeutic response and survival rates (Cance et al., 1990). In other studies, altered pRB expression is more commonly associated with the invasive phenotype of bladder cancer (Presti et al., 1991; Cairns et al., 1991) and non-small cell lung cancer (Xu et al., 1991). The above observations indicate that in tumours other than retinoblastoma, RB1 deletions are likely to be involved in tumour progression.

1.5 The role of cyclin dependent kinase inhibitors (CKIs) in cell cycle regulation

The activity of CDKs is controlled both by their association with cyclins and the complex phosphorylation and dephosphorylation of the CDK polypeptide itself, hence providing another tier of control (Norbury and Nurse, 1992).

The fact that the cyclin D-CDK complex mediates the phosphorylation of pRB predicts that the prevention of this phosphorylation event can be executed by mechanisms which either impinge on the pRB itself or through inhibition of CDK activity. Studies to date suggest that the latter mechanism seems to be more likely. The identification of CDK inhibitors (CKIs) over the last two years has introduced another level of regulation within an already complex network. These inhibitory proteins either associate with the cyclin-CDK complex or bind to the CDK subunit alone resulting in the impediment of its kinase activity, hence providing the cell with another set of brakes in the cell cycle. Some CKIs are modulated in response to extracellular signals such as TGF-β, cyclic AMP (cAMP) and contact inhibition, others appear to function in intrinsic steps of the cell cycle such as mediating radiation induced growth arrest and apoptosis, while a few others function as tumour suppressors.

p21 and p16 were the first CKIs found in the mammalian system. The discovery of these proteins made an outstanding impact on cell cycle research due to their links with the tumour suppressor genes p53 and RB1. The emergence of p21 and p16 was followed by the discovery of other cell cycle inhibitory proteins. They can be loosely grouped into: (a) the p15/p16/p18/p19 group which specifically inhibit cyclin D-CDK4 and cyclin D-CDK6 complexes, and (b) the p21/p27/p57 group which interact with all the known cyclin-CDK complexes.
p21 is the best characterised of the CKIs. It was discovered in several laboratories using different approaches which explained the variety of names by which it is known. Harper et al (1993) identified a 21kDa protein which binds to cyclin-CDK2 complexes in vitro and called it Cip1, for CDK interacting protein. The gene was also cloned as a p53-regulated transcript by El-Deiry et al (1993) and designated as WAF1. p21 was also identified because expression of its gene (called SDI1 in this case) is increased in senescent cells (Noda et al., 1994). The physiological role of p21 was inferred from studies involving mutant cells defective in p53 which fail to transcribe the p21 gene (El-Deiry et al., 1993; Dulic et al., 1994). It now appears that DNA damage causes activation of p53, which in turn transcriptionally activates the expression of p21, leading to transient cell cycle arrest by inhibiting CDKs (El-Deiry et al., 1994). More recently, p21 was shown to bind to proliferating cell nuclear antigen (PCNA) via its C-terminal portion and inhibits PCNA-dependent DNA replication suggesting that p21 can inhibit cell cycle proliferation by two independent mechanisms (Luo et al., 1995).

p27, also called Kip1, has a significant sequence homology to p21. It was noted to be elevated during contact inhibition of cells and following treatment of cells with TGF-β (Polyak et al., 1994). It is postulated that p27 binds to cyclin E-CDK2 and cyclin D-CDK4 complexes and inhibits the kinase activities in vitro in a stoichiometric manner (Polyak et al., 1994). It also inhibits CDK2, CDK4 and CDK6 by preventing their phosphorylation by CAK (CDK-activating kinase) (Polyak et al., 1994, Toyoshima and Hunter, 1994). Overexpression of p27 has been shown to arrest cells in G1 (Toyoshima and Hunter, 1994).

p57, also known as Kip2, is another p21 and p27 homologue although with a more restricted tissue expression pattern (Lee et al., 1995). The current interest in p57 is the fact that the gene is localised on chromosome 11p15.5 which is a region associated with both sporadic cancer and the Beckwith-Weidemann Syndrome, a genetic condition with characteristic dysmorphic features and the propensity to develop cancers. These observations implicate p57 as a tumour suppressor (Matsuoka et al., 1995).

Recently, several studies have shown that CKIs may provide the brakes necessary for withdrawal from the cell cycle as cells develop. p21 has been shown to be expressed as muscle cells stop dividing and differentiate (Guo et al., 1995). Induction of p21 has also been shown to occur during
differentiation of HL60 cells (Jiang et al., 1994) and hepatoma cells (Steinman et al., 1994).

1.6  **p16 and p15 genes**

Although p21 is the best characterised of the CKIs, it was p16 however which grabbed the major headlines initially when researchers claimed that it was a tumour suppressor with a role that could equal or even surpass that of p53 (Marx, 1994; Kamb et al., 1994).

The **p16** gene (also known as **INK4, MTS1, or CDKN2**) is localised on chromosome 9p21. It has been known for some time that 9p21 is a frequent site of allelic loss in a wide variety of tumours and cell lines (reviewed in chapter 5). These observations have led to the hypothesis that 9p21 contains a tumour suppressor (or suppressors) which may have a role in the pathogenesis of many tumour types. Xiong et al (1992) noted a 16kDa protein which co-immunoprecipitated with CDK4 in some transformed cells. They also noted that the complexes lacked a cyclin and PCNA. Serrano et al (1994) later showed p16 to be a potent inhibitor of cyclin D-CDK4 kinase.

The gene itself was isolated independently during positional cloning of genes in a region of human chromosome 9p21, a region which is frequently deleted in melanomas and gliomas (Holland et al., 1994; Olapade et al., 1992). Through an analysis of deletions and rearrangements present in these tumour cell lines, a new putative tumour suppressor in 9p21, the **p16** gene was identified (Kamb et al., 1994). A homologous gene, now known as **p15**, was also mapped about 25kb centromeric to **p16**. The genomic organisation of **p16** and **p15** will be discussed in chapter 5. **p15** could also possibly be another tumour suppressor gene as studies on tumour cell lines and primary tumours have shown deletions of **p15** in a subset of them (Kamb et al., 1994; Okamoto et al., 1995).

The status of p16 as a major tumour suppressor is still being disputed. Despite this current controversy, its role in controlling cell growth via CDK inhibition is unchallenged, and some primary tumours indeed have nonsense mutations and deletions within the **p16** gene. Its contribution to tumorigenesis, however, may not be as widespread as originally anticipated.
Recently, another level for regulating p16 protein function in human cancers was unravelled. Merlo et al (1995) reported that methylation of the CpG island within exon 1 can result in the transcriptional silencing of the p16 gene and they showed this to be present in 20% of different primary neoplasms.

1.6.1 p16 function in the cell cycle

p16 was shown initially to inhibit specifically CDK4 (Serrano et al., 1994). It achieves this by competing with cyclin D1 for CDK4 binding sites, hence preventing the kinase activity required for the phosphorylation of pRB. This cascade of events will potentially cause a cell cycle arrest in G1 (Figure 1.6). Studies in cell lines have confirmed these predictions (Koh et al., 1995). Despite this, there are still questions on how p16 is itself regulated. Tam et al (1994) have shown that p16 expression peaks during the S phase which suggests that it is probably required only at the G1-S transition at the time when CDK4 kinase activity is no longer necessary.

This model also predicts that deletions or inactivation of p16 would result in deregulated kinase activity which could potentially lead to persistent phosphorylation of pRB and carcinogenesis. On the other hand overexpression of p16 would instead inhibit cell growth.

The intimate link between pRB and p16 invites one to speculate on the changes in p16 expression in cells where pRB is inactivated. This scenario will inevitably lead to other questions relating to changes in the modulation of CDK4 and cyclin D-CDK complexes. The prediction is that there will be no cyclin D-CDK complexes present and, logically, p16 would probably be overexpressed to try and exert some inhibition on the uncontrolled rate of cell proliferation. Some of this issues will be addressed further in the later chapters.

Most of the initial studies to answer some of the questions above, have been carried out using cell lines. For example, it has been shown that in cells lacking pRB, no cyclin D-CDK complexes are present (Serrano et al., 1994). It has also been reported that there is a reciprocal relationship between p16 and pRB expression in cell lines (Parry et al., 1995). The big question here is how far do studies with cell lines correlate with what actually happens in primary cells. Cell lines by definition are cells which have been immortalised and are capable of continuous propagation in culture. By virtue of this, they are prone
Figure 1.6: p16 inhibits CDK4/CDK6 to prevent the phosphorylation of pRB and progression of cells from G1 into the S phase of the cell cycle.
to secondary changes in culture, both in terms of mutations and cell behaviour. Although it is an ideal and mechanistic approach to analyse genetic changes as well as protein-protein interaction, the data obtained do not always reflect accurately events in vivo. The gold standard for unravelling deregulated molecular events in a transformed cell is through the analysis of gene and protein expression in primary tumours.

1.7 Oncogenic potential of G₁ checkpoint control

An intricate balance is maintained between p16, D cyclins, CDK and pRB in a normal cycling cell and these components are targets for deregulation. Disruption of any of these components would be potentially oncogenic. The status of pRB as a tumour suppressor gene has been established and will be further explored in the following chapters. The next obvious prediction would be that deregulated expression of the other three components could be potentially oncogenic as well.

The most compelling data so far pertain to D cyclins where amplification and overexpression of the protein has been reported in several cell lines and primary tumours. The cyclin D1 gene was first identified as the PRAD1 gene juxtaposed to the parathyroid gene by inversion in parathyroid adenomas (Motokura et al., 1991). Translocation of IgH gene to the cyclin D1 gene has been reported in certain B-cell lymphomas (de Boer et al., 1993). These observations suggest that cyclin D1 gene is the target gene which is transcriptionally activated in these rearrangements. However, the most prevalent disruption at the cyclin D1 locus is DNA amplification, which occurs in breast carcinomas (15%), hepatocellular carcinomas and squamous cell carcinomas (50%) (Keyomarsi et al., 1993; Zhang et al., 1993; Callender et al., 1994). Amplification of the putative cyclin D1 oncogene is accompanied by overexpression of its mRNA and protein (Lammie et al., 1991; Gillett et al., 1994; Tsuruta et al., 1994). Furthermore, mice carrying a cyclin D1 transgene driven by the promoter of the mouse mammary tumour virus develop mammary hyperplasia and adenocarcinoma (Wang et al., 1994). When cyclin D1 overexpression is targeted to the lymphoid system and coupled with c-myc overexpression, the transgenic mice develop lymphomas (Bodrug et al., 1994; Lovec et al., 1994). It is probable that overexpression of cyclin D1 results in deregulation of the G₁ checkpoint through excessive phosphorylation of pRB. The actual mechanism of oncogenicity of cyclin D1 however, may not be that simple.
Amplification of CDK4 has been reported to occur in human malignancies, notably in sarcomas and gliomas (Khatib et al., 1993; He et al., 1994; Schmidt et al., 1994). Interestingly, He et al observed that the gliomas with CDK4 amplification had intact p16 suggesting another mechanism for dampening the actions of p16.

Loss of function of the CDK inhibitors would be expected to cause increased kinase activity and to promote cell cycle progression, hence they are attractive candidates for tumour suppressor genes. p16 has so far been reported to be frequently deleted in many tumour cells lines. Studies on primary tumours are beginning to suggest that its importance may be limited to a subset of these malignancies. This will be further discussed in chapter 5. The intriguing paradox observed between p16 and pRB provides an obvious challenge to analyse their status in primary tumours and investigate the modulatory changes which occur between them in a malignant setting.

In summary, the various oncogenic events discussed above all seem to converge towards an identical end point which is the deprivation of the services of pRB at the G₁ checkpoint control, either through its functional inactivation (as a result of sequestration or deregulated phosphorylation) or genetic inactivation (due to rearrangements or point mutations). It is probable that deregulation of only one of the components in the G₁ checkpoint, is sufficient to tilt the balance towards neoplasia.

1.8 Aims of study

The main aim of studies presented in this thesis was to define the role of two tumour suppressor genes, RB1 and p16, in AML. Analysis at the level of DNA, RNA and protein expression was carried out where feasible. A summary of the specific aims are listed below:-

i. to analyse possible rearrangements of the RB1 gene in AML using a large series of patients and normal controls.

ii. to analyse the expression of pRB in AML by Western Blotting and to correlate the findings with analysis at the DNA level.
iii. to ascertain whether inactivation of pRB has any influence on the outcome of disease in AML.

iv. to analyse AML samples for homozygous deletions and rearrangements of the p16 gene.

v. to analyse p16 protein expression in AML patients and to look for correlation between the inactivation of pRB and the function of p16 in these patients.

vi. to analyse the levels of CDK4 in samples from patients with abnormal pRB and abnormal p16 to see whether these levels correlate with the currently accepted functions and interplay of these proteins.

vii. to provide a model based on the experimental findings for leukaemogenesis or leukaemia progression of AML.
CHAPTER TWO

MATERIALS AND METHODS

2.1 PATIENTS AND NORMAL CONTROLS

A total of 106 AML patients were included in the RB1 gene study. For the pRB study, 103 samples were analysed. Majority of these patients were part of the UK MRC AML trial 10 and 11 while others were patients treated at the University College Hospital, London. The samples used for DNA and protein analyses do not entirely overlap because of non-availability of protein samples for some of the patients and protein degradation in others. The patients were aged between 16-70 years old. Only one patient was in relapse while the others were at presentation. The diagnosis of AML was based on morphological and cytochemical criteria of bone marrow aspirates and peripheral blood smears according to the FAB classification. Eighteen haematologically normal individuals were included as normal controls for the Southern blot Analysis. Nine of these were used as controls for the western blotting analysis. These controls were mothers attending the antenatal clinic follow-up and laboratory staff members.

2.2 DNA EXTRACTION

Reagents
Ficoll-Hypaque (Nycomed, Norway), Phosphate-Buffer Saline (Sigma, UK), Proteinase K (Sigma), Phenol (Rathburn Chemicals Ltd., Scotland), Chloroform (BDH, UK), Tris-EDTA, pH 7.4, (BDH) and 100% Ethanol (BDH).

Method
Mononuclear cells from either peripheral blood or bone marrow were isolated by Ficoll-Hypaque centrifugation according to the manufacturers instructions (Nycomed, Norway). High molecular weight DNA was prepared by proteinase K/detergent digestion, phenol-chloroform extraction and ethanol precipitation (Sambrook et al., 1989). Purity and concentration of DNA were measured by spectrophotometry at 260nm and 280nm ($A_{260} = 50\mu g/ml$ DNA).
2.3 RNA EXTRACTION

Reagents

GTC (250ml)
Guanidium thiocyanate (118g), 0.75M Na-citrate [pH 7] (8.35ml), 20% Na-lauryl-sarcosine (6.25ml) and DEPC-treated water to a final volume of 250ml. This was then filtered through 0.45μm acrodisc filters (Sigma, UK) and finally 1.8ml of β-mercaptoethanol was added prior to use.

CsCl₂ (250ml)
Caesium chloride (240g), 0.5M EDTA, [pH 7] (5ml) and DEPC-treated water to a final volume of 250ml. This was dissolved at 37°C overnight and then autoclaved. The final density was 1.69g/ml.

Na-Acetate (for precipitation)
2M Na-Acetate, [pH 8 and autoclaved] (1.5ml), 10% (w/v) Sodium dodecyl sulphate (0.1ml) and DEPC-treated water to a final volume of 10ml.

Method
For the RNA extraction, 50 x 10⁸ mononuclear cells were lysed in 3ml of GTC (Fluka, Switzerland). The DNA was sheared by passing the GTC-cell lysate several times through a syringe with a 23G needle. Five millilitres of CsCl₂ was pipetted into each ultracentrifuge tube (Beckman, UK). The GTC-cell lysate was then layered onto the CsCl₂ and ultracentrifuged at 27000 rpm for 22 hours at 20°C. The supernatants were drained and the bottom of each ultracentrifuge tube containing the RNA pellet was sliced off using a hot scalpel blade. 200μl of NaAc/ethanol was added to the vitreous pellet and left for a few minutes to precipitate. The precipitate was transferred into 1.5ml eppendorf tubes containing 0.9ml NaAc/ethanol. This was left to precipitate further overnight at 4°C. The samples were spun in a micro-centrifuge for 20 mins and the supernatants drained. A final wash with 70% ethanol was performed followed by a final spin for 20 mins. The RNA pellet was dissolved in 50-200μl of DEPC-treated water. The optical density of each sample was read at 260nm and 280nm. An OD₂₆₀ of 1 is equivalent to a RNA concentration of 40μg/ml. The RNA samples were stored at -80°C until used.
2.4 PREPARATION OF PROTEIN LYSATES

Mononuclear cells were isolated from either peripheral blood or bone marrow by the same method described above. After washing the cells in PBS, crude protein lysates were prepared by lysing 10 x 10^7 cells in 500μl of 250mM Tris-HCl (pH 6.8), 0.15M NaCl, 4% (w/v) Sodium Dodecyl Sulphate (SDS), 5mM dithiothreitol (DTT) and 0.05% (w/v) bromophenol blue. The lysates were boiled for 10 minutes and then stored at -20°C until used.

2.5 SOUTHERN BLOTTING ANALYSIS

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction Enzymes</td>
<td>Boehringer Mannheim, Germany</td>
</tr>
<tr>
<td>Hybond-N+ membranes</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>[α-32P]dCTP or [α-32P]dATP</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>Inverse field gel electrophoresis system</td>
<td>Bio-Rad Laboratories Inc., CA</td>
</tr>
<tr>
<td>Salmon sperm DNA</td>
<td>Sigma, UK</td>
</tr>
</tbody>
</table>

20x SSPE (175.3g NaCl, 27.6g NaH₂PO₄·H₂O and 7.4g EDTA per litre of solution)

100x Denhardt's reagent

(1g Ficoll, 1g Bovine Serum Albumin Fraction V and 1g Polyvinyl-pyrolidone, dissolved in 50ml of distilled water, then filtered through a 0.45μm acrodisc filter. Stored at -20°C till used)

10x TAE (242g Tris, 57.1ml glacial acetic acid, 100ml of 0.5M EDTA [pH 8.0] per litre of solution)

Each patient sample containing high molecular weight DNA (5-15μg) was digested overnight with 20U of restriction enzyme at 37°C. The DNA samples were then separated through a 0.6% agarose gel in 1x Tris-acetate-EDTA buffer (TAE) using an inverse field gel electrophoresis system (Bio-Rad Laboratories Inc., CA) over 5 hours. The DNA was then blotted onto Hybond N+ filters (Amersham, UK) using the manufacturer's alkaline blotting method. This method will denature the DNA into single strands during the transfer. The other advantage of this method is that no fixation step is required. The filters were prehybridised in 5x SSPE, 5x Denhardt's, 0.1% SDS and 100μg/ml salmon sperm DNA for a minimum of 4 hours at 65°C. Hybridisation was
carried out overnight at 65°C in the same solution containing a labelled probe. Both prehybridisation and hybridisation were carried out in a shaking water-bath.

The probes were labelled with either \([\alpha-^{32}\text{P}]\text{dCTP}\) or \([\alpha-^{32}\text{P}]\text{dATP}\) using the random priming method (Boehringer Mannheim, Germany). The labelled probes were then passed through a spun column containing Sephadex G50 (Pharmacia Bioprocess, Sweden) before being added to the hybridisation solution. After hybridisation overnight, the filters were washed with 2x SSC, 0.1% SDS three times at room temperature for 20 minutes each, 0.5x SSC, 0.1% SDS at 65°C for 30 minutes and a final high stringency wash with 0.2X SSC, 0.1% SDS at 65°C for 30 minutes. Filters can either be exposed overnight to phosphoimager plates (for subsequent quantification) or to Kodak XOMAT films using intensifying screens for 5-14 days. The filters were then stripped by washing in 0.4N NaOH for 30 minutes at 65°C followed by 0.1X SSC, 0.1% SDS for 15 minutes at 65°C for another 15 minutes before proceeding to hybridisation with another probe.

2.6 SDS-PAGE and WESTERN BLOTTING

**Materials**

| Acrylamide/Bis 37.5:1 (30%) | Biorad, Hemel Hampstead, UK |
| TEMED | Biorad, Hemel Hampstead, UK |
| Ammonium persulphate | Biorad, Hemel Hampstead, UK |
| Molecular weight rainbow markers | Amersham, Bucks, UK |
| Hybond-C Extra (nitrocellulose membrane) | Amersham, Bucks, UK |

**Separating gel preparation:**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>6% (for pRB)</th>
<th>Volume (ml)</th>
<th>15% (for p16)</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>5.35</td>
<td>2.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5M Tris-HCl pH 8.8</td>
<td>2.5</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrylamide/Bis 30%</td>
<td>2</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% (w/v) Ammonium persulphate</td>
<td>0.075</td>
<td>0.075</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>0.0075</td>
<td>0.0075</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Stacking gel preparation:

Reagents

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>3.05</td>
</tr>
<tr>
<td>1.5M Tris-HCl (pH 8.8)</td>
<td>1.25</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>Acrylamide/Bis 30%</td>
<td>0.65</td>
</tr>
<tr>
<td>10% (w/v) Ammonium persulphate</td>
<td>0.375</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.075</td>
</tr>
</tbody>
</table>

5x Electrode running buffer, pH 8.3

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>9.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>43.2</td>
</tr>
<tr>
<td>SDS</td>
<td>3.0</td>
</tr>
<tr>
<td>Water</td>
<td>to a final volume 500ml</td>
</tr>
</tbody>
</table>

Western transfer buffer

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3.0 g/l</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.8 g/l</td>
</tr>
<tr>
<td>Methanol</td>
<td>20%</td>
</tr>
</tbody>
</table>

Method

The separating gel was prepared and poured into a minigel apparatus (Hoefer, UK) which was set up according to the manufacturer's instructions. This was overlayed with water to straighten the upper level of the gel. Once set, the water was poured off and the space above the gel dried with a piece of filter paper. The stacking gel was then layered onto the separating gel (30µl of bromophenol blue was added to aid visualisation of the when loading), and a 10-well comb inserted into place. Once set, the comb was removed and the wells flushed with running buffer using a syringe and needle. This is to remove unpolymerised polyacrylamide.

Western Blot Analysis. 15µl of each protein sample in buffer (approximately 3 x 10^5 cells) were loaded and seperated by SDS-PAGE (6% for pRB, 10% for actin and tubulin and 15% for p16 westerns). Electrophoresis was carried out at a constant 100V for 60-120 minutes depending on the concentration of the gel. After electrophoresis, the proteins were transferred to Hybond-C Extra
membranes (Amersham) using semi-dry electro-transfer method. The membranes were then blocked overnight in PBST (Phosphate buffered saline with 0.05% [v/v] Tween 20) with 10% (w/v) skimmed milk. They were then washed in PBST 3 times for 15 minutes each followed by overnight incubation at 4°C with the primary antibody (see text for concentration). After 3 washes with PBST, the membranes were incubated with the secondary antibody (peroxidase conjugated immunoglobulins) for 1 hour. Following this, another 3 washes with PBST were performed. Detection of the protein bands were carried out using the Electro-Chemiluminescence (ECL) method (Amersham UK). In this technique, the membranes were exposed to the chemiluminescence mixture for 1 minute. Films were then exposed at intervals of 15 seconds to 2 minutes until maximum saturation of the film has occurred.

2.7 IMMUNOPRECIPITATION

**Materials**

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP-40</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Phenylmethylsulfonylfluoride (PMSF)</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Disuccinimidyl suberate (DSS)</td>
<td>Boehringer Mannheim, Germany</td>
</tr>
<tr>
<td>Protein-A-sepharose</td>
<td>Sigma, Poole, UK</td>
</tr>
</tbody>
</table>

**Lysis Buffer**

137mM NaCl, 20mM Tris (pH 8), 1mM MgCl₂, 1mM CaCl₂, 1% NP40, 10% (v/v) Glycerol, 1mM Na orthovanadate, 1mM β-glycerophosphate, 1mM NaF, 5mM pyrophosphate, 2mM EDTA, 1mM PMSF, 10μg/ml aprotinin, 10μg/ml leupeptin and 10μg/ml pepstatin.

**Method**

Cells were centrifuged and resuspended in 1ml of ice cold lysis buffer (with protease inhibitors) and incubated for 30-60 minutes on ice. All subsequent steps were then carried out in the cold room at 4°C on ice. Samples were centrifuged at 12000g for 10 minutes in a microcentrifuge (MSE) and the supernatant was transferred to a fresh eppendorf tube. The antibody was
added and the contents were then mixed. Incubation was carried out on a rotator in the cold room for 1 hour. At the end of incubation, 50μl of 50% protein A sepharose was added and incubated on the rotator for another hour. The samples were then pulsed briefly at 12000g and the resulting pellet washed in lysis buffer 4 times. The final pellet was resuspended in 60μl of 2x sample buffer and heated at 95°C for 10 minutes. Samples were then either run on a SDS-polyacrylamide gel immediately or stored at -20°C until used.

2.8 CELL CULTURE

Materials
Tissue culture flasks Becton Dickinson, Oxford
RPMI 1640 + L-glutamine Gibco, Paisley, UK
Fetal Calf Serum ICN, High Wycombe, UK
Trypsin/EDTA solution Gibco, Paisley, UK

Cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue of origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL60</td>
<td>APML</td>
<td>Collins et al. 1977</td>
</tr>
<tr>
<td>U937</td>
<td>Histiocytic Lymphoma</td>
<td>Sundstrom &amp; Nilsson, 1976</td>
</tr>
<tr>
<td>TF-1</td>
<td>Erythroleukaemia</td>
<td>Kitamura et al., 1989</td>
</tr>
<tr>
<td>Daudi</td>
<td>B cell lymphoma</td>
<td>Silverman et al., 1982</td>
</tr>
<tr>
<td>K562</td>
<td>CML in blast transformation</td>
<td>Lozzio &amp; Lozzio, 1975</td>
</tr>
<tr>
<td>J6</td>
<td>T-cell leukaemia</td>
<td>Gordon Peters, 1995</td>
</tr>
<tr>
<td>WERI-1</td>
<td>Retinoblastoma</td>
<td>McFall et al., 1978</td>
</tr>
</tbody>
</table>

Except for the J6 and WERI-1 cell lines, the others were routinely maintained in the laboratory. The J6 cells were used as the negative control for the p16 experiments while the WERI-1 cells were negative controls for the RB experiments.

All cell lines were non-adherent and maintained in suspension cultures in RPMI 1640 supplemented with 10% FCS (heat inactivated at 55°C for 3 minutes) and incubated in a 5% CO₂-containing atmosphere. Cells were split or refed twice a week. Antibiotics were not routinely added to the cell cultures. Protein samples from these cells were prepared by taking 10^7 cells and pelleting them by centrifugation at 1200rpm for 5 minutes. 500μl of 2x SDS Laemmli sample buffer was added. Samples were then boiled for 10 minutes and kept at -20°C until used.
2.9 POLYMERASE CHAIN REACTION

Materials

- 10x Buffer Promega, UK
- 25mM MgCl₂ Promega, UK
- dNTPs (deoxynucleotides) Promega, UK
- Taq Polymerase (5000 U/ml) Promega, UK
- Primers Oswell DNA, Edinburgh, UK
- Thermocycler Hybaid, UK
- Mineral Oil Sigma, Poole, UK

Method
The PCR reaction used in this thesis would adhere to the following example. Since each pair of primers would require its own optimisation, further developments on the technique will be elaborated in the appropriate chapters.

Typical example of PCR reaction mixture (for a 20μl reaction volume)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>12.4 (depends on the amount of DNA or cDNA)</td>
</tr>
<tr>
<td>10x Buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.4</td>
</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>0.4</td>
</tr>
<tr>
<td>3' primers (200ng/μl)</td>
<td>0.4</td>
</tr>
<tr>
<td>5' primers (200ng/μl)</td>
<td>0.4</td>
</tr>
<tr>
<td>DNA or cDNA</td>
<td>4.0 (depends on concentration)</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5 U (for each 20μl reaction)</td>
</tr>
</tbody>
</table>

A mastermix of the reagents was made (minus the template) so as to ensure identical concentrations of each reagent in each PCR reaction vessel. Filtered pipet tips and special PCR pipettes were used for all PCR reactions. Sixteen microlitres of the mastermix were pipetted into each labelled tube. DNA or cDNA was then added and mixed by pipetting up and down. A drop of mineral oil is added and the tubes were capped and briefly spun in a microcentrifuge. A hot-start was used in all the PCR experiments for this thesis. The tubes were placed in the thermocycler and the block heated up to 95°C for 3
minutes. The block temperature was then lowered and held at 85°C. The Taq polymerase was added at this point and the amplification cycles were then commenced. Basically they consist of denaturation at 95°C for 3 minutes, annealing and extension. The temperature and duration for the annealing and extension depended on the primers used. Details of these are given in the relevant chapters. A final extension at 72°C was carried out for 5 minutes. The PCR products were then electrophoresed through an ethidium bromide gel and visualised under the UV light to check for their products.

2.10 REVERSE-TRANSCRIPTION-PCR (RT-PCR)

One microgram of total cellular RNA was reverse transcribed using 250ng oligo dT as primer (Promega, UK) in a total volume of 20μl containing 1x Taq polymerase buffer, 5.25mM MgCl₂, 1mM of each deoxynucleoside triphosphate, 20U RNAse inhibitor and 3.75U AMV reverse transcriptase (Promega, UK). Reaction mixtures were incubated at 42°C for 1 hour and followed by 95°C for 5 min. The resulting cDNA was stable for up to several weeks at 4°C. An aliquot of the RT reaction was then used for the PCR.

2.11 Sequencing of PCR and RT-PCR products

PCR or RT-PCR products were electrophoresed through a 0.8% low melting point agarose gel (Sigma, UK) and purified (Wizard PCR preparation, Promega, UK). Sequencing reactions were then performed using the cycle sequencing method (fmol Cycle Sequencing, Promega, UK) according to the manufacturer's instructions. The sequencing gels contained 6% polyacrylamide (sequencing grade) and electrophoresis was carried out in 0.5x Tris-borate-EDTA buffer at 1200volts for 2-3 hours using the Bio-Rad sequencing gel system. Gels were then transferred onto 3MM chromatography paper (Whatmann, UK), dried for 45 minutes and then exposed to hyperfilms (Amersham, UK) overnight.
CHAPTER THREE

ANALYSIS OF REARRANGEMENTS OF THE RB1 GENE BY SOUTHERN BLOTTING

3.1 INTRODUCTION

The RB1 genomic locus, localised on chromosome 13q14, is complex. It consists of 27 exons dispersed within 180 kilobases (kb) of genomic DNA. The complete genomic sequence has been published (Toguchida et al., 1993). The genomic organisation of the gene with the restriction sites for EcoRI and HindIII is shown in Figure 3.1(A and B). The positions of the 27 exons were assigned by restriction mapping and subsequently confirmed by sequencing (Lee et al., 1987a; Friend et al., 1987). Most of the 5' untranslated region and the coding sequence for the N-terminus of the RB protein are contained in exon 1. The last exon is the largest (1889 base pairs), and contains the C-terminal coding sequence as well as a large 3' untranslated region. The two smallest exons are exons 15 and 24 consisting of 32bp and 31bp respectively. Intron 15 is the smallest (80bp), in contrast to intron 17 which spans approximately 70,000bp. The cDNA is 4.7kb in length. There is a GC-rich region (greater than 82%) at the extreme 5' end of the cDNA.

The RB1 gene promoter is located just 5' of exon 1 (Goodrich and Lee, 1993) and extends approximately 1600bp in length. It contains regulatory elements for the transcription factors E2F, ATF and SP1 (Ohtani-Fujita et al., 1994; Gill et al., 1994). Any mutations within these sites can dramatically decrease the RB1 promoter activity. In fact two cases of retinoblastoma were found to have point mutations within the promoter region (Sakai et al., 1991). It has also been shown that CpG methylation inactivates RB1 promoter activity, suggesting that one mechanism for inactivation of the RB1 gene is through hypermethylation of the 5' end of the RB1 gene (Ohtani-Fujita et al., 1993).

One of the transcription factors that interacts with pRB is E2F, and its transcriptional activation is negatively regulated by pRB (Hiebert et al. 1993; La Thangue et al., 1993). There is an E2F site in the RB1 promoter located directly downstream of the essential retinoblastoma binding factor 1 (RBF-1) and ATF sites (Ouellette et al., 1992). This domain has been designated as the RB-E2F site. It has been shown in several cell lines that a deletion of this site results in activation, not suppression, of RB1 promoter activity, indicating
Figure 3.1A: Genomic organisation of the human retinoblastoma susceptibility gene showing *EcoRI* restriction sites and approximate sizes (in kilobases) of restriction fragments detected with cDNA probe.
Figure 3.1B: Genomic organisation of the human retinoblastoma susceptibility gene showing HindIII restriction sites and approximate sizes (in kilobases) of restriction fragments detected with cDNA probe
that the RB-E2F site acts as a silencer element (Ohtani-Fujita et al., 1994). Factor(s) that bind to the E2F site of the RB1 promoter negatively regulate the promoter activity.

Rearrangements of the RB1 gene have been found in a broad range of malignancies. As listed in Table 3.1, the frequency of rearrangement as detected by Southern blotting analysis is higher in some tumour types than in others. The extent of overall mutation is probably higher since point mutations and small deletions are not detected by Southern blotting. The common occurrence of rearrangements in various tumour types suggests a pleiotropic contribution of RB1 in carcinogenesis.

Table 3.1: Frequency of RB1 gene rearrangements in various primary solid tumours

<table>
<thead>
<tr>
<th>Type of malignancy</th>
<th>Study</th>
<th>Frequency of gene rearrangements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinoblastoma</td>
<td>Fung et al., 1987</td>
<td>16/40 (40%)</td>
</tr>
<tr>
<td></td>
<td>Kloss et al., 1991</td>
<td>15/92 (16%)</td>
</tr>
<tr>
<td></td>
<td>Blanquet et al., 1995</td>
<td>46/232 (20%)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>Toguchida et al., 1988</td>
<td>13/30 (43%)</td>
</tr>
<tr>
<td></td>
<td>Wunder et al., 1991</td>
<td>6/14 (43%)</td>
</tr>
<tr>
<td></td>
<td>Wadayama et al., 1995</td>
<td>18/63 (28%)</td>
</tr>
<tr>
<td>Small cell lung cancer</td>
<td>Harbour et al., 1988</td>
<td>1/8 (13%)</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>Miyamoto et al., 1995</td>
<td>8/30 (27%)</td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>Henson et al., 1994</td>
<td>16/54 (30%)</td>
</tr>
<tr>
<td>Soft tissue sarcoma</td>
<td>Reissmann et al., 1989</td>
<td>4/29 (14%)</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>T’Ang et al., 1988</td>
<td>3/41 (7%)</td>
</tr>
</tbody>
</table>

In haemopoietic malignancies there is an apparent lack of DNA analysis data. Many have chosen the much easier task of analysing the protein expression. Chen et al. (1990) found RB1 gene rearrangement in 1 out of 52 cases of acute lymphoblastic leukaemia (ALL) while Ginsberg et al. (1991) reported rearrangements in 1 of 26 T-ALL cases and 2 of 55 non-Hodgkin’s lymphoma (NHL) cases. In a study using interphase cytogenetics and fluorescence in-situ hybridisation (FISH), 11 of 35 cases (31%) of chronic lymphoid leukaemia (CLL) had monoallelic RB1 gene deletion, (Stilgenbauer et al., 1993). Weide et al. (1992) analysed 45 cases of high grade NHL by Southern blotting and
found none with rearrangements. Western blotting however showed that 26 of the cases (58%) had no RB protein. For chronic myeloid leukaemia (CML), Ahuja et al (1991) found 8 out of 68 cases with gene rearrangements.

For acute myeloid leukaemia (AML), the frequency of RB1 gene rearrangements is controversial. The following table highlights the conflicting results from studies done so far.

<table>
<thead>
<tr>
<th>Study</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chen et al., 1990</td>
<td>0/69 cases</td>
</tr>
<tr>
<td>Ahuja et al., 1991</td>
<td>5/54 cases (4/15 M4 and M5)</td>
</tr>
<tr>
<td>Tang et al., 1992</td>
<td>0/24 cases</td>
</tr>
</tbody>
</table>

Although the numbers involved were relatively small, Ahuja et al claimed that the 27% frequency of rearrangements in monocytic leukaemias was significant and may indicate lineage specificity. No studies to date have offered more conclusive data than these three groups.

The aim of the study in this chapter was to determine the actual frequency of rearrangements of the RB1 gene in AML patients. Southern blotting was used because it is one of the most efficient techniques for studying gross rearrangements of a gene especially one which is as large and complex as in the case of the RB1 gene. Some of the apparent weaknesses of the previous studies include the small number of patients analysed, the inadequate number of normal controls screened, and the lack of data on polymorphisms within the gene in both normal individuals and patients. These pitfalls might lead to errors in the determination of the frequency of rearrangements.

3.2 MATERIALS AND METHODS:

3.2.1 AML patients and normal controls

A total of 106 AML patients were analysed. The patients' age ranged from 16-70 years. The diagnosis of AML was based on morphological and cytochemical criteria of bone marrow aspirates and peripheral blood smears. The breakdown of the patients according to their FAB types is shown in Table 3.3. None of the patients were known to have chromosome 13 abnormalities.
Eighteen haematologically normal controls were also analysed, either women attending an antenatal clinic or laboratory staff members.

Table 3.3: Breakdown of AML patients according their FAB types

<table>
<thead>
<tr>
<th>FAB type</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>15</td>
</tr>
<tr>
<td>M2</td>
<td>15</td>
</tr>
<tr>
<td>M3</td>
<td>16</td>
</tr>
<tr>
<td>M4</td>
<td>40</td>
</tr>
<tr>
<td>M5</td>
<td>20</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>106</strong></td>
</tr>
</tbody>
</table>

3.2.2 Southern blotting analysis and Polymorphisms

Southern blotting is a powerful and efficient tool for detecting gene rearrangements. The bands detected by the technique are restriction fragments arising from the digestion of genomic DNA using specific restriction enzymes. These fragments are detected by probing with a radioactive-labelled probe containing complementary sequences. However, restriction fragments which do not contain exonic sequences would not be detected by a cDNA probe.

Differences in the band configuration on a Southern blot are due to changes in the DNA sequence that result in the gain or loss of a restriction enzyme site, a deletion in the region between two such restriction sites or a duplication of a restriction fragment. These changes can either be the result of normal genetic variation or due to a rearrangement or mutation. The presence of genetic heterogeneity between individuals has been recognised for a long time especially in regions which do not encode for proteins and those not involved in regulatory functions. This normal variation of the genetic sequence is loosely termed genetic polymorphism. Variations resulting in changes in configuration of bands on a Southern blot are called Restriction Fragment Length Polymorphisms (RFLPs) (Weatherall, 1991). There are also other polymorphisms which involve point mutations that cannot be detected by currently available restriction enzymes and consequently are not detected by Southern blotting. They can however be detected by other techniques such as Single Strand Conformation Polymorphism or by direct sequencing of DNA.
There are also regions of the genome in which the length of DNA between specific restriction sites varies considerably between different individuals and again this variability is inherited. The different sizes of these fragments arise from variable number of tandem repeats (VNTR) and are present in many genes. They are valuable as genetic markers. Interestingly, the *RB1* gene has a VNTR located in the intron between exons 17 and 18 (Wiggs *et al.*, 1988).

There are RFLPs which correlate with a disease phenotype. One of the best known examples is the sickle cell gene which is the result of a single base substitution resulting in the loss of a *MsuI* restriction site. There are also many β-thalassaemia mutations which are directly detectable by restriction enzyme analysis (Weatherall, 1991). But most RFLPs do not result in a phenotypic change.

The objective of Southern blotting is simply to detect any changes in the normal germline pattern obtained from a DNA sample. It is difficult to differentiate a harmless change in a restriction fragment caused by the presence of a polymorphic site from a true rearrangement, especially when analysing samples from patients with an underlying disease. In some cases the polymorphisms are well defined in normal individuals and shown not to result in phenotypic changes. But in most genes, the full extent of polymorphisms involving restriction sites are not known. For the *RB1* gene, the best characterised polymorphic site is the *BamH* site in intron 1 (Figure 3.4) (Bookstein *et al.*, 1990). Unfortunately, the full extent of the polymorphic sites for the other restriction enzymes used in this study is not known.

The other potential problem which can arise in Southern blotting is the presence of artefactual bands. These may arise from unrelated cross hybridising sequences or from non-specific binding, especially within GC-rich regions.

There are a number of approaches to avoid spurious interpretation of results: (1) screening a number of normal controls in addition to the patient samples, (2) repeating the analysis for samples showing abnormal bands (3) confirming those with truly rearranged bands by analysing pRB protein expression and (4) utilising more than one restriction enzyme digest. In developing the technique and the approach to screening the AML patients, 18 normal controls were also analysed to establish both normal germline patterns as well as any polymorphisms present. Any abnormal patterns found were repeated
at least twice to be certain that they are not artefacts. Four restriction enzymes, namely \textit{EcoRI}, \textit{HindIII}, \textit{BamHI} and \textit{BglII}, were used to maximise the detection of rearrangements.

3.2.3 Optimisation of conditions for \textit{RB1} Southerns

The general technique of Southern blotting has remained much the same since it was published by Ed Southern (1975). In this pioneering paper, two specific technical problems were highlighted in the conclusions. One was the problem of separating large fragments of DNA and the other was the inefficiency of hybridisation to small fragments. The advent of inverse field gel electrophoresis and pulse field gel electrophoresis have succeeded in overcoming the first problem, but the second problem remains a difficulty. It is almost always necessary to optimise the conditions for each probe used.

There were many aspects of this technique that needed to be fine tuned to get good quality results that would ensure accurate interpretation of the bands. It is especially vital for the \textit{RB1} gene due to its large size and complex genomic organisation as well as the apparent heterogeneity of mutations shown in studies so far.

The following parameters were examined:-
1. \textit{RB1} cDNA probes to be used
2. amount of genomic DNA to be loaded
3. type of membrane for DNA transfer
4. germline and negative controls
5. [\(\alpha^{32}\text{P}\)]dATP versus [\(\alpha^{32}\text{P}\)]dCTP for labelling probes
6. hybridisation protocol
7. sensitivity of the technique

3.2.2.1 Testing different \textit{RB1} cDNA fragments for probes and establishing the germline bands

The recommended length for a probe is 100-1000bp (Bio-Rad-Instruction Manual). The cDNA of the \textit{RB1} gene is 4.7kb in length. Using this as a probe would potentially result in 11 bands arising from \textit{HindIII} digest and 11 from the \textit{EcoRI} digest. There would also be several overlapping bands because of the similarity in their sizes. Consequently, most studies have used 2 fragments from the full length cDNA and designated them as the 5' probe (0.9kb) and the
3' probe (3.8kb) (Figure 3.2). These fragments were obtained by digesting the cDNA with EcoRI which cuts it through exon 9. By using these two probes, the number of bands appearing on an autoradiograph for each enzyme digest is suitably reduced and overlapping bands kept to a minimum. The usefulness is further appreciated in cases of deletions where pin-pointing the location of the deletion is made much easier.

The other problem with the RB1 cDNA is the high GC-content of its 5' end which tends to give a high background due to non-specific binding. To test this, 2 identical blots were prepared and one was probed with the full sized 0.9kb 5' probe and the other was probed with a 0.67kb probe (probe C, Figure 3.2) which was prepared by excising the GC-rich sequence using the restriction enzyme Hpal. The use of this truncated 5' probe significantly decreased the high background obtained using the full length 0.9kb probe.

However, using the smaller 5' probe would mean that the 10kb and 4.6kb fragments of the EcoRI and BamHI digests respectively, would escape analysis. To avoid this, both the 0.9kb and 0.67kb probes were used for the EcoRI and BamHI digests.

3.2.2.2 Determining the amount of genomic DNA to be used in digest

This experiment was conducted to determine the optimum amount of DNA required to detect all the bands. The theory behind this is that more DNA means a higher copy number of each restriction fragment generated by an enzyme, thus improving the detection of smaller fragments. The drawback is that it increases the likelihood of non-specific background bands. For this optimisation, four different amounts of DNA from one control were used: 3, 5, 8 and 10μg. These were digested with HindIII and Southern blotting was done using the 3' probe. The results revealed that using either 8 or 10μg DNA gave better detection of the smaller fragments.

3.2.2.3 Establishing germline and negative controls

A haematologically normal individual was included as the germline control for each Southern blotting done. For a negative control, the WERI-1 cell line was used. This cell line originated from the retinal cells of a patient with retinoblastoma (McFall et al., 1978) and lacks the RB1 gene completely.
Figure 3.2: *RB1* cDNA probes used in Southern blotting

Exons

DNA

cDNA

BamHI  HpaI  EcoRI  BamHI

Exons 1  9  27

C

0.67kb

A  0.9kb  3.8kb  B

5' probe  3' probe
3.2.2.4 Comparing two different membranes for blotting

Two nylon membranes from two manufacturers, Hybond-N+ membrane (Amersham, UK) and Zetaprobe membrane (Bio-Rad) were compared. The Hybond-N+ membranes gave marginally better quality bands on the eventual autoradiograph.

3.2.2.5 \([\alpha^{-32P}]dATP\) versus \([\alpha^{-32P}]dCTP\) for labelling probes

The RB1 cDNA sequence contains twice as many adenines as compared to cytosines. Theoretically, labelling with \([\alpha^{-32P}]dATP\) should result in a higher specific activity when compared to using the same amount of \([\alpha^{-32P}]dCTP\).

This experiment confirmed that there is a significant improvement in the intensity of the bands produced when the probes were labelled with \([\alpha^{-32P}]dATP\).

3.2.2.6 Comparing three different hybridisation protocols

Many hybridisation protocols have been published and also advocated by manufacturers of certain membranes. These protocols vary in the composition of hybridisation solution used and the temperature for hybridisation. For example the protocol using formamide allows hybridisation to take place at 42°C whilst most other protocols require hybridisation at 65°C.

Three different protocols were tested. The composition of the hybridisation solutions and the hybridisation temperatures were as follows:-

**Formamide protocol:-**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>50% (w/v)</td>
</tr>
<tr>
<td>(Na_2HPO_4)</td>
<td>0.12M</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.25M</td>
</tr>
<tr>
<td>SDS</td>
<td>7% (w/v)</td>
</tr>
</tbody>
</table>

Hybridisation temperature: 42°C
**Standard Protocol** (as suggested by Bio-Rad Life Science Group, USA)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>0.25M</td>
</tr>
<tr>
<td>SDS</td>
<td>7% (w/v)</td>
</tr>
</tbody>
</table>

Hybridisation temperature: 65°C

**SSPE protocol** (Amersham, UK)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSPE</td>
<td>5x</td>
</tr>
<tr>
<td>Denhardt's reagent</td>
<td>5x</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5% (w/v)</td>
</tr>
<tr>
<td>Single stranded salmon sperm DNA</td>
<td>100μg/ml</td>
</tr>
</tbody>
</table>

Hybridisation temperature: 65°C

The two major differences between these hybridisation protocols are the nature of the blocking agent used and the temperature for hybridisation. The first two protocols use a high concentration of SDS to affect the blocking while the last protocol uses single stranded salmon sperm DNA, Denhardt's reagent and a much lower concentration of SDS to achieve this. The second difference is the hybridisation temperature. The formamide protocol allows hybridisation to take place at 42°C because formamide effectively reduces the melting temperature (Tm) of the probe duplex. The results showed that the SSPE protocol gave the best results.

**Summary of optimisation experiments**

At the end of the optimisation experiments, the following conditions were chosen for the subsequent Southern blotting:

i. Probes: 0.67kb (5') and 3.8kb (3') for all digests.
   0.9kb (5') probe for the EcoRI and BamHI digests

ii. DNA for loading: 8μg

iii. Membrane: Hybond-N+ (Amersham)

iv. Blotting: Alkaline blotting (which removed the need for fixation)

v. Radioactive label: [α-32P]dATP

vi. Hybridisation protocol: 5x SSPE, 5x Denhardt's, 0.5% (w/v) SDS, 100μg/ml of salmon sperm DNA, with hybridisation at 65°C.
3.2.4 Testing the sensitivity of the Southern blotting technique

To test the sensitivity of detection of the RB1 bands, the 4.6kb polymorphic fragment from the BamHI digest was utilised. As mentioned previously, this is a well known polymorphism within intron 1 (Bookstein et al, 1990) and an individual can either be heterozygous, homozygous positive or homozygous negative for the site, hence causing the presence or absence of the 4.6kb band (Figure 3.4). To conduct this sensitivity experiment, a homozygous positive DNA sample and a heterozygous DNA sample were selected, their DNA concentrations quantified using a spectrophotometer and then equalised. These final concentrations were confirmed by spectrophotometric analysis and also by running equal amounts on an agarose gel. Following this, a series of mixtures of the DNA with varying ratios of each sample were made. These DNA mixtures were allowed to mix thoroughly overnight, digested with BamHI and fractionated through a 0.6% agarose gel. The Southern blotting analysis was done using the 5' probe as described.

3.2.5 Southern blotting analysis of 18 normal controls and 106 AML patients

Eighteen haematologically normal controls and 106 AML patients were analysed. A known germline normal control was included in each set of samples electrophoresed. The negative control was included from time to time to ensure specificity of the bands.

3.3 RESULTS

3.3.1 Germline configuration of bands in normal controls

The germline configuration of bands for each enzyme digest when probed with either the 5' or the 3' probe is shown in Table 3.4. Their corresponding positions on the autoradiographs are shown in Figure 3.3A and Figure 3.3B. There were several bands which were technically difficult to detect. One possible reason is that the exonic sequence within the restriction fragment to which the probes can hybridise is small. Examples include the 4.5kb band for the HindIII digest with the 3' probe (Figure 3.3B), the 2.5kb band for the EcoRI digest with the 5' probe (Figure 3.3A) and the 3.8kb band for the BamHI digest.
Figure 3.3A: Germline bands of *RB1* in normal controls detected by Southern blotting analysis (sizes in kilobases)

5' probe

- EcoRI
- HindIII
- BamHI
- BglII
Figure 3.3B: Germline bands of *RB1* in normal controls detected by Southern blotting analysis
(sizes in kilobases)

3' probe

- **EcoRI**
  - 7.0
  - 5.6
  - 3.8
  - 2.8
  - 2.4
  - 1.2

- **HindIII**
  - 10
  - 7.5
  - 6.2
  - 6.2
  - 5.5
  - 4.5
  - 2.1

- **BamHI**
  - >12
  - 9.5
  - 5.5
  - 3.8

- **BgII**
  - >12
  - 12
  - 5
  - 3.8
  - 3.0
  - 2.4
  - 2.2
  - 2.0
Table 3.4: RB1 Germline bands detected by Southern blotting

5' probe

<table>
<thead>
<tr>
<th>EcoRI</th>
<th>HindIII</th>
<th>BamHI</th>
<th>BglII</th>
</tr>
</thead>
<tbody>
<tr>
<td>10kb</td>
<td>14kb</td>
<td>5.5kb*</td>
<td>&gt;12kb (2 bands)</td>
</tr>
<tr>
<td>3.4</td>
<td>7</td>
<td>4.6**</td>
<td>7.5</td>
</tr>
<tr>
<td>2.5++</td>
<td>6.5</td>
<td>2.2</td>
<td>4.5</td>
</tr>
<tr>
<td>1.8</td>
<td>1.5</td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td>1.3</td>
<td>1.2</td>
<td></td>
<td>1.4</td>
</tr>
</tbody>
</table>

3' probe

<table>
<thead>
<tr>
<th>EcoRI</th>
<th>HindIII</th>
<th>BamHI</th>
<th>BglII</th>
</tr>
</thead>
<tbody>
<tr>
<td>7kb</td>
<td>10.5kb</td>
<td>&gt;12kb (2 bands)</td>
<td>12</td>
</tr>
<tr>
<td>5.6</td>
<td>7.5</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>3.8</td>
<td>6.2</td>
<td>5.5</td>
<td>3.8</td>
</tr>
<tr>
<td>2.8</td>
<td>5.5</td>
<td>3.8+/++</td>
<td>3</td>
</tr>
<tr>
<td>2.4</td>
<td>4.5++</td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>1.2</td>
<td>2.1</td>
<td></td>
<td>2.2</td>
</tr>
</tbody>
</table>

+ Denotes a band labelled as abnormal by previous studies
++ Denotes bands which are difficult to detect
* Denotes a known cross-hybridising band
** Denotes a known polymorphic band

3.3.2 Polymorphisms in the RB1 gene in normal controls

An additional band which was detected in a single enzyme digest without giving rise to abnormalities in other digests was considered to be due to a restriction enzyme polymorphism. Both common and rare polymorphisms were seen in the normal controls. The BamHI polymorphic site in intron 1 was present in twelve of the 18 normal controls screened. The frequency of this site for 18 normal controls and 106 AML patients is summarised in Figure 3.4. The actual allelic frequencies (as calculated by the formula $x^2 + 2xy + y^2 = 1$, where $x$ is a positive allele with the polymorphic site present and $y$ is a negative allele where it is absent) are $x=0.7$ and $y=0.3$. It is interesting to note that the 43% frequency for heterozygotes concurred with a study by Bookstein et al (1990) although involving a much smaller number of individuals.
**Figure 3.4:** *BamHI* polymorphism in the *RB1* gene in 18 normals and 106 AML patients

![Diagram showing *BamHI* polymorphism in the *RB1* gene.]

<table>
<thead>
<tr>
<th>Homozygous positive (+/+</th>
<th>Heterozygous (+/-)</th>
<th>Homozygous negative (-/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6kb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5kb</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No of individuals</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous positive (+/+)</td>
<td>61</td>
</tr>
<tr>
<td>Heterozygous (+/-)</td>
<td>53</td>
</tr>
<tr>
<td>Homozygous negative (-/-)</td>
<td>10</td>
</tr>
</tbody>
</table>
Another common polymorphism was seen in the EcoRI digests when the 3' probe was used. Three of the normal controls showed this pattern (Figure 3.5). This polymorphism was seen in 18% of the AML patients. Two other normal controls each had a rare polymorphic band each (Figure 3.5). One was a band from the BglII digest with the 3' probe and the other from an EcoRI digest at the 3' end of the gene. These polymorphisms were not observed in any of the AML patients.

3.3.3 Sensitivity of detection of bands by Southern blotting

Figure 3.6 shows the autoradiograph of the sensitivity experiment which utilised the polymorphic BamHI site in intron 1. The 4.6kb band can be seen to decrease in intensity when the proportion of DNA with this polymorphic site decreases in amount. The other important finding was that 2.5% of the DNA carrying this polymorphic site was sufficient for the corresponding band to be detected (lane 8).

3.3.4 Southern blotting results of 106 AML patients

At the completion of the preliminary analysis of the 106 AML patients, a total of 15 samples showed the presence of novel bands (excluding the polymorphisms mentioned above). None of the patients showed any deletion of bands. All had normal germline patterns in addition to the extra bands. Table 3.5 shows a detailed list of the patients and their respective novel bands. The sizes of the bands as well as the digests in which they appear are also listed. The most striking feature of this data was the presence of identical extra bands in 7 of these cases. All of them had a 5kb band in the EcoRI digest and a 4.5kb band in the HindIII digest when probed with the 5' end of the cDNA. Five of these cases are represented in Figure 3.7 (arrowed lanes). The implication was that these bands could be due to a common mutation/polymorphism within the 5' end of the \textit{RB1} gene. One of these 7 cases also had an additional band of 2.5kb in size with the HindIII digest at the 5' end of the gene (Figure 3.7, patient 7). This 2.5kb band was also seen in 2 other samples (Figure 3.7, patients 2 and 8).
Figure 3.5: Polymorphisms in the RB1 gene

Common polymorphisms
(detected in both AML patients and normal controls)

-/-  +/+  +/-

4.6kb

BamHI:
5' probe

12kb  10kb

EcoRI:
3' probe

Rare polymorphisms
(each detected in a single individual)

BglII:
3' probe

EcoRI:
3' probe

5.5kb

4.5kb
Figure 3.6: Sensitivity of Southern blotting technique using *BamH*I polymorphic site as marker

Percentage of DNA with polymorphic 4.6kb band
Figure 3.7: Representative Southern blots showing rearranged bands consistently detected in 5 AML patients using digestion with both EcoRI and HindIII

Individuals with rearranged bands are indicated by arrows
C=germline control

EcoRI digests

HindIII digests

AML patients (n=15)
Table 3.5: AML patients with their respective additional bands seen on different enzyme digests

<table>
<thead>
<tr>
<th>Patient No</th>
<th>5' EcoRI</th>
<th>5' HindIII</th>
<th>5' HindIII</th>
<th>3' EcoRI</th>
<th>3' HindIII</th>
<th>3' BglII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5kb</td>
<td>4.5kb</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5kb</td>
<td>4.5kb</td>
<td></td>
<td></td>
<td></td>
<td>2.5kb</td>
</tr>
<tr>
<td>3</td>
<td>5kb</td>
<td>4.5kb</td>
<td>2.5kb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5kb</td>
<td>4.5kb</td>
<td></td>
<td></td>
<td>2.5kb</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5kb</td>
<td>4.5kb</td>
<td></td>
<td></td>
<td>2.5kb</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5kb</td>
<td>4.5kb</td>
<td></td>
<td></td>
<td>2.5kb</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>5kb</td>
<td>4.5kb</td>
<td></td>
<td></td>
<td>2.5kb</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>2.5kb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>2.5kb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>2.5kb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td>2.5kb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>2.5kb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>4kb</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td>8kb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2kb</td>
</tr>
</tbody>
</table>

A repeat analysis was carried out on all 15 samples with rearranged bands and all the abnormal bands were successfully reproduced. It was tentatively decided that the 7 cases (patients 1-7, Table 3.5) with more than one abnormal band detected were true rearrangements and the site for this common mutation would be further investigated. The results of these additional experiments are discussed in the next section.

Another common novel band, 2.5kb in size, arising from HindIII digests (3' probe) was seen in 8 patients (Figure 3.8). Five of them were the same patients with the possible common mutation at the 5' end of the gene. Finally, three other patients showed a unique additional band each (Figure 3.8). One had a 8kb band from the EcoRI digest (3' probe), another showed the presence of a 4kb band from the HindIII digest (5' probe) and the third showed a 1.2kb band from the BglII digest (3' probe). These bands were attributed to polymorphisms as they were only seen with single enzyme digests.

The status of the results after this initial screening was 7 out of 106 AML patients had rearrangements (8.5%) and 8 with polymorphic bands. To be
Figure 3.8: Novel bands detected in AML patients in single enzyme digests

AML patients (n=3)

**HindIII:**
- 3' probe

AML patients (n=2)

**BglII:**
- 3' probe

AML patients (n=4)

**HindIII:**
- 5' probe

2 AML patients

**EcoRI:**
- 3' probe

2.5kb

1.2kb

8kb
absolutely certain that the bands seen in the 7 cases were not artefacts or due
to cross hybridising sequences, another repeat analysis was performed. The
result showed consistency of the 2.5kb band (HindIII, 5' probe) but there was
variability of both the 5kb (EcoRI, 5' probe) and 4.5kb (HindIII, 5' probe)
bands. To investigate this further, 4 sets of experiments were carried out
involving these 7 samples.

3.3.5 Further investigative analysis of the 7 samples with a possible
common mutation/polymorphism at the 5' end of the RB1 gene

This potential 'hot-spot' for the mutation/polymorphism could either be in the
promoter region, within exons 1-9 or within introns.

3.3.5.1 PCR amplification of the promoter region

The 1.6kb promoter region was amplified using the Polymerase Chain
Reaction (PCR). The PCR technique is described in Chapter 2. The primers
which were designed from the promoter sequence (Lee et al., 1993) are as
follows: PF(5'-3'), GAGCTCTTGCTTAATACTC (sense) [nucleotides -1585
to -1565] and PR(5'-3'), GGCCAAAAAGAGTCCCCTGCA (anti-sense)
[nucleotides -20 to -10]. Thirty-five cycles of amplification were carried out
using an annealing temperature of 60°C. All 7 patients showed the expected
1.6kb band. To test whether there were any new EcoRI or HindIII sites within
the promoter sequence of these patients, PCR products of two samples were
electrophoresed through low melting point agarose, purified (Wizard PCR
Preps, Promega UK), and digested with either EcoRI or HindIII. To control for
the EcoRI restriction digest, the 4.7kb RB1 full length cDNA which contained
an EcoRI site was used. The PCR products from the patients remained intact
after the digestion, indicating that there were no new EcoRI or HindIII sites
within them whereas the control DNA was split into 2 fragments as expected.
The results from this experiment suggested that the promoter region was not
the likely site of the common mutation/polymorphism.

3.3.5.2 Southern blotting of samples using the RB1 promoter fragment
as a probe

A representative blot containing all the 7 samples with the common mutations
was hybridised using the RB1 promoter PCR fragment as the probe. From the
genomic map containing the restriction sites (Figure 3.1), the only band
expected from a HindIII digest on a Southern blot analysis was a single band of 14kb. As the autoradiograph shows (Figure 3.10), all the samples analysed contained only the 14 kb band. The common 4.5kb band was not present. Hence, it could be concluded that the common mutation/polymorphism was not likely to be within the promoter region.

3.3.5.3 RT-PCR of exons 1-9

RT-PCR analysis of the coding region from exon 1 to exon 9 was performed for 5 of the 7 samples. Reverse transcription and PCR were carried out according to the methods described in chapter 2. The primers for the PCR, designed from the coding sequence (Goodrich and Lee, 1993), were 1F: (5'-3') TTCCGTTTTTTCAGGGGAC (sense) [nucleotides 154-174] and 9R: (5'-3') CTCTGGAAGTCCATTAGATGT (anti-sense) [nucleotides 972-992]. All 5 RNA samples analysed showed the expected size of the coding sequence from exon 1 to exon 9 which is 0.9kb. Digestion of these RT-PCR products with EcoRI or HindIII failed to reveal any new restriction sites. The results of this experiment suggest that the common mutation was not likely to be within the coding region of exons 1 to 9. It does not however exclude the possibility that the altered sequence could be contained within the 5' untranslated region or the introns.

3.3.5.4 Southern blotting analysis using 3 different probes

The third and final analysis done on these nine samples utilised Southern blotting again. Three different probes were prepared by performing PCR from three different regions within exons 1-9. Primers were designed based on the coding sequence of the RB1 gene. These probes were labelled as probe D (exons 1-2), probe E (exons 3-6) and probe F (exons 7-9) (Figure 3.9). The PCR products were sequenced according to the methods described in chapter 2 to confirm that they contained the correct sequences for the respective exons. These PCR probes together with the full length 5' probe were used separately to hybridise 4 identical membranes containing two of the samples with the common mutation. The autoradiographs are shown in Figure 3.11(A and B). Surprisingly, neither of the common 5.0kb (EcoRI) nor the 4.5kb (HindIII) bands were present. This experiment was repeated using two other samples but again the abnormal bands were not detected. Finally, the original membranes containing the abnormal bands were reprobed with the full length 0.9kb probe. This time the common bands could not be detected. The
Figure 3.9: PCR probes used in Southern blotting analysis of AML patients with the 'common' rearrangements detected in the EcoRI and HindIII digests.
Figure 3.10: Southern blotting analysis of 7 AML samples with common rearrangements using HindIII digests and the promoter fragment as the probe.
Figure 3.11A: Southern blotting analysis using 4 different probes of 2 AML patients with previously detected common rearranged bands

* EcoRI digests *

Key:
- Denotes germline bands
- Denotes 'common' rearranged band
- Denotes position of expected band

Probe D

Probe E

Probe F

0.9kb probe

0.9kb probe

Denotes germline control

Denotes negative control (WERI-1 cells)
Figure 3.11B: Southern blotting analysis using 4 different probes of 2AML patients with previously detected common rearranged bands

Key:
- Denotes germline bands
- Denotes 'common' rearranged band
- Denotes position of expected band
- Denotes germline control
- Denotes negative control (WERI-1 cells)

Original membrane

0.9kb probe

C^G Pt

C^G C^- Pt Pt

C^G C^- Pt Pt

C^G C^- Pt Pt

Probe D

HindIII digests

Probe E

Probe F
consolation was that the 2.5kb band seen in one of the 7 patients was consistently detected by probe D, hence indicating that it is most likely due to a common polymorphism within the intronic region from exon 3 to exon 6.

To summarise the results, of the 106 AML patients analysed by Southern blotting, none had gross rearrangements of the \textit{RB1} gene. Fourteen patients consistently showed the presence of a novel band in a single enzyme digest, hence these were considered to be restriction enzyme polymorphisms.

\textbf{3.4 DISCUSSION}

The principal objective of this chapter was to establish conclusive data on the frequency of rearrangements of the \textit{RB1} gene in patients with AML using Southern blotting analysis. The strength of the approach was in the number of patients analysed, the number of normal controls used and the number of repeat analyses performed. The final data showed that none of the patients had true rearrangements and in addition these studies highlighted several other important sources of difficulty in this type of analysis. First was the disappearance of some abnormal band patterns despite being reproduced initially at least twice, second was the reproducibility of artefactual bands and finally, perhaps most importantly, was the frequency of polymorphic bands in this large and complex gene. I shall attempt to discuss these points not only in the context of my data but with supporting evidence from other studies.

As mentioned before, Southern blotting analysis is a powerful tool for analysing gene structure and for detection of gross rearrangements and major deletions within a gene. The possible alterations in bands obtained can either be one of the following: the absence of normal germline bands which indicates homozygous deletions, reduced intensity of bands which suggests heterozygous deletions and finally, additional bands which may either indicate rearrangements involving restriction sites or polymorphic bands. Another mechanism for the appearance of novel bands is the presence of cross-hybridising sequences, although there is currently no evidence for sequences structurally related to the \textit{RB1} gene in the human genome (Belka \textit{et al.}, 1991).

\textit{Difficulties in the interpretation of data}

The analysis of the \textit{RB1} gene by Southern blotting was made difficult by both its large size and its complex genomic structure. Because of this, certain fragments were not easily detected on a Southern blot. Absence of these
bands could easily have been interpreted as deletions if care was not taken to repeat any abnormal result several times. To add to this difficulty, there is a GC-rich region in the 5' end of the cDNA which increased the occurrence of non-specific bands. Although this was solved by probing with a truncated version of the 5' probe, the 0.9kb probe was still required to analyse exon 1. Another problem was the lack of knowledge on the full extent of the polymorphic sites within the RB1 gene thus making it difficult to explain the additional bands. It was easy and tempting to label novel bands as rearrangements but the fallacy of this was that these bands could merely represent polymorphisms. There were also reported technical difficulties in obtaining certain bands on the Southern blot (Lee et al., 1991). This could be attributed to the variable Tm (melting temperature) of some of the smaller exonic fragments annealing to the probe cDNA as well as to the varying stringency of wash required after hybridisation. For example, many studies have not been able to show the 4.5kb band in the HindIII digest when probed with the 3' probe (Figure 3.6). Others have failed to show the smaller 2.5kb band on the EcoRI digest when probed with the 5' probe. In addition, there are claims that certain bands were abnormal when they were actually either germline bands or a polymorphism (Ahuja et al., 1991).

Therefore, it was crucial that in analysing the RB1 gene, abnormal results should be repeated more than once, and any abnormal band detected in one enzyme digest must be shown to result in rearrangement in a different enzyme digest. It was also vital that an adequate number of normal controls were screened, not only to establish the normal germline bands for each digest but also to look for the presence of possible polymorphisms. This simple advice applies not only for RB1 but when screening for any gene. Only then it is possible to obtain conclusive and reliable data on the frequency of rearrangement of a particular gene in a certain disease.

Comparison with previous data
After screening 106 AML patients, none were found to have deleted bands. Fourteen patients consistently showed the presence of additional bands and further analysis suggested that they were all likely to be due to polymorphisms rather than arising from mutations. This result differs significantly from the study by Ahuja et al (1991) which claimed that 5 out of 54 AML cases studied by similar methods had rearrangements of the RB1 gene. Four of these 5 cases were either of the M4 or M5 FAB types hence prompting a further claim that RB1 gene mutations were more common in the monocytic leukaemias
(27%). I believe that the findings on monocytic leukaemias were purely coincidental and the overall data is not convincing. The number of patients with monocytic leukaemias analysed were small (15 cases compared to 60 cases studied here). There was a lack of normal controls screened and there was ambiguity in some of the bands claimed to be abnormal. Some of the abnormal samples had novel bands, but there was no attempt to exclude the possibility that they were polymorphic bands. In fact, one of the bands labelled as abnormal was actually a normal germline band. Another sample with reduced band intensities was claimed to have a heterozygous deletion of the gene, however, this could have been due to reduced DNA loading. It was also not stated how many times the abnormal results were reproduced in repeat experiments.

**Polymorphisms in the RB1 gene**

The word *polymorphic*, which literally means "many forms", has received many definitions by geneticists. The popular definition of polymorphism is the occurrence of a variation of the normal in >1% of the population that cannot be explained or maintained by mutation alone (McKusick 1994). Another form of this definition states that a locus is polymorphic if there are two or more alleles, each of which occurs with a frequency of 1% or more (Thompson et al., 1994). It is arguable though that it is quite difficult to differentiate polymorphism from mutation, which is defined simply as a change in the genetic sequence, when studying samples with an underlying disease, unless the polymorphism is known and well defined in normal individuals. A more appropriate definition is a functional definition which is the alteration of the genomic sequence without affecting the function of the expressed protein. For the purpose of interpretation of Southern blotting results, consensus has it that a band is more likely to be polymorphic if it only appears in a single enzyme digest. Likewise, if a sample shows abnormal bands in more than one enzyme digest, then the bands are more likely to be truly rearranged. Although this criteria is arbitrary, it is reasonable and valid for the interpretation of Southern blotting results. As mentioned earlier, the total mutation rate is likely to be higher because of alterations which are not detected by this technique.

One of the problems in this RB1 study was the lack of knowledge of the full extent of polymorphic sites within the gene. Only one of the enzymes involved, the BamHI, is known to have a polymorphic site within the RB1 gene. The polymorphic sites for EcoRI, HindIII or BglII have not been fully
characterised. Although the normal controls did help in defining the germline bands as well as uncovering two rare polymorphic sites, the numbers screened were too small to cover all the polymorphisms within this gene. The question was how to differentiate polymorphism from actual rearrangement when both of them present as novel bands. Hence, the simple working criteria for defining polymorphisms and rearrangements for the interpretation of Southern blot data was justifiable.

The BamHⅠ polymorphism has repeatedly been mentioned in previous sections. Fifty one percent of the samples screened were heterozygous for this polymorphism. The significance of this polymorphism within the context of AML is limited. However for patients with retinoblastoma tumours this polymorphic site can be used as a genetic marker to follow the transmission of alleles through generations of a family. The other common polymorphism found was an EcoRI site situated within the 3' end of the gene. Of the 106 patients, 18% showed this polymorphic pattern. Two of the 18 normal controls had a unique polymorphic band each. These polymorphisms have not been reported before and could be said to be rare. Three unique polymorphic bands were seen in 3 patients. Three other patients had a common polymorphic HindⅢ site in an intron within the region from exon 3 to exon 6. Another common polymorphic HindⅢ site was found at the 3' end of the gene. Eight patients had this polymorphism. Interestingly, neither of the HindⅢ polymorphisms were detected in the 18 normal controls. The significant occurrence of polymorphisms underlined the importance of careful interpretation of Southern blotting results.

**Sensitivity of detection and possible contamination by normal cells**

The practical aim of any screening technique is to reduce the number of false negatives to a minimum and to maximise the detection of true positives. A sensitive technique is one which would achieve these targets. However, the internal limitations of any technique will restrict the sensitivity from reaching 100%. For Southern blotting, it is a question of whether an abnormal sequence of DNA can be detected amongst the many other normal alleles. Studies have claimed and shown that the sensitivity of detection using the Southern blot technique is between 1-5%.

The sensitivity of detection experiment was carried out not only to establish the sensitivity of the technique in the author's hand but also to allay some concerns about 'contamination' by non-leukaemic cells in the leukaemic
samples. The 2.5% detection level confirmed that the sensitivity of the technique was in the range reported by others. More importantly, as the ratio of abnormal DNA to normal DNA content is equivalent to the ratio of blast cells to normal haemopoietic cells in patients samples, the result meant that the Southern blot technique should detect any abnormal band arising from only 2.5% of blast cells. Having said that, there is a possibility that an abnormal band would not be detected at all should the recognition sequences be too small for the cDNA probe to detect. The result of the sensitivity experiment also indicate that the presence of only 2.5% of normal cells in the patients samples is sufficient to give rise to germline bands on the Southerns. This brought the issue of contamination of leukaemic blood samples by normal cells into perspective and will be further discussed later.

**The false 'common mutation' in 7 patients**

Seven of the cases screened initially showed identical additional bands in two different enzyme digests. By the working criteria, these were defined as true rearrangements. The presence of identical rearrangements in these 7 patients suggested the possible presence of a mutational 'hot-spot' within the gene. In fact, a similar 4.5kb band from the HindIII digest has been described before in a breast cancer cell line (T’Ang et al., 1988) and they concluded that this band was due to a duplication of a segment of the gene, resulting in novel bands in both the HindIII and EcoRI digests. However, the specific location of this rearrangement was not pursued by the group.

There were several arguments against the belief that a mutational 'hot-spot' was present. Firstly, this form of abnormal band configuration has only been described in the study mentioned above despite other studies on the RB1 gene. Secondly, one of these patients also had another non-germline band (the 2.5kb band) in the HindIII digest. It would seem unlikely that a common set of mutations would also contain another site of mutation unless there was a double mutation in this particular patient. Thirdly, this concept of a mutational 'hot-spot' within the RB1 gene, although very attractive, has not been conclusively proven to exist despite sporadic reports. Studies which claimed that the RB1 'pocket-region' was the seat for this hot-spot have not been substantiated sufficiently by evidence at the DNA level. Shimizu et al (1992) analysed 24 retinoblastoma patients by PCR-SSCP and found 57% of the mutations occurred within the coding area for the pocket region of the protein. However, two bigger studies involving 106 and 232 unrelated retinoblastoma patients respectively using the same techniques have found
that mutations are spread almost evenly throughout the gene (Lohmann et al., 1994; Blanquet et al., 1995). Yet a study analysing human sarcomas found deletions to occur mainly in the 5' end, well outside the pocket region (Reismann et al., 1989). However, none of the mutations matched the Southern blot pattern detected in the 7 AML patients in my study. Although the concept of mutational 'hot-spot' fits very well with the activity within the pocket region of the RB protein, more convincing DNA data are required to show that this is the case in vivo. Nevertheless, the presence of these bands in the AML samples warranted further investigations which were undertaken.

It was possible to work out the probable location for this common mutation at the 5' end of the RB1 gene by examining the genomic map containing the restriction sites for the enzymes EcoRI and HindIII (Figure 3.1).

The possible molecular mechanisms which could result in these extra bands were:

i. the deletion of a fragment between two restriction sites
ii. the loss of a restriction site either by mutation or a deletion
iii. the creation of a new restriction site through mutation

The third event is highly unlikely here since the chances of a new EcoRI site and a new HindIII site occurring independently in the same 7 samples is very slim. It was also improbable that they occurred simultaneously from a single mutation since the cutting sites for these enzymes are too dissimilar (A'AGCTT for HindIII and G'AAATTC for EcoRI). The most likely explanation then would be a deletional event between two restriction sites or one involving a restriction site. Another requirement was that this event would simultaneously result in a new band in both the EcoRI and HindIII digest. From the genomic map (Figure 3.1) this event could arise either from a deletion within the promoter region of the gene, the region between exons 3 and 6 and the region between exons 7 and 9. In other words it could be anywhere within the 5' end of the gene.

The third analysis of the 7 samples demonstrated a variability in the detection of the common bands. Hence, the status of the 'hot-spot' was severely questioned. It was vital then not only to prove that these bands were real but also to locate the site involved in the gene. It was this rationalisation that prompted the investigative analysis to pinpoint this critical site by PCR, RT-
PCR and Southern blot analysis. PCR analysis of the promoter and the RT-PCR analysis of exons 1-9 failed to confirm the presence of this 'hot-spot'. The Southern blot analysis using the 3 different probes provided convincing evidence that the identical rearranged bands were artefactual probably arising from cross-hybridising bands which appeared only under certain optimal experimental conditions. The reason why these bands were reproducible in the initial 3 analyses remains unclear. Certainly, the most important message of this whole affair was that abnormal bands seen on a Southern blot should and must be reproduced consistently and proven beyond doubt to be real before being labelled as true rearrangements.

**Presence of germline bands in all samples**

The germline pattern was seen in all the samples analysed. This could be explained by the simple fact that there were no deletions at all in any of the samples. However, the major problem with analysing leukaemic blood samples, in contrast to cell lines, is that it is virtually impossible to obtain a pure population of blast cells from the Ficoll-Hypaque method of mononuclear cell preparation. Analysis of cytospins prepared from the mononuclear cell fraction of some selected samples showed that the proportion of blasts in the leukaemic samples vary between 80-100%. The purity depends much on the blasts content of the initial peripheral blood sample which ranges between 30-100%. Inevitably there would be some 'contamination' by normal cells in the mononuclear cell fraction. It is fair to say that the isolated DNA would then contain a small amount of normal DNA as well. The other possible cause of a false positive result is the presence of a heterogenous blast population with some blasts containing normal \(RB1\) and others with abnormal \(RB1\). This scenario is consistent with the multistep model of leukaemogenesis whereby a leukaemic cell subclone acquires a further mutation, in this case \(RB1\), and may contribute to leukaemia progression. This heterogeneity in tumour cell population has been shown to occur using histochemical studies of human sarcomas in which 40% of the tumours analysed showed heterogeneity in the RB protein expressed (Cance et al., 1990).

There are several methods to overcome the problem of normal cell contamination of samples. One is through a better technique of blast cells separation, for example by using monoclonal antibodies and magnetic beads. The other approach is through quantification of a band or bands against a normal internal control, preferably one analysed on the same Southern blot. Reduction of intensities when compared to the normal reference band could
then be calibrated and determined to be either due to a heterozygous deletion or a homozygous deletion. Within the context of leukaemic samples, this interpretation is valid only if the exact proportion of normal cells in the sample is known. For some genes this method of quantification is easily done but unfortunately for the \textit{RB1} gene, there are too many bands to quantify and other than using the germline bands as reference points, there is no suitable control. In addition, some of the germline bands are very sensitive to low stringency wash leading to very faint bands and I have seen fluctuations in the intensities of bands, in the same sample, from one blot to another. I believe this approach of quantification for the \textit{RB1} gene is not practical. Hence, for bands with reduced intensity, unless they are highly reproducible and can be properly quantified against a normal reference band, nothing can be said about the possibility of heterozygous deletions or homozygous deletions in a sample contaminated by normal cells. For a band that is completely absent, provided that it is reproducible, then it can be safely said to be homozygously deleted.

In summary, my data showed that gross rearrangement of the \textit{RB1} gene is very rare in AML. Polymorphisms of the \textit{RB1} were commonly seen and can be misinterpreted as rearrangements. The Southern blotting technique despite its sensitivity and specificity in screening for gross structural aberrations, is actually a relatively crude method for detecting gene inactivation. Point mutations and minor deletions will not be identified by this technique. Even for retinoblastoma tumours, only 10-20\% have gross rearrangements of an \textit{RB1} allele detectable by Southern blot analysis (Yandell \textit{et al.}, 1989) and only 70\% show loss of the other allele (Zhu \textit{et al.}, 1989), yet loss of function of the \textit{RB1} gene is believed to occur in 100\% of retinoblastomas. The PCR-SSCP method is able to detect many point mutations or minor deletions. However for the \textit{RB1} gene, this would involve screening 27 different PCR fragments for each sample to cover the exons alone. Any abnormal samples would then have to be sequenced. This is a mammoth task if it is to be performed on a large series of samples. Even if a point mutation is revealed, it would not reflect on the functionality of the protein expressed. It was for this reason that the next step taken was to analyse for RB protein expression in the AML patients using western blotting.
CHAPTER FOUR

ANALYSIS OF PROTEIN EXPRESSION OF THE RETINOBLASTOMA GENE IN PATIENTS WITH ACUTE MYELOID LEUKAEMIA

4.1 INTRODUCTION

The retinoblastoma protein (pRB) is a nuclear phosphoprotein which is ubiquitously expressed in most cultured cells and normal tissues (Lee et al., 1987a, Bernards et al., 1989). It consists of 928 amino acids and the apparent molecular weight as assessed by denaturing polyacrylamide gel electrophoresis ranges from 110-116kDa. The difference in migration is caused by changes in phosphorylation of pRB which occurs at different stages of the cell cycle (Burke et al., 1992). pRB is hypophosphorylated in the G0/G1 phase of the cell cycle, is sequentially phosphorylated as the cell progresses through G1 and remains in this state until mitosis is completed. On western blotting, the hypophosphorylated form runs at 110kDa while the fully phosphorylated form migrates at 116kDa.

There is an important domain within the protein structure which has been labelled as the 'pocket region' (Figure 4.1). It consists of two non-contiguous sub-domains spanning the region from amino acids 393 to 572 and 648 to 774. This pocket region is the binding site for certain transforming oncoproteins encoded by DNA tumour viruses: the SV40 large T antigen, the adenovirus E1A protein, and the human papilloma virus E7 protein (DeCaprio et al., 1988, Whyte et al., 1989, Dyson et al., 1989, Ewen et al., 1989). The pRB pocket region has also been shown to be a target for some naturally occurring mutations (Horowitz et al., 1990; Kaye et al., 1990; Qin et al., 1992; Kato et al., 1994).

The C-terminal region contains the binding site for E2F which is one of several transcription factors known to bind with pRB (Chellappan et al., 1991). Absence or inactivation of pRB releases E2F hence allowing the transcription of genes required for entry into S phase (reviewed in chapter 1).

Although the C-terminal region has been the main focus of pRB research, the N-terminus moiety may also modulate the growth suppressive properties of pRB. Xu et al (1994) observed in their study that an N-terminal truncated pRB,
Figure 4.1: Retinoblastoma protein structure showing the multiple phosphorylation sites and the binding domains

(Adapted from Goodrich and Lee, 1993)
94kDa in size, exerted a more potent cell growth suppression as compared to the full-length pRB protein in a diverse group of cell lines examined.

Although the exact relationship between the biochemical properties of pRB and its biological activity in tumour suppression is unknown, there are however important observations made from studies so far (reviewed in chapter 1) which can be summarised as follows: (a) phosphorylation of pRB is necessary for the progression of a normal cell from G1 into the S phase, (b) the binding of certain viral oncoproteins to pRB results in the impairment of the growth inhibitory function of pRB leading to immortalisation of infected cells, (c) pRB acts as a transcriptional repressor of genes involved in cell proliferation through its binding with the transcription factor E2F, (d) pRB binds to a host of other transcriptions factors and is also capable of exerting both positive and negative regulation of certain gene promoters (e) pRB has a role in differentiation, and (f) pRB may also play a part in apoptosis.

Hence a simplistic view would be that pRB sits at a critical and important point in regulatory pathways controlling a cell's fate. Loss of pRB function removes a significant part of this regulation thus depriving the cell of an important mechanism for braking cell proliferation through the modulation of gene expression (Weinberg, 1995). It is easy to understand that mutations or abnormal regulation resulting in a defective pRB protein, or its absence would then confer a cell with a growth advantage as well as losing its ability to differentiate. Hence, alterations in pRB could possibly initiate a cancer or contribute to increasing the grade of malignancy of a preexisting cancer.

The evidence that pRB inactivation contributes to the evolution of cancer has been provided by studies which showed the common occurrence of rearrangements at the DNA level (reviewed in chapter three) and the absence of protein expression in a broad range of malignancies. Table 4.1 shows some of the malignancies where pRB inactivation has been reported.

Among the haematological malignancies studied, loss of pRB has been found to be common in chronic lymphoid leukaemia (42%) (Kornblau et al., 1994) and high grade non-Hodgkin's lymphoma (58%) (Weide et al., 1994). Preudhomme et al (1994) however analysed 37 patients with myelodysplasia and found none with loss of pRB.
Table 4.1: Frequency of pRB inactivation in solid tumours

<table>
<thead>
<tr>
<th>Type of malignancy</th>
<th>Study</th>
<th>Frequency of inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcomas</td>
<td>Cance et al., 1990</td>
<td>31/44 (70%)</td>
</tr>
<tr>
<td>Non-small cell lung Cancer</td>
<td>Xu et al., 1991</td>
<td>10/36 (28%)</td>
</tr>
<tr>
<td>Low grade Bladder Cancer</td>
<td>Presti et al., 1991</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>High grade Bladder Cancer</td>
<td>Presti et al., 1991</td>
<td>21/26 (81%)</td>
</tr>
<tr>
<td>Breast Cancer</td>
<td>Pietilainen et al., 1995</td>
<td>75/205 (37%)</td>
</tr>
</tbody>
</table>

In acute myeloid leukaemia, loss of pRB function has been reported by at least 4 groups (Table 4.2). However only one coupled the protein studies with DNA analysis. Despite lacking parallel data at the DNA level, the similarity of these results suggests a significant role of pRB inactivation in AML. It is interesting to note the study by Weide et al which showed a high frequency of pRB inactivation in monocytic leukaemias. This concurred with the data at the DNA level (Ahuja et al., 1991) which showed rearrangements to be common in the monocytic leukaemias.

Table 4.2: Studies of pRB expression in Acute Myeloid Leukaemia

<table>
<thead>
<tr>
<th>Study</th>
<th>Frequency of abnormal pRB</th>
<th>Southern blotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tang et al (1992)</td>
<td>5/18 cases (28%)</td>
<td>All normal</td>
</tr>
<tr>
<td>Kornblau et al (1994)</td>
<td>22/113 cases (19%)</td>
<td>Not done</td>
</tr>
<tr>
<td>Weide et al (1993)</td>
<td>11/20 M4 and M5 (55%)</td>
<td>Not done</td>
</tr>
<tr>
<td>Zhu et al (1994)</td>
<td>11/39 cases (30%)</td>
<td>Not done</td>
</tr>
</tbody>
</table>

The aims of the studies presented in this chapter were to consolidate the above findings using a large series of patients, to confirm whether the frequency of inactivation is commoner in monocytic leukaemias, to correlate the findings at the DNA level to the protein expression and to ascertain whether inactivation of pRB has any influence on the outcome of disease in AML.

4.2 MATERIALS AND METHODS

A total of 103 AML patients of varying FAB types were analysed. All except one were samples obtained at presentation. The one exception was a sample taken when the patient became refractory to treatment (patient 86). Nine
haematologically normal control samples were also studied. The Daudi cell line and the WERI-1 cell lines (McFall et al., 1977) were used as positive and negative controls respectively. All protein samples were crude total cell lysates prepared according to methods described in chapter 2.

4.2.1 The western blotting technique

The standard technique is described in detail in chapter 2 (Harlow and Lane, 1988). For the pRB westerns, 6% SDS-polyacrylamide gels were used. Protein from 3 x 10^5 cells was loaded into each lane and electrophoresis was carried out at a constant voltage of 100volts. Pre-stained markers (Rainbow markers, Amersham, UK) were used as protein size standards. The samples were run until the bromophenol blue reached the bottom edge of the gel. The gel was then blotted onto Hybond-C extra membranes (Amersham, UK) by the semi-dry electro-transfer method as described. The anti-pRB antibody PMG3-245 (Pharmingen USA) was used at a concentration of 0.1µg/ml. This mouse monoclonal antibody recognises an epitope between amino acids 300-380 in the RB protein. The peroxidase conjugated secondary antibody was used at a concentration of 0.05µg/ml. All samples which had no detectable pRB protein on the western blot were confirmed with a repeat analysis using another antibody, the Pab-2 anti-RB antibody (Oncogene Science, UK) which recognises an epitope at the C-terminal end of the protein. This is a rabbit polyclonal antibody and a concentration of 0.1µg/ml was used. Both anti-actin and anti-tubulin antibodies were used to control for loading and to assess protein integrity.

4.2.2 Showing the different phosphorylation states of pRB during the cell cycle

Daudi cells were grown in the presence or absence of α-interferon (300U/ml for 48 hours) which arrests the cells in the G1 phase of the cell cycle (Burke et al., 1992). Protein lysates were made and western blotting performed.

4.3 RESULTS

4.3.1 pRB expression in cell lines

The western blotting analysis of the 2 cell lines using two different antibodies is shown in Figure 4.2A. The Daudi cells showed both the hypo- and
Figure 4.2A: pRB expression in Daudi and WERI-1 cell lines using 2 different anti-pRB antibodies

PMG3-245 antibody

Pab-2 (C-terminal) antibody

116kDa
110kDa

a  b  c

Lanes:
a  Daudi cells
b  WERI-1 cells
c  Mononuclear cells

Figure 4.2B: Different phosphorylation states of pRB in Daudi cells

116kDa
110kDa

a  b

Lanes:
a  Daudi cells without interferon
   (showing the hyperphosphorylated form of pRB)

b  Daudi cells with interferon
   (showing the hypophosphorylated form of pRB)
hyperphosphorylated forms of pRB (lane a) using either of the antibodies. The WERI-1 cell line showed the complete absence of pRB expression (lane b) and it was used as the negative control during subsequent analysis of patients samples.

The different states of phosphorylation were then shown separately by the Daudi cells cultured in the presence and absence of α-interferon. In the presence of interferon, cells were arrested in G1 hence they expressed mainly the hypophosphorylated form of pRB (Figure 4.2B, lane b). Cycling Daudi cells instead showed the presence of 116kDa band which represents the hyperphosphorylated form of pRB (Figure 4.2B, lane a).

4.3.2 Protein loading and integrity in the AML samples

To test for protein loading and integrity, the status of actin in all the samples were analysed. The actin antibody detects a 44kDa band on the western blot. The intensity of this band correlates with the amount of protein loaded onto the gel. It must be mentioned here that quantification is mainly visual and comparative. Although in terms of cell numbers, the same amount was loaded into each lane, the actin bands would help to correct further the amount of loading when interpreting the intensity of pRB bands.

To reflect the protein integrity, the actin band should also be single and intact. Seventeen samples showed fragmentation of the actin band into multiple bands (Figure 4.3A, representative blot) suggesting the presence of protein degradation (Tang et al., 1993). It was also possible that they were due to different isoforms of actin. In fact, four different isoforms of actin are known to exist. To test this phenomenon further, fresh mononuclear cells were prepared from a normal individual and aliquots were taken at different time intervals: 24 hours, 48 hours at 37°C and 72 hours at 37°C. These aliquots of cells were lysed with sample buffer and a western blotting done. The actin bands from these samples are shown in Figure 4.3B. The samples at 24 and 48 hours showed the multiple actin bands whilst that at 72 hours had only the 44kDa band. Although the results of this test were not conclusive as to the nature of the multiple actin bands, it was decided that the AML samples showing this pattern should be excluded from the final data. To be certain of the integrity of the samples, tubulin westerns were also done for all samples. Only those with single actin and tubulin bands were included in the final analysis.
Figure 4.3A: Actin western of AML samples showing multiple actin bands

Figure 4.3B: Evaluation of multiple actin bands for possible degradation
4.3.3 pRB expression in normal controls and AML patients

All 9 normal controls showed the presence of normal pRB. A variable pattern of protein expression was found in the remaining 86 AML cases (Table 4.3). Forty-four samples each showed a single and intact 110kDa pRB band. Since the intensity of these are comparatively equal to the Daudi cells and the normal controls after correction for loading, they were interpreted as normal. A complete absence of the pRB band was seen in 20 cases (represented by lanes i and j, Figure 4.4). Repeat analysis using the Pab-2 antibody performed on all samples with no pRB, showed that the results were reproducible (represented by lanes a-g, Figure 4.5B). The corresponding actin bands were intact. No other bands were seen in these samples. Altered pRB bands were detectable in 7 others (represented by lanes e-g, Figure 4.4). All of them had the 110kDa band present as well and the altered bands appear to be truncated bands with sizes varying between 90-100kDa.

Table 4.3: Spectrum of abnormal protein expression in AML patients

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total absence of RB protein</td>
<td>20</td>
</tr>
<tr>
<td>Altered pRB band size</td>
<td>7</td>
</tr>
<tr>
<td>Reduced intensity of pRB band</td>
<td>8</td>
</tr>
<tr>
<td>Hyperphosphorylated pRB band present</td>
<td>7</td>
</tr>
<tr>
<td>Normal pRB expression</td>
<td>44</td>
</tr>
<tr>
<td>TOTAL</td>
<td>86</td>
</tr>
</tbody>
</table>

Seven samples showed a markedly diminished amount of pRB band. As shown in Figure 4.4 (lanes I and m), the 110kDa bands were barely detectable in these samples. Their corresponding actin bands can be seen to be intact and approximately constant. A comparison of the intensities of the pRB bands to a normal control (lane c) suggests that there is at least a 5x reduction in intensity. Interestingly, 7 other samples showed the presence of both the hypophosphorylated and hyperphosphorylated forms of pRB (Figure 4.4, lanes h and k). The intensity of the hyperphosphorylated bands appear less than the hypophosphorylated bands in all the 7 cases.

Unfortunately, no remission protein samples were available for any of the patients with abnormal pRB. However, a protein sample was available for patient 86 when he became refractory to treatment. The results showed the
Figure 4.4: Representative western blot showing the spectrum of pRB expression in AML patients

Lanes:
a  Daudi cells without interferon (cycling)
b  Daudi cells with interferon (non-cycling)
c  Normal control
d  AML patient with normal pRB band
e-g  AML patients with altered pRB bands
h&k  AML patients with both hypo- and hyperphosphorylated pRB bands
i-j  AML patients with absent pRB
l-m  AML patients with reduced intensity of pRB bands
Figure 4.5: Western blot of 8 AML patients using Pab-2 anti-RB antibody

Lanes:

a-g  AML patients with undetectable pRB
h    Patient no.86 during follow-up showing undetectable pRB
i    Patient no.86 at presentation showing intact pRB
presence of a normal pRB band at presentation but the band was undetectable using a sample obtained when the patient became refractory to treatment (Figure 4.5, lane h and i).

4.3.4 Complete remission and survival data of AML patients versus pRB status

The complete remission and survival data were obtained from the Medical Research Council (MRC), Oxford, and University College Hospital, London. The data was available for 77 of the 86 patients analysed. The patients had either received the DAT or the ADE treatment protocol. Table 4.4 shows the comparison of data between patients with normal and abnormal pRB.

<table>
<thead>
<tr>
<th></th>
<th>Abnormal pRB</th>
<th>Normal pRB</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete remission rate</td>
<td>15/25 (60%)</td>
<td>39/55 (71%)</td>
<td>0.19</td>
</tr>
<tr>
<td>Median survival (days)</td>
<td>195</td>
<td>376</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Although the frequency of complete remission is lower in the group with abnormal pRB as compared to the group with normal pRB, statistical analysis using Fisher's test however proved that this difference was not significant (P value =0.19). The survival data was analysed by plotting the Kaplan-Meier survival curves for those with normal and abnormal pRB. The survival curves are shown in Figure 4.6. The median survival in days for both groups are shown in Table 4.4. The group with normal pRB appear to have a longer median survival, however statistical analysis using the log-rank test suggest that this difference is not significant (P value = 0.28).

4.4 DISCUSSION

Loss of RB protein expression or the presence of a truncated protein product by western immunoblotting has proven to be a sensitive indicator of RB mutation in several types of cultured tumour cells (Lee et al., 1987b; Shew et
Figure 4.6: Survival of AML patients: comparison between abnormal pRB and normal pRB groups

P = 0.277
al., 1990; Horowitz et al., 1990). This correlation has been extended to studies in primary tumours which showed similar findings (Bookstein et al., 1990). The possible patterns of protein expression detected on a western blot analysis could be one of the following: normal expression, reduced expression, overexpression and the expression of a truncated protein.

All the patterns mentioned, except for overexpression, were seen in the AML protein samples analysed. These patterns can be due to diverse mechanisms operating at the DNA or protein synthesis level. Total absence of protein can be due to gross deletions of the gene or a single point mutation occurring early on in the gene. This point mutation must result in a premature stop codon or a frameshift that completely alters the whole protein sequence. A point mutation occurring further along the gene may or may not result in a truncated protein. This depends on whether an mRNA is successfully transcribed or not. Even if a shortened transcript is produced, the translated protein may not be sufficiently stable to be detected on a western blot.

**pRB absence in AML patients**
The results from the western blotting showed that 23% (20/86) of the AML patients screened had no detectable pRB. Significant protein degradation could be excluded as the samples had intact actin and tubulin. This percentage of pRB inactivation, although lower than in previous studies, reaffirmed the high incidence of abnormal pRB expression in AML, implying a role for the gene in the pathogenesis of this leukaemia.

The underlying molecular lesions causing the complete absence of the RB protein in these 20 samples are unclear since no gross rearrangements were seen at the DNA level by Southern blotting analysis (chapter 3). It is possible that this loss of pRB was as a result of point mutations or small deletions within the gene causing aberrations in the mRNA transcript. Such transcripts have been shown to be extremely unstable and to result in failure of expression of the protein (Kato et al., 1994). Further evidence for these postulations were seen in a study involving 106 retinoblastoma patients (Lohmann et al., 1994) where 20 cases were found to have small deletions (1-18bp) and the majority of them resulted in the premature truncation of the transcripts. There was however no protein analysis done. Yandell et al (1989) found point mutations in 7 retinoblastoma patients, 4 of which resulted in new premature stop codons while the other 3 created abnormal splice signals leading to aberrant splicing during biogenesis of the mRNA transcripts. It
would have been interesting to see the status of the mRNA transcripts by Northern blot analysis in these 20 AML patients but unfortunately RNA samples were not available. An alternative explanation to account for the total absence of pRB without any rearrangements at the DNA level was a mutation occurring in a regulatory region of the gene. The promoter region of RB1 has many regulatory sequences (Goodrich and Lee, 1993; Ohtani-Fujita et al., 1994) and mutations involving any of these sites could result in a complete failure of RNA expression. A deletion in the promoter region has been found to occur in a prostate carcinoma case (Bookstein et al., 1990) where a 103bp deletion in the promoter region resulted in the total inactivation of the protein. Two cases of retinoblastoma have also been reported to have point mutations within the promoter region (Sakai et al., 1991).

Thirdly, it is possible that absence of pRB represents disordered post-translational regulation rather than a defect in pRB itself. This could be an abnormality in the relevant transcription factors with failure of production or increased degradation of the protein. Again, mRNA studies might be informative by showing the presence of intact RB1 transcripts in the absence of protein expression.

Finally, it was also possible that some of the samples with apparent normal pRB bands could actually have proteins which are non-functional especially if the lesions occurred within the pocket region of the gene. For example, a single amino acid substitution in exon 21 in a small cell lung carcinoma cell line (Kaye et al., 1990) resulted in a normal sized pRB on a western blot but was defective both in phosphorylation as well as in binding to viral oncoproteins. It is likely therefore that the frequency of 23% is an underestimate of the actual extent of pRB inactivation in AML.

**Patients with altered pRB bands**

The presence of altered band sizes in 7 samples warrants comment. In all these 7 cases, the normal 110kDa band was also present. This could be due to the presence of contaminating normal cells although unlike the RB1 Southern, no sensitivity testing was done for the western blotting. There were two possibilities to explain the mechanism behind the altered bands. One was the occurrence of protein degradation in the samples although the presence of normal actin and tubulin bands in all these 7 cases made this unlikely. The more plausible explanation for these altered bands was the presence of truncated protein products resulting from mutations or deletions within the
coding sequence. For example, the SAOS-2 cells, an osteosarcoma cell line, has deletions in the exons 21-27, which accounted for its truncated pRB of 95kD in size. Sample g in Figure 4.4, showed a 90kDa band in addition to the normal pRB band. This interpretation could be confirmed by purifying the truncated protein and determining its N-terminal peptide sequence but this is potentially very difficult to do. Alternatively, functional tests could be performed by analysing the ability of these proteins to bind oncoproteins such as E2F in band shift assays.

Reduced pRB expression in AML samples
Eight patients showed a markedly reduced expression of pRB. This was based on the observation that the intensities of the bands were much less than the normal samples, after correcting for loading. It was however decided not to interpret this as abnormal pRB expression since the normal level of pRB in a cell varies throughout the cell cycle and from one cell type to another. It has been shown that the levels of pRB can increase up to 8-fold during the cell cycle (Buchkovich et al., 1989). Hence, in these 8 cases, it could be possible that there are more quiescent cells than in the others. There is however one study which has performed quantification on cases with reduced intensity of pRB bands. Kornblau et al. (1992, 1994) in their studies on pRB expression in AML and CLL, used an arbitrary normal range which they defined as being within 2SD of the mean intensity of a series of normal mononuclear cell fractions when measured on the densitometer. In a surprising contradiction, Kornblau’s group reported in a follow-up study that some AML cells suspected of having mutant \textit{RB1} genes on the basis of low levels of pRB have an increase in the levels of normally phosphorylated pRB when stimulated with mitogen (Zhang \textit{et al.}, 1995). Furthermore, the normal reference range in the mononuclear cell fraction might not be comparable to the actual normal range for the leukaemic blasts concerned. The use of this arbitrary normal range for pRB, I believe, must be exercised with great caution.

Presence of hyperphosphorylated pRB in AML patients
Seven AML patients (8%) showed the presence of the hyperphosphorylated form of pRB. This form of pRB implied that there were a significant amount of blasts which were cycling. Kornblau \textit{et al.} (1994) found that 30% of AML patients had the hyperphosphorylated forms of pRB, and these patients were found to be within the group with better prognosis. It was probable that the presence of a higher proportion of blasts which were cycling resulted in an
increased response to chemotherapy. There was no apparent difference in the seven patients in my study with regard to their complete remission rates.

To summarise at this point, the data on the western blotting showed that 20 of the 86 AML patients had no detectable pRB and another 7 with altered proteins making a total of 31% with abnormal expression. There was no preponderance of abnormalities in patients with M4 and M5 FAB types (Table 4.5), in contrast to the data from Ahuja et al and Weide et al. On the other hand 50% of patients with M2 and M3 in my study had abnormal pRB which corroborated the data by Kornblau et al (1994) who analysed 113 patients. The difference in frequencies between the FAB types was not statistically significant (P values = 0.12 to 0.34).

Table 4.5: Frequency of pRB abnormalities in AML cases according to their FAB types

<table>
<thead>
<tr>
<th>FAB type</th>
<th>Number of cases analysed</th>
<th>Number with abnormal pRB</th>
<th>Frequency of pRB abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>13</td>
<td>3</td>
<td>23%</td>
</tr>
<tr>
<td>M2</td>
<td>22</td>
<td>11</td>
<td>50%</td>
</tr>
<tr>
<td>M3</td>
<td>4</td>
<td>2</td>
<td>50%</td>
</tr>
<tr>
<td>M4</td>
<td>20</td>
<td>5</td>
<td>25%</td>
</tr>
<tr>
<td>M5</td>
<td>8</td>
<td>3</td>
<td>38%</td>
</tr>
<tr>
<td>M6</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Not known</td>
<td>18</td>
<td>3</td>
<td>17%</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>27</td>
<td>31%</td>
</tr>
</tbody>
</table>

The overall frequency of inactivation consolidated the data from previous studies. The frequency obtained was probably an underestimate due to several reasons. Firstly, as mentioned above, there could still be functional abnormalities in those samples with apparent normal sized pRB on the western blots. Secondly, the antibodies used recognise only certain epitopes of the protein. Any changes in pRB occurring outside this locus might not have resulted in an abnormal band. Thirdly, those with reduced levels of pRB could possibly have no pRB expression since the bands detected might be due to the contribution from the contaminating normal cells. The other possibility of this reduced expression is the presence of two subpopulations of leukaemic
blasts, one with normal pRB and another with no pRB as a result of a clonal selection. This heterogeneity in pRB expression has been shown to be present in immunohistochemical studies of human sarcomas (Cance et al., 1991).

The inevitable question at this stage is how does inactivation of pRB fit into the biology of leukaemogenesis. It is clear that mutation of both alleles resulting in pRB inactivation is required for the development of retinoblastoma (Knudson et al., 1971, Cavanee et al., 1983). All retinoblastoma tumours or cell lines analysed so far have shown no intact pRB (Horowitz et al., 1990). However, this phenomenon occurs only in a subset of other malignancies, including leukaemia. It has also been shown that pRB inactivation leads to more invasive forms of cancer and poorer response to treatment. The tissue specificities for the initiation and progression of cancer resulting from RB mutations suggest that initiation and progression may be separate processes.

Both alleles of the RB1 gene have to be mutated to inactivate the protein. Individuals with germline mutations of both alleles of the RB1 gene have a >90% chance of developing retinoblastoma. They also have >200 times the normal risk of developing osteosarcoma in the second decade of life but have normal risk of other malignancies including leukaemia. Those with only one germline mutation are normal except for their susceptibility to develop cancer. Retinoblastoma only arise when somatic mutation occurs in the other allele in developing retinal cells as opposed to mature retinal cells. But allelic loss occurs in other cell types too and yet these patients with non-hereditary retinoblastomas do not have a predisposition to other cancers. Hence, the absence of pRB in the other tissues must either have no effect at all or just cell death, but not uncontrolled proliferation.

On the other hand, many types of malignancies to which individuals with germ-line mutations are not predisposed, show the loss of an RB1 allele (Borg et al., 1992). Although the actual frequency of a single allelic loss in these tumours is hard to define, it is known that a subset of them has mutations involving both alleles, hence showing as rearrangements on a Southern blot or undetectable pRB bands on a western blot. An example of these phenomenon is that shown by parathyroid adenoma which is known to progress to parathyroid carcinoma. Cryns et al (1995) showed that pRB was absent in all 5 carcinomas analysed yet only 1 of 19 adenomas had no detectable pRB.
Putting the above points into perspective, a suitable model for the mechanism of pRB involvement in tumorigenesis in cancers other than retinoblastomas would be that other genetic events resulted in the initiation of these tumours. Loss of functional pRB occurs later and may contribute to their progression (Figure 4.7). pRB inactivation might therefore only occur in a subset of these tumours, as evidenced by studies in primary tumours as well as the 20 AML cases analysed here. The findings from a study by Preudhomme et al (1994) which showed that pRB inactivation is very rare in myelodysplasia further suggests that RB1 mutation is a late event in leukaemogenesis.

Those with RB1 mutations might show different tumour behaviour. Studies have shown that tumours with no pRB expression generally have either a high grade phenotype or a more invasive lesion (Cance et al., 1990; Presti et al., 1991). With regard to haematological malignancies, Towatari et al (1991) reported in their study that a change in pRB expression occurred mainly during the transformation phase of CML. Five cases with megakaryoblastic crisis were found to have no detectable pRB in contrast to the normal expression during the chronic stage. Zhu et al (1994) analysed the growth characteristics of AML blasts from 39 cases and found that those with no pRB form the major percentage showing autonomous growth in blast colony assays. These findings suggest that the inactivation of pRB could have caused the acquisition of autocrine growth characteristics by allowing the expression of other genes initially repressed by pRB. It is interesting to note the case of patient no.86 in my study who became refractory to treatment and showed a change in pRB expression during the course of the disease. A larger follow-up study is required to compare pRB status during presentation and relapse, so as to substantiate this observation.

Taken together, these findings suggest strongly that pRB is most likely to be involved in the progression of some cases of AML. pRB inactivation would also be likely to lead to a more severe disease. Although none of the patients studied here had any remission protein samples available, at least one study has looked into the prognostic effects of altered pRB expression. Kornblau et al (1994) analysed 113 newly diagnosed AML treated with the same therapeutic regimen and showed that the median survival was 12 weeks for those with low and undetectable pRB expression compared to 40 weeks for
Figure 4.7: Possible role of *RB1* mutation in leukaemia progression

- Progenitor cell
- Oncogene activation
- Leukaemia
- *RB1* mutation
- Leukaemia progression
- No *RB1* mutation
those with high levels of pRB expression (P value = 0.02). The multivariate analysis done by this group indicated that low pRB level was an independent prognostic factor predictive of poor survival after allowing for other known prognostic factors. My data also showed that the frequency of complete remission and median survival were better in those with normal pRB compared to those with abnormal pRB. However these differences were statistically not significant. Since it was not possible to perform the multivariate analysis due to lack of information on the other aspects of patients' characteristics and other disease-related factors, my data does not invalidate the findings by Kornblau et al. It however highlights another difficulty in terms of assessing a prognostic marker for a disease. It is most likely that inactivation of pRB is only one of many factors which can influence the clinical response and disease outcome and that the contribution maybe more significant in some patients than in others. By the same extension, inactivation of pRB may have more impact and potentially damaging in some tumours than in others. Studies in patients with breast carcinoma have shown that inactivation of pRB, although present in 35% of cases, did not predict for a poor outcome of disease (Pietilainen et al., 1995). In contrast, studies bladder carcinoma indicated that those with inactivation of pRB had more invasive tumours and poorer clinical response than those with normal pRB (Presti et al., 1991).

It would be understandable that absence of pRB could result in either a more invasive form of malignancy or a reduced survival rate. Inactivation of RB in these malignant cells will effectively remove another barrier in cell cycle control hence conferring these already actively proliferating cells with yet another growth advantage.

In summary, 31% of the AML cases studied had abnormalities in pRB. The results suggest a role of pRB in acute myeloid leukaemia. The exact contribution to the pathogenesis of AML remains unclear although it is most likely to be in the progression rather than initiation of disease. My data did not confirm nor disprove that inactivation of pRB could be used as a prognostic indicator of disease severity in AML. It is clear that better prospective studies are required to investigate this further. Another future aim is to study whether the reintroduction of either a normal RB1 gene or the protein itself in patients with no pRB can halt leukaemia progression thus allowing more effective chemotherapy for better survival. This novel target of cancer therapy for tumours with deficient pRB has been explored in cell lines and animal models.
Finally, looking from the cell cycle perspective, pRB is just one component of a complex regulatory circuit which requires an interplay with other protein molecules such as cyclins, CDKs and CKIs. Abnormalities in any of these proteins could have the same effect as pRB abnormalities and so could contribute to the pathogenesis of AML. It was in the light of this speculation that the next step taken was to analyse the p16, a CDK4 and CDK6 inhibitor, for gene rearrangements and for protein expression in AML.
CHAPTER FIVE

THE ANALYSIS OF DELETIONS AND REARRANGEMENTS IN THE p16 GENE IN AML PATIENTS USING MULTIPLEX PCR AND QUANTITATIVE SOUTHERN BLOTTING

5.1 INTRODUCTION

The phosphorylation of pRB is considered to be a requirement for the transition of normal cells from the G1 phase to the S phase of the cell cycle. For pRB, the major kinases responsible for its phosphorylation are CDK4 and CDK6 working in association with D-type cyclins. Recently another group of regulators was discovered. These are molecules which function as negative regulators by inhibiting the activity of CDKs, hence they are called CDK inhibitors (CKIs). One of the earliest CKIs to be discovered was p16 (Kamb et al., 1994; Nobori et al., 1994) which specifically binds to CDK4 and CDK6 (Parry et al., 1995), thereby inhibiting the ability of these kinases to interact with cyclin D1. This inhibition of CDK4 activity prevents the phosphorylation of pRB, halting the cell cycle progression from G1 into S phase (Serrano et al., 1994; Lukas et al., 1995) (reviewed in chapter 1). Loss of p16 function should result in unregulated kinase activity, leading to persistent phosphorylation and uncontrolled cellular proliferation. Hence, the p16 gene is a good candidate for a tumour suppressor gene.

The launching pad for the p16 gene was a study by Kamb et al. (1994) which showed the presence of homozygous deletions in >50% of 290 cell lines using a PCR approach. Nobori et al. (1994) also reported a similar frequency in a study involving 46 cell lines. The findings implied that the p16 protein could possibly function as a major tumour suppressor, perhaps equalling or exceeding the central role long attributed to p53. This triggered a series of studies in primary tumours. This chapter and the subsequent two chapters report studies analysing the involvement of the p16 gene and its protein expression in AML. The findings are later correlated with the data on pRB expression in AML and offer some in vivo evidence of the interaction between these two proteins in the setting of a malignancy.

Genomic organisation of the p16 gene

The locus 9p21 has been reported to be frequently deleted in many tumour types including melanomas, leukaemias, gliomas and bladder carcinoma.
(Fountain et al., 1992; Diaz et al., 1990; Olapade et al., 1992; Miyao et al., 1993). Kamb et al (1994) and Nobori et al (1994) identified a putative tumour suppressor gene within this locus, \( p16 \) (also known as \( MTS1 \) and \( CDKN2 \)) which encodes a 16kDa protein. The \( p16 \) gene consists of 3 exons whose sizes are 126, 307 and 11bp respectively (Figure 5.1). There is a CpG island at the 5' end which is believed to play a role in the transcriptional control of the gene (Merlo et al., 1995). In addition to the homozygous deletions found in the whole gene, Kamb et al (1994) also showed that point mutations and small deletions are commonest in exon 2. This was confirmed by another study from the same group (Liu et al., 1995) involving 154 cell lines. No mutations were found in exon 3. There is another gene, called \( MTS2 \) or \( p15 \), located approximately 25 kilobases centromeric to the \( p16 \) gene (Figure 5.1) (Kamb et al., 1994). The cDNA sequences of \( p16 \) and \( p15 \) are 44% identical in the first 150 nucleotides and 97% identical in the following 243 nucleotides (Hannon et al., 1994) before diverging. \( p15 \) is also capable of inhibiting cyclin D-CDK4 activity and mutations of the gene have been found to accompany \( p16 \) deletions in some tumours (Okuda et al., 1995; Jen et al., 1994).

### \( p16 \) gene deletions in primary tumours

Table 5.1 shows the frequency of \( p16 \) gene deletions in primary tumours. Except for oesophageal tumours, pancreatic tumours and glioblastomas, the frequency of involvement is much less than that demonstrated in cell lines.

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Study</th>
<th>Frequency of deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic carcinoma</td>
<td>Caldas et al., 1994</td>
<td>10/27 (37%)</td>
</tr>
<tr>
<td>Oesophageal cancer</td>
<td>Mori et al., 1994</td>
<td>14/27 (52%)</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>Schmidt et al., 1994</td>
<td>19/46 (41%)</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>Jen et al., 1994</td>
<td>26/38 (68%)</td>
</tr>
<tr>
<td>Bladder carcinoma</td>
<td>Spruck et al., 1994</td>
<td>6/31 (19%)</td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td>Jen et al., 1994</td>
<td>0/25 (0%)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Ohta et al., 1995</td>
<td>0/30 (0%)</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>Okamoto et al., 1995</td>
<td>4/22 (18%)</td>
</tr>
</tbody>
</table>

In haematological malignancies, a disparity of involvement is again apparent. Table 5.2 shows the frequency of homozygous deletions detected in
Figure 5.1: Genomic organisation of the \textit{p16} gene

Chromosome 9p21 locus

\textit{p16} gene \hspace{1cm} \textit{p15} gene

Exon

126bp \hspace{1cm} 307 \hspace{1cm} 11

\(25\text{kb}\)
studies done so far. There appears to be a preponderance of deletions in lymphoid neoplasms as compared to myeloid lineages.

Table 5.2: \textit{p16} gene deletions in haemopoietic malignancies

<table>
<thead>
<tr>
<th>Type of leukaemia</th>
<th>Study</th>
<th>Frequency of deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>Ogawa \textit{et al.}, 1994</td>
<td>4/14 (29%)</td>
</tr>
<tr>
<td>ALL</td>
<td>Okuda \textit{et al.}, 1995</td>
<td>18/43 (42%)</td>
</tr>
<tr>
<td>T-ALL</td>
<td>Hebert \textit{et al.}, 1994</td>
<td>16/24 (66%)</td>
</tr>
<tr>
<td>T-ALL</td>
<td>Otsuki \textit{et al.}, 1995</td>
<td>8/23 (35%)</td>
</tr>
<tr>
<td>B-ALL</td>
<td>Hebert \textit{et al.}, 1994</td>
<td>1/31 (3%)</td>
</tr>
<tr>
<td>B-ALL</td>
<td>Otsuki \textit{et al.}, 1995</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td>CLL</td>
<td>Otsuki \textit{et al.}, 1995</td>
<td>0/34 (0%)</td>
</tr>
<tr>
<td>CML-BC</td>
<td>Ogawa \textit{et al.}, 1994</td>
<td>0/13 (0%)</td>
</tr>
<tr>
<td>AML</td>
<td>Ogawa \textit{et al.}, 1994</td>
<td>0/45 (0%)</td>
</tr>
<tr>
<td>AML</td>
<td>Sill \textit{et al.}, 1995</td>
<td>1/25 (2%)</td>
</tr>
</tbody>
</table>

\textit{The rationale for using the Polymerase Chain Reaction (PCR) in p16 gene studies}

The first few studies describing the involvement of \textit{p16} in malignancies utilised the PCR technique to directly amplify exons 1 and 2 from genomic DNA (Kamb \textit{et al.}, 1994, Nobori \textit{et al.}, 1994). Since the \textit{p16} gene is relatively small this approach is appropriate for initial screening purposes. However, most tumour tissues will also contain normal tissue components and the contribution of this normal component to the DNA that is amplified can give rise to false positive results. In fact when the studies on primary tumours reported a much reduced frequency of deletions against the findings of the original report by Kamb \textit{et al} (1994), it was suggested that the samples analysed may have been contaminated by normal cells (Kamb \textit{et al.}, 1995). It is hence vital to design the experimental methods so as to allow the most accurate assessment of the tumour population rather than the contaminating normal tissue.

In the case of leukaemic blood samples, the presence of non-leukaemic cells is inevitable considering that the mononuclear cell fraction obtained by Ficoll density centrifugation would also contain normal monocytes, lymphocytes and
possibly some granulocytes. The contribution of this non-leukaemic fraction in
the total DNA of the sample was taken into consideration when deciding on
the approach to use for screening the AML samples. The other important but
less quantifiable issue was the possibility of a p16 deletions occurring only in
a sub-population of the leukaemic cells due to a clonal selection. This again
might give rise to a positive result.

Hence, whatever the approach used, quantification should be done to ensure
that the contribution from the non-leukaemic cells be accounted for. For
quantification to be valid, the reduction in signal intensity obtained must truly
represent the actual decrease in the number of cells carrying a deletion.
Furthermore this correlation must be linear. My method of choice for this study
initially was PCR. Despite the obvious risk of amplifying DNA from non-
leukaemic cells, the attraction for using PCR in this study was overwhelming.
Firstly, the potency of PCR would allow rapid screening of samples. Secondly,
with good optimisation, the high specificity which this technique offers will
remove the problems of non-specific bands and high background eminent in
Southern blotting.

One approach to control for the presence of non-leukaemic cells during
analysis for deletions is by using a multiplex PCR, which is the simultaneous
amplification of 2 or more loci in a single PCR reaction vessel (Burgart et al.,
1992). The advantage of doing this is that it allows for an assay of a gene of
interest when compared to an internal control. In other words, it provides for
semi-quantitative analysis of the PCR and its products (Abbs et al., 1992). Of
course this approach does not absolutely remove the problem of the non-
leukaemic cells totally but, with proper calibration of both the gene of interest
and the control as well as proper sensitivity testing, the problems of
contaminating non-leukaemic cells could possibly be reduced to a minimum.

The other advantage of a multiplex PCR is it allows the simultaneous
screening of different exons in a gene. Many studies have utilised this
multiplex PCR approach as a rapid detection method for mutations in the
cystic fibrosis gene (Cremonesi et al., 1992), the globin gene (Fortina et al.,
1992), the p53 gene (Runnebaum et al., 1994), the RB1 gene (Lohmann et
al., 1994), for detection of bcr-abi fusion mRNAs (Zhang et al., 1995) and for
determination of myeloid lineage of leukaemic cells (Crisan, 1994).
For the \textit{p}16 gene analysis in AML, the use of multiplex PCR was therefore intended to provide a rapid screening method for detecting homozygous deletions, to significantly reduce the number of false negatives should they occur and most importantly to account for the presence of non-leukaemic cells.

\textit{The Polymerase Chain Reaction}

The polymerase chain reaction (PCR) is an \textit{in vitro} enzymatic synthesis and amplification of specific DNA sequences (Saiki \textit{et al.}, 1985; Mullis \textit{et al.}, 1987). It is a powerful technique which requires only picogram quantities of starting template DNA which go through 30-40 cycles of amplification, to obtain sufficient amount of product for interpretation or further analysis.

However, like any other technique, PCR is not without its pitfalls. Because of its extremely potent amplification potential, any contaminating DNA in a sample will be amplified giving rise to unwanted bands. Contamination is mainly from exogenous genomes, plasmids or PCR products (Kwok \textit{et al.}, 1989; Kitchin \textit{et al.}, 1990). In the context of leukaemic samples, the 'contamination' is from non-leukaemic cells as well. This is especially crucial when looking for deletions as any contaminating DNA will result in a false positive data. In the first group of samples which were analysed the blast proportion was >95%, but the 5% population of non-leukaemic cells which could still potentially be amplified. Non-specific bands may also appear as a result of primer mismatching. Inefficient amplification will cause either a false negative result or a much reduced amount of PCR product. This is vital when quantification is required. However most of these problems can be overcome or reduced both by fine-tuning or optimisation of each reaction and with the use of appropriate positive and negative controls for every PCR reaction.

For a PCR to be quantitative or at least semi-quantitative, there must be an internal control within the same PCR reaction vessel and for quantification to be valid, the PCR should be within the exponential phase of amplification. Mathematical analysis has shown that the PCR reaches a plateau after 28.6 cycles (Cha \textit{et al} 1993). Hence, for quantitative PCR, reactions should not be allowed to reach 29 cycles.
5.2. MATERIALS AND METHODS

5.2.1 Setting up the multiplex PCR

The first step in setting up the PCR using a new set of primers was to optimise the reaction i.e to find out the best conditions for generating PCR products which were specific (the sole amplification of the locus of interest), with a high yield (efficient) and of a high fidelity (negligible amount of mispriming and Taq induced errors). Studies have shown that the overall efficacy of PCR is influenced by numerous components of the reaction. Parameters such as the annealing temperature, buffer conditions especially Mg²⁺ concentration, the design of the primers, concentration of the primers and the DNA polymerases used all need to be taken into consideration and optimised. The need to optimise was even greater in setting up the multiplex reaction where more than one set of primers were used in a single reaction tube.

5.2.1.1 PCR of exon 1 or 2 of the \textit{p16} gene

Primers for the \textit{p16} exons

Two sets of genomic primers were used: one pair flanking exon 1 and the other flanking exon 2 (Kamb \textit{et al.}, 1994). Exon 3 was not included in the analysis since the majority of deletions have been reported to involve exons 1 and 2.

The primers sequences are (5'-3'):-

\begin{align*}
1F & : \text{GAAGAAAGAGGAGGGGCTG} \\
1R & : \text{GCGCTACCTGATTCCAATTC} \\
2F & : \text{GGAAATTGGAAACTGGAAGC} \\
2R & : \text{TCTGAGCTTTGGAAGCTCT}
\end{align*}

The expected sizes for the PCR products with the above intronic primers were 320bp and 500bp for the exon 1 and 2 fragments respectively. Genomic DNA of 25 AML patients with high blast counts (>95%) were analysed. 100ng of DNA was used as the template for a 20μl PCR reaction, carried out according to methods described in chapter 2 (page 50). Experiments were carried out to determine the optimum annealing temperatures for both sets of primers and the results showed that these were 60°C and 64°C for the exon 1 and exon 2 primers respectively. Using a similar approach, the optimum Mg²⁺ concentration for both primers was ascertained as 2.5mM.
Figure 5.2(A and B) shows a representative gel of exon 1 and exon 2 PCR products of 10 AML patients. Patient no.6 showed absence of the exon 2 band, but this was actually a false negative result as a repeat analysis later revealed that this sample had a single and intact exon 2 band.

To confirm that the products obtained from the PCR truly represented the fragments of the two exons of the p16 gene, they were electrophoresed through a 0.8% low melting point agarose gel, purified and sequenced using methods described in chapter 2 (page 51). The results confirmed that the PCR products contained the correct p16 exonic sequences.

5.2.1.2 Multiplex PCR of exon 1 or 2 with an internal control

The next step was to set up a multiplex PCR reaction using either exon 1 or exon 2 primers with a suitable internal control. A fragment on the β-globin gene was selected for this purpose. The primer sequences for the globin fragment were: GF: (5'-3'), ACACA ACTGTGTCACTAGC and GR: (5'-3'), CCACTTGACCTATCTCAAC (Saiki et al., 1985). Optimisation of the globin primers was carried out and the annealing temperature was found to be equally efficient between 58-64°C. This was a crucial advantage since the annealing temperatures for both exons 1 and 2 were within this range. This is important when doing a multiplex PCR since annealing temperatures which differ greatly from one primer set to another will affect the efficacy of the PCR. The globin primers gave rise to a PCR product of about 250bp in length which allowed clear electrophoretic separation from the 340bp and 500bp PCR products of exon 1 and 2 respectively.

The reaction mixture for the multiplex reactions was as follows:-

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume in μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>11.6</td>
</tr>
<tr>
<td>10x Buffer (with 15mM MgCl₂)</td>
<td>2.0</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>0.4</td>
</tr>
<tr>
<td>dNTPs (deoxynucleotides) [10mM]</td>
<td>0.4</td>
</tr>
<tr>
<td>3' exon 1 or 2 primers (200ng/μl)</td>
<td>0.4</td>
</tr>
<tr>
<td>5' exon 1 or 2 primers (200ng/μl)</td>
<td>0.4</td>
</tr>
<tr>
<td>3' globin primers (200ng/μl)</td>
<td>0.4</td>
</tr>
<tr>
<td>5' globin primers (200ng/μl)</td>
<td>0.4</td>
</tr>
<tr>
<td>DNA (100ng)</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Figure 5.2: PCR of exon 1 or 2 of the *p16* gene

A  

**exon 1**

340bp

B  

**exon 2**

500bp

lanes:
1-10  AML patients
J6  J6 cells with p16 deletion
NC  Normal control
As this was still at the optimisation stage, thirty cycles of amplification were carried out using an annealing temperature of 60°C for exon 1 analysis and 64°C for exon 2 analysis.

The products were run through a 2% agarose gel, stained with ethidium bromide and visualised with UV light. Figure 5.3A shows a representative gel of the PCR products of exon 1 together with the globin fragment as an internal control while Figure 5.3B shows the corresponding result for exon 2. It must be stressed here that at this stage, assessment of the bands were purely visual. The negative control, the J6 cell line, is in the last lane for both gels and it showed an intact globin fragment with absence of both the p16 exonic fragments. Patient no.2 in Figure 5.3B showed an absence of the exon 2 with an intact globin fragment. This sample had showed an intact exon 2 band (Figure 5.2B) in an earlier PCR analysis.

5.2.1.3 Multiplex PCR of both exons 1 and 2 with an internal control

This experiment was carried out to develop a rapid screening method to analyse both exons simultaneously in a single reaction tube. The same approach as above was used but this time both exon 1 and exon 2 together with the globin primers as the internal control were added to the PCR reaction.

The PCR reaction mixture contained:-

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume in µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>10.0</td>
</tr>
<tr>
<td>10x Buffer (with 15mM MgCl₂)</td>
<td>2.0</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>0.8</td>
</tr>
<tr>
<td>dNTPs (deoxynucleotides) (10mM)</td>
<td>0.8</td>
</tr>
<tr>
<td>3' exon 1 primers (200ng/µl)</td>
<td>0.4</td>
</tr>
<tr>
<td>5' exon 1 primers (200ng/µl)</td>
<td>0.4</td>
</tr>
<tr>
<td>3' exon 2 primers (200ng/µl)</td>
<td>0.4</td>
</tr>
<tr>
<td>5' exon 2 primers (200ng/µl)</td>
<td>0.4</td>
</tr>
<tr>
<td>3' globin primers (200ng/µl)</td>
<td>0.4</td>
</tr>
<tr>
<td>5' globin primers (200ng/µl)</td>
<td>0.4</td>
</tr>
<tr>
<td>DNA (100ng)</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Figure 5.3: Multiplex PCR of exon 1 or exon 2 using globin as internal control

A

1 2 3 4 5 6 7 8 9 10

exon1 340bp
globin 250bp

AML (n=10)

B

1 2 3 4 5 6 7 8 9 10

exon2 500bp
globin 250bp

AML (n=10)

NC Normal control
J6 J6 cells with p16 deletion
Twenty-five AML samples and one normal control were analysed. The negative control was DNA from the J6 cell line (Parry et al., 1995) which has a deletion of the p16 gene. Thirty cycles of amplification were carried out using an annealing temperature of 60°C, which was the lower annealing temperature for the two exons. PCR products were then electrophoresed through a 2% agarose gel and viewed under UV light.

The results of multiplex PCR using both exon 1 and exon 2 primers together with the globin control are shown in Figure 5.4. This representative gel contained a normal control (NC), 10 AML samples (numbered 1-10) and the negative control, the J6 cell line. Patient no.2 showed absence of exon 1 and exon 2 fragments with an intact globin band. This sample had originally shown intact exon 1 and exon 2 bands in section 5.2.1.3 (Figure 5.2A and 5.2B). All the ten samples here showed intact exons 1 and 2 in a repeat multiplex PCR. These results show that false negative results can occur despite using an internal control.

Of the 25 samples analysed, all showed intact exon 1 and exon 2 PCR products. The globin control fragment was intact in all samples and the negative control only showed the globin fragment.

5.2.1.4 Evaluating the contribution of non-leukaemic cells in the PCR analysis of leukaemic samples

The results from the previous section showed that the multiplex PCR had been successfully optimised and should allow rapid screening of AML patients samples. Provided that abnormal results were reproducible, there should not be a problem in the interpretation of presence or absence of bands. However, it still did not exclude the possibility that the amplification was derived from the 'contaminating' DNA of non-leukaemic cells. It was crucial that this issue be resolved and evaluated before more samples were analysed.

This experiment was designed to evaluate the contribution of non-leukaemic cells in the leukaemic sample within the multiplex PCR analysis as well as to confirm the validity of using this approach for the subsequent screening of AML samples. To conduct this experiment, DNA from the J6 T-cell line and a normal control sample containing an intact p16 gene were selected and their concentrations quantified. Both these DNA solutions were then adjusted by serial dilutions to reach an equal final concentration of 100ng/μl. This final
Figure 5.4: Multiplex PCR of exons 1 and 2 of the \textit{p16} gene using a globin fragment as an internal control

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal control (NC)</td>
</tr>
<tr>
<td>2</td>
<td>J6 cells with \textit{p16} deletion</td>
</tr>
<tr>
<td>3-10</td>
<td>AML patients (n=10)</td>
</tr>
</tbody>
</table>

- **exon 1**
- **exon 2**
- **globin**
concentration was reconfirmed by spectrophotometric measurements and also by running equivalent amounts on an agarose gel.

A set of DNA mixtures was then made by mixing both DNA samples in varying ratios. A multiplex PCR reaction was set up using exon 2 primers as well as the globin primers in one reaction vessel. The PCR on this occasion was carried out for 28 cycles only, to allow for semi-quantitative interpretation. The result of this experiment is shown in Figure 5.5.

A decreasing intensity of the p16 exon 2 bands was observed as the concentration of the p16\(^{\text{vve}}\) DNA decreases. The globin bands were almost constant throughout confirming that the total amount of DNA in each reaction was approximately equal. The most alarming observation was the presence of a signal even when the p16\(^{\text{vve}}\) DNA present in the mixture was only 1%. It was also apparent that the samples with one and two percent p16\(^{\text{vve}}\) DNA had intensities which did not reflect their proportions to the total DNA in the sample. In other words, for homozygous deletions to be diagnosed reliably in the leukaemic samples using PCR, the blast content must approach 100%. Since this level of purity could not be guaranteed in the samples studied, an alternative approach using Southern blotting analysis was subsequently developed and used.

5.2.2 Quantitative Southern blotting analysis

5.2.2.1 Introduction

This technique involved quantification of bands detected by Southern blotting analysis. There was still the problem of 'contamination' by non-leukaemic cells to be considered. The major difference here was the absence of exogenous amplification involved in this technique and the concurrent problems unique to the PCR are avoided. The loading amount of DNA can be controlled by a reference band i.e a gene which is unrelated to the one being investigated, as in PCR, but there is no amplification of the control band involved.

The Southern blotting methods used for the analysis of rearrangements of the RB1 gene have been similarly applied. The only major modification was the introduction of another probe within the same hybridisation solution to act as an internal control as well as a reference band for quantification. The quantification aspect of this approach was crucial. The signal intensity of a
Figure 5.5: Amplification of non-leukaemic cells in leukaemic samples by PCR analysis

100 75 50 20 10 5 2 1 0

Percentage of p16+ve DNA
band must be demonstrated to be directly proportional to the amount of normal DNA alleles present. Provided that the intensity of the control band remained constant, it would then be possible to deduce homozygous or heterozygous deletions even in the presence of non-leukaemic cells.

Optimisation of the conditions was easier since most of the general conditions for hybridisation have been established from chapter 3. The other advantage was that the \textit{p16} gene is much smaller and the genomic organisation less complex than the \textit{RB1} gene. The following experiments were carried out as part of the optimisation of the technique before the screening of AML samples were performed.

5.2.2.2 Preparing the \textit{p16} probe

The exon 2 PCR product was used as the probe for the Southern blot analysis. This probe has also been used in some other studies (Ogawa \textit{et al.}, 1994; Nobori \textit{et al.}, 1994). The probe is approximately 500bp in length. The exon 2 probe was amplified from a haematologically normal sample and the PCR product purified and sequenced to confirm that it contained the correct sequence. Probe labelling was carried out by the random priming method using [\(\alpha\)-32P]dATP as the radioisotope.

5.2.2.3 Southern blot analysis using different enzyme digests and the incorporation of an internal control

The \textit{c-fms} gene was chosen as the internal control. The gene which is localised on chromosome 5, encodes the receptor for macrophage colony stimulating factor (M-CSF). One might question the suitability of this gene as an internal control, since there are studies which showed the occurrence of \textit{c-fms} mutations of the gene in myeloid malignancies especially in those with 5q deletion (Boulton \textit{et al.}, 1991). None of the AML patients analysed here were known to be 5q-. The only potential problem which could have occurred was in the case of a sample with heterozygous deletions in both the \textit{c-fms} and the \textit{p16} gene which would then give rise to apparently normal relative intensities for both bands on a Southern blot.

The \textit{c-fms} probe was obtained by \textit{BamH}I digestion of a plasmid containing a 3kb cDNA \textit{c-fms} clone (kindly provided by Dr. J. Wainscoat, Oxford, UK). The reason for selecting this probe as an internal control in our quantitative
Southerns was simply because it gave a single band with the \textit{EcoRI} (2.5kb fragment) (Boultwood \textit{et al}., 1991). Since the \textit{p16} probe also gave a single band (4.2kb) with the \textit{EcoRI} digest on the Southern blot (Kamb \textit{et al}., 1994), the simultaneous use of these two probes would, if successful, give rise to two bands, hence allowing quantification to be done by comparing the ratio of these two bands against a set of normal individuals.

Southern blotting analysis was carried out using the conditions described in chapter 3. Four different enzyme digests (\textit{BamHI}, \textit{BglII}, \textit{EcoRI} and \textit{HindIII}) were used to establish the germline band configurations for each enzyme as well as to choose the appropriate enzyme for the quantitative Southern blotting analysis when screening the patients samples. The band configuration detected with both the \textit{c-fms} and \textit{p16} probes for each of the enzymes is shown in Figure 5.6. The most intense band in each digest correspond to the \textit{c-fms} band, whilst the fainter bands represent the \textit{p15} and \textit{p16} bands. The \textit{EcoRI} digest gave rise to 3 bands. A 4.2kb band representing the \textit{p16} gene, a 6.4kb band representing the homologous \textit{p15} gene (Kamb \textit{et al}., 1994; Ogawa \textit{et al}., 1994) and a 2.5kb band representing the \textit{c-fms} band. It was decided to use the \textit{EcoRI} digests for subsequent Southern blotting analysis of the AML samples to simplify the quantification of bands.

\subsection*{5.2.2.4 Quantification of bands}

For the purpose of quantification, filters were exposed to phosphorimager plates overnight and evaluated on a Fujimax bas 1000 phosphorimager (Fuji Photo Film Co, Japan). The signals were quantified using a Whole Band Analyzer software (Millipore) run on a Sun Sparc workstation. The filters were also exposed to pre-flashed XAR-5 film (Kodak, Rochester NY).

\subsection*{5.2.2.5 Optimisation of quantitative Southern blotting analysis}

The conditions for hybridisation were identical to the \textit{RB1} Southerns. The only requirement here was to establish the relative amounts of each probe to be used and the random priming procedure.

The \textit{c-fms} probe is 3kb in length while that of \textit{p16} is much shorter at 0.5kb. A Southern blotting analysis was performed using approximately equal amounts of the probes (50ng). Figure 5.7A showed the autoradiograph of this experiment.
Figure 5.6: Germline bands detected in 4 different enzyme digests using \textit{p16} and \textit{c-fms} probes
Figure 5.7: Quantitative Southern blotting: optimisation of p16 and c-fms probes

A

(p15) 6.4kb —►
(p16) 4.2kb —►
(c-fms) 2.5kb —►

Ratio of intensities between p16 to c-fms bands is 20:80

Normal controls (n=5)

B

(p15) 6.4kb —►
(p16) 4.2kb —►
(c-fms) 2.5kb —►

Ratio optimised to 40:60 by decreasing the amount of c-fms and increasing the radioactivity for p16 labeling

Normal controls (n=5)
Quantification of the relative intensities between the p16 and c-fms band was carried out by selecting these two bands using the image analyser software (Millipore) and assigning the total intensity to be 100% for each lane. The relative intensities for each band were then obtained and they gave a ratio of 20:80. Although this ratio is acceptable for subsequent screening and quantification of patients samples, it would be preferable that the intensities were approximately equal. Two modifications were made i.e decreasing the amount of c-fms probe against the p16 probe and reducing the amount of radioisotope used for the random priming of c-fms probe.

The other aspect of optimisation was in the random priming of the probes itself. These could either be done together in a single random priming reaction tube or alternatively carried out separately and mixed together later into the hybridisation solution. Both methods were done and the results show that there was not much difference between the two (data not shown). However it was decided to prime the probes separately so as to avoid undue competition between the two priming processes within a single reaction tube.

The following random priming mixtures were used for the c-fms and the p16 probes:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amount in µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-fms probe</td>
<td>1 (20ng equivalent)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>11</td>
</tr>
<tr>
<td>Reaction buffer (with random hexamers)</td>
<td>2</td>
</tr>
<tr>
<td>dCTG</td>
<td>3</td>
</tr>
<tr>
<td>[α-P32]dATP</td>
<td>2</td>
</tr>
<tr>
<td>Klenow enzyme (10U/µl)</td>
<td>1</td>
</tr>
<tr>
<td>p16 probe</td>
<td>6 (50ng equivalent)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5</td>
</tr>
<tr>
<td>Reaction buffer (with random hexamers)</td>
<td>2</td>
</tr>
<tr>
<td>dCTG</td>
<td>3</td>
</tr>
<tr>
<td>[α-P32]dATP</td>
<td>3</td>
</tr>
<tr>
<td>Klenow enzyme (10U/µl)</td>
<td>1</td>
</tr>
</tbody>
</table>

Using these protocols, the resulting ratio of the p16 to the c-fms band was effectively reduced to 40:60 (Figure 5.7B).
5.2.2.6 Testing the sensitivity of quantitative Southern blotting

This experiment was performed to test for the sensitivity of the quantitative Southern blot analysis. As mentioned in section 5.2.2.1 it was crucial to prove that using the system optimised in the previous sections, a linear relationship could be obtained consistently between the signal intensity of the \( p16 \) band and the amount of DNA loading. This will allow a proper calibration to be calculated mathematically so that interpretation of homozygous or heterozygous deletions can be made with confidence even in the presence of some non-leukaemic cells in the samples analysed.

The J6 T-cell line (Parry et al., 1995) which is \( p16 \) negative (\( p16^{-ve} \)) and a \( p16 \) positive (\( p16^{+ve} \)) normal individual were chosen for this purpose. Their respective DNA were mixed in varying proportions. Southern blotting was then performed using the same methods described above. The intensities of the 4.2kb \( p16 \) band and the 2.5kb \( c-fms \) band were measured using a phosphorimager and a ratio of their intensities was obtained for each sample.

The results of this sensitivity experiment are shown in Figure 5.8. There is a linear progression of the relative intensity of the \( p16 \) bands as the amount of \( p16^{+ve} \) DNA increases as shown by the graph. The sensitivity of \( p16 \) detection was 2.5% of the total. In other words 2.5% of \( p16^{+ve} \) DNA is sufficient to give rise to a \( p16 \) signal. It also means that the presence of 2.5% non-leukaemic cells can give rise to a false positive result should the rest of the cells be \( p16^{-ve} \). However, since the band is quantifiable, and there is a linear relationship between signal intensity and amount of \( p16^{+ve} \) DNA, interpretation is possible. This whole process of optimisation emphasizes the importance of quantitative Southern blotting when looking for gene deletions in leukaemic samples as some degree of contamination by normal cells is inevitable.

5.2.2.7 Quantitative Southern blotting analysis of 18 normal controls

Eighteen haematologically normal controls were analysed to establish the normal range of intensities of the \( p16 \) band against \( c-fms \) bands. Quantification revealed that the intensity of the \( p16 \) band for each sample ranged between 35-45% of the total intensities of both \( p16 \) and \( c-fms \) bands.
Figure 5.8: Southern blot and graph showing the sensitivity of \textit{p16} detection and linearity of signal intensity in proportion to \textit{p16+ve DNA}.
5.2.2.8 Quantitative Southern blotting analysis of 76 AML patients

A total of 76 AML patients of varying FAB types were analysed. For each set of Southern blotting, a normal sample was used as the positive control. A negative control, the J6 cell line, was used intermittently to ensure specificity of the \( p16 \) bands. The bands were quantified using methods described above.

5.3 RESULTS

Quantitative Southern blotting results of 76 AML patients

A total of 76 patients with AML of different FAB types were investigated. None of the AML samples showed absence of the \( p16 \) or \( p15 \) bands. No additional bands were detected, indicating that there were no gross rearrangements. The \( c-fms \) internal control was also intact throughout. All the AML samples had intensities of the \( p16 \) band ranging between 35-45% of the total intensity of both \( p16 \) and \( c-fms \) bands. This range was identical to that found in the 18 normal controls. Therefore none of the samples had homozygous deletion of the \( p16 \) gene. Since there was no sample with a reduction of intensity well outside the normal range, heterozygous deletions were also unlikely. All samples also showed the presence of the \( p15 \) band. A representative blot containing DNA from three AML patients together with \( p16^{+ve} \) and \( p16^{-ve} \) controls is shown in Figure 5.9.

5.4 DISCUSSION

The study by Kamb \textit{et al} (1994) showing homozygous deletions of \( p16 \) in \( >50\% \) of 290 cell lines, including leukaemic cell lines, was done using PCR. Samples with no \( p16 \) bands were interpreted as having homozygous deletions of the \( p16 \) gene. A subset of those without any abnormalities in the PCR was directly sequenced and some were found to have small mutations and deletions. This prompted a claim that the actual frequency of involvement of the \( p16 \) gene is more than 75%. The results of studies on primary tumours (Table 5.1), including this report on AML patients, showed that homozygous deletions in primary tumours were not as common in contrast to cell lines. One reason for this discrepancy could be that the frequent mutations seen in cell lines were the result of secondary events arising in culture. The possibility of non-cancerous cells in the primary tumour material giving rise to false
Figure 5.9: Representative Southern blot of 3 AML patients using EcoRI digest and probed with $p16$ and c-fms probes

Lanes:
- a Normal control
- b WERI (pRB -ve) cell line
- c J6 cell line ($p16$ -ve)
- d-f AML patients
positive results by PCR was mentioned by Kamb (Kamb, 1995) when reiterating this criticism by others on his findings.

The problem with AML samples in this study was that exclusion of non-leukaemic cells was difficult to perform especially when a large study is undertaken. In an attempt to overcome this problem, a multiplex PCR approach using an internal control was initially used for the screening of deletions in the \textit{p16} gene.

\textbf{The use of PCR in screening for mutations}

Although the PCR is widely used as a screening method for the detection of deletions and mutations, caution should be applied when interpreting PCR data more so when dealing with negative results than in positive ones. Negative PCR findings could be just simply due to a failed PCR which in turn can be due to many different causes. Even if a PCR reaction is repeatedly negative, the failure to obtain a product does not absolutely mean that a deletion is present in the sample. An internal control should be included in the same PCR reaction vessel showing that other unrelated genes can be amplified simultaneously. Even including an internal control does not absolutely safeguard against the occurrence of a false negative, as shown by sample 6 in Figure 5.2B and sample 2 in Figure 5.3B. It is mandatory that repeatedly negative results in the PCR suggesting deletions are confirmed by Southern blotting or absence of protein expression by western blotting. Only then can one be certain of the validity of the final data.

Multiplex PCR of two or more exons simultaneously must be interpreted with even more caution since there are many more variables involved. Preferential amplification from one set of primers at the expense of the others is a real problem (Walsh \textit{et al.}, 1992). The chances of non-specific primer annealing and primer dimerisation are also increased. Conditions must be optimised to suit all sets of primers involved and an internal control must again be included.

The AML samples chosen for the multiplex PCR were those whose blast cell content were >90\%. All 25 samples analysed showed no deletions of any exons at all after 3 repeat reactions. However some false negatives did occur. Had these samples been persistently negative, it would have been necessary to confirm the results using other methods such as Southern blotting. The impression at this stage was that multiplex PCR could be useful for the rapid
screening of multiple loci in a gene, provided that an internal control is used in all reactions and negative results repeated and confirmed by other methods.

The validity of using the PCR technique as described here for the screening of deletions in the p16 gene in leukaemic samples was severely challenged by the experiment which showed that 1% of p16+ DNA was sufficient to give rise to a PCR product with an intensity which arguably didn't appear to be proportionately reduced. It was thus considered that this approach was inappropriate for the study of leukaemic samples which are inevitably 'contaminated' by non-leukaemic cells. Despite this, Sill et al (1995) used a similar approach to screen CML patients and diagnosed homozygous deletions in the p16 gene if the ratio of the p16 to the control band was less than that found in the 75% p16-loss control sample. In this study mixing experiments were performed and showed a penultimate dilution of p16 loss at 75%, which contrasts with my data that a mere 1% of p16+ DNA was sufficient to give rise to a PCR product. The reason for this discrepancy is not clear.

**Quantitative Southern Blotting**

The predominant advantage of using Southern blotting as compared to PCR is the absence of the amplification factor and its related problems of efficacy and exogenous contamination. Hence, the internal control band in a Southern blot reflects accurately the total amount of DNA.

Screening of the normal samples showed that the intensity of the p16 band was between 35-45% of the total. A linear correlation between p16 signal and DNA loading has been demonstrated (Figure 5.8). Hence, a leukaemic sample with a homozygous deletion of the p16 gene would have no p16 band at all on a Southern blot while retaining the c-fms band. If there were to be a 10% contamination by non-leukaemic cells, the intensity would theoretically be 3.5-4.5% of the total. Likewise, a heterozygous deletion of p16 in a sample with 0% contamination would give an intensity between 17.5-22.5%. Table 5.3 shows the predicted relative intensities of the p16 bands in both homozygous and heterozygous deletions, and the changes affected by varying contamination with non-leukaemic cells. These expected values correlated well with the actual values found in the sensitivity testing.
Table 5.3: Predicted range of intensity of \textit{p16} band in homozygous and heterozygous deletions (based on a 35-45\% intensity in normal controls)

<table>
<thead>
<tr>
<th>% Contamination</th>
<th>\textbf{Homozygous deletion}</th>
<th>\textbf{Heterozygous deletion}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range of intensity (%)</td>
<td>Range of intensity (%)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>17.5-22.5</td>
</tr>
<tr>
<td>10</td>
<td>3.5-4.5</td>
<td>19.25-24.25</td>
</tr>
<tr>
<td>20</td>
<td>7-9</td>
<td>21-27</td>
</tr>
<tr>
<td>30</td>
<td>10.5-13.5</td>
<td>22.75-29.25</td>
</tr>
<tr>
<td>40</td>
<td>14-18</td>
<td>24.5-31.5</td>
</tr>
</tbody>
</table>

The results show that none of the 76 AML patients screened had homozygous deletion of the \textit{p16} gene. None of the samples had an intensity outside the normal range indicating that heterozygous deletions were also rare. These conclusions are valid even accounting for the presence of up to 40\% non-leukaemic cells in the mononuclear cell fraction.

The results obtained were in contrast to the data on cell lines. At the time of completion of this study, other groups analysing other tumours also reported on this conflicting frequency of deletions in primary tumours when compared to cell lines (Table 5.1). The tumour suppressive properties of \textit{p16} are well established but the significance of its contribution to the genesis of many cancers is still unclear. The possible reasons to explain for this disparity include: (a) deletions are restricted only to a subset of primary tumours; (b) different mechanisms could be involved in the inactivation of \textit{p16} in different tumours, such as point mutations and small deletions, mutations in the promoter region or within the non-coding regions; (c) \textit{p16} deletions are restricted to the late stages of tumour progression; (d) \textit{p16} being deleted as a consequence of cell culture; (e) \textit{p16} deletions were masked by the contamination of non-cancerous cells in tumour samples; and (f) the possible presence of other tumour suppressor genes at the locus 9p21.

Further studies are required to confirm some of the above hypotheses. One of the important lessons here was that it is dangerous to extrapolate data from analysis of cell lines to the events occurring \textit{in vivo} in primary tumours.

The rarity of deletions of \textit{p16} in AML was also in contrast to the relatively common occurrence in lymphoid neoplasms (Table 5.2). Further studies
would be required to see whether this phenomenon reflects a lineage specificity for \( p16 \) deletions in leukaemia.

In summary, homozygous deletions and gross rearrangements of the \( p16 \) gene are rare in acute myeloid leukaemia. The results concurred with other recent studies done so far in AML (Ogawa et al., 1994; Quesnel et al., 1995; Sill et al., 1995), using similar methods of screening. Nevertheless, as pointed out before, the Southern blotting technique will not be able to detect small deletions, point mutations or any involvement in the promoter region of the gene. There could still be a possibility of these mechanisms of \( p16 \) inactivation in AML. The search for point mutations using SSCP analysis in the \( p16 \) gene will be described in the next chapter.
CHAPTER SIX

ANALYSIS OF THE $p16$ GENE FOR POINT MUTATIONS AND POLYMORPHISMS USING THE RT-PCR-SSCP TECHNIQUE

6.1 INTRODUCTION

The early studies analysing the involvement $p16$ gene in primary tumours looked mainly for homozygous deletions. This was understandable due to the initial report by Kamb et al (1994) and Nobori et al (1994) which showed a high frequency of deletions in cell lines. It was inevitable that when only a subset of primary tumours showed gross deletions of the gene, later studies also incorporated the analysis for point mutations in these tumours.

In a follow-up to the original report on $p16$ by Kamb et al, the group analysed 154 cell lines which were not homozygously deleted for $p16$ and found 18% had mutations (Liu et al., 1995). The spectrum of mutations found include missense, termination, frameshift and splicing defects. The majority of these occurred in exon 2 and no mutations were found in exon 3. Although there were no protein analyses done, it is probable that these mutations result in the inactivation of the $p16$ protein.

In studies involving uncultured tumours, Okamoto et al (1995) found that apart from 4 cases with homozygous deletions, 2 out of 22 cases of metastatic non-small cell lung carcinoma (NSCLC) had insertions in the $p16$ gene causing premature stop codons in exon 2, while Hayashi et al (1994) found 30% of NSCLC analysed by SSCP had mutations. In haemopoietic malignancies, Otsuki et al (1995) analysed 117 primary lymphoid malignancies using the same methods and found none with an abnormal SSCP pattern, although a subset of these samples had gross deletions by Southern blotting.

The purpose of the study presented here was to search for other mechanisms which could inactivate the $p16$ gene in AML by using the single strand conformation polymorphism (SSCP) analysis. Having found no deletions or gross rearrangements at the DNA level using quantitative Southern blotting, the occurrence of point mutations within the coding sequence of the $p16$ gene was still a possibility. It would also be important to investigate for the presence of genetic polymorphisms in the gene.
Single Strand Conformation Polymorphism analysis

Analysis by SSCP is considered to be a simple, fast, efficient and powerful technique to detect point mutations and polymorphisms in genetic disorders (Orita et al., 1987). The basis for this technique is shown in Figure 6.1. When a double stranded DNA is denatured and run through a non-denaturing gel, each of the strands will assume a unique folded conformation stabilised by intrastrand interaction. Any mutation on either of these strands will result in a change of conformation when compared to the normal allelic conformation. These conformational changes are seen as mobility shifts on the non-denaturing gel electrophoresis. The mutations underlying the abnormal bands are then characterized by DNA sequencing. However, the effect of sequence change on electrophoretic mobility is unpredictable (Orita et al., 1989). Hence it may be possible that some of the sequence changes may not appreciably affect the mobility.

The sensitivity of SSCP in detecting point mutations is dependent on several factors. These include the length of the fragment, the extent of cross-linking concentration of acrylamide, the presence of glycerol, the temperature during electrophoresis and buffer concentration (Orita et al., 1989; Hayashi et al., 1989). The most crucial parameter which could affect the sensitivity of the technique is the gel temperature which is dependent on the ambient temperature as well as the wattage at which the gel is run. Changes in temperature will affect the stability of intra-strand bonds responsible for the unique conformation of each allele.

The current consensus is that the PCR products must be run in different gel conditions to ensure the maximum sensitivity (Hayashi et al., 1989; Smith et al., 1992). The most sensitive condition for SSCP analysis vary from one PCR fragment to another and the optimisation is mainly empirical. Studies on the familial hypercholesterolaemia gene, have shown that using three different gel conditions the sensitivity of this technique approached 95% (Leren et al., 1993).

In this study, three gel conditions were chosen, based on extensive experience obtained within the laboratory for detecting sequence changes in the GM-CSF receptor α and β chains (Wagner et al., 1994).

The SSCP technique would also detect point mutations which do not alter the protein sequence. These conservative mutations are part of genetic
Figure 6.1: Basis of Single Strand Conformation Polymorphism (SSCP) analysis

Normal

\[ \text{G} \beta \text{C} \]
\[ \text{T} \alpha \text{A} \]
\[ \text{C} \gamma \text{G} \]
\[ \text{G} \rho \text{C} \]

Radioactive PCR or RT-PCR products

Single base change

\[ \text{G} \beta \text{C} \]
\[ \text{T} \alpha \text{A} \]
\[ \text{C} \gamma \text{G} \]
\[ \text{G} \rho \text{C} \]

Heat Denaturation

Non-denaturing gel electrophoresis

Bands seen on autoradiograph
polymorphisms which are due to individual variation. They are equally important to identify to avoid inaccuracy in interpretations of results. Okamoto et al (1995) described 5 cases with polymorphisms in the p16 gene in their analysis of lung cancers. All five resulted in no change in the protein sequence. Hence, any abnormalities found with the SSCP analysis should be sequenced to see whether they are true mutations or polymorphisms.

6.2 MATERIALS AND METHODS

RNA samples of 25 AML patients with >90% blast counts were analysed. One haematologically normal control and 2 cell lines (TF1 and HL60) were also studied.

6.2.1 RT-PCR

The RT-PCR technique is described in chapter 2 (page 51). For the RT-PCR of the p16 cDNA, one microgram of total cellular RNA was reverse transcribed using 250ng oligo dT as primer (Promega, UK) in a total volume of 20μl containing 1x Taq polymerase buffer, 5.25mM MgCl₂, 1mM of each deoxynucleoside triphosphate, 20U RNase inhibitor and 3.75U AMV reverse transcriptase (Promega, UK). Reaction mixtures were incubated at 42°C for 1 hour and at 95°C for 5 min. Four microliters of the RT reaction were used for the radioactive PCR in a total volume of 20μl containing 0.5U Taq polymerase, 1x Taq polymerase buffer, 2.5mM MgCl₂, 0.2mM deoxynucleoside triphosphates (Promega, UK), 80ng of each primer and 0.2μl of [α-³²P]dATP (6000 Ci/mmol; Amersham UK). The optimal size of PCR fragments for SSCP analysis is believed to be less than 400bp (Hayashi et al., 1991). As the template for PCR is cDNA, exonic primers were designed to create fragments of optimal sizes which covered the entire coding sequence. Primers for exon 1 were (5'-3'): 1F, AGCATGGAGCCTTCGGCTGAC (sense) and 1R, CTGGATCGGCTCCGACC GTA (antisense), giving a PCR product of 128bp in size. Primers for exon 2 and 3 were (5'-3'): 2F, TACGGTGAGGCGATCGACG (sense) and 2R, CAATCGGGGTGGTGCAGGG (antisense), giving a PCR product of 340bp in size. Amplification was performed using the following parameters: 95°C for 30 sec, 62°C (Exon 1) 64°C (Exon 2 and 3) for 30 sec, and 72°C for 45 sec, for a total of 30 cycles followed by a final extension at 72°C for 5 min.
6.2.2 SSCP analysis

One microliter aliquots of radioactive PCR products were added to mixtures of 10μl of 0.1% SDS, 10mM EDTA and 11μl denaturing solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Samples were boiled for 5 minutes, then chilled on ice and 5μl loaded onto non-denaturing polyacrylamide gels (sequencing size, 6% polyacrylamide, crosslinker ratio 37:1, 1x Tris-borate-EDTA with or without 10% glycerol). Samples were electrophoresed under three different conditions to increase the probability of detecting mutations. These running conditions were: (1) 10% glycerol, room temperature, 17mA for 15 hours; (2) no glycerol, room temperature, 15W for 5 hours; (3) no glycerol, 4°C, 10mA for 15 hours. Gels were then dried and exposed overnight at room temperature with an intensifying screen using XAR-5 film (Kodak, UK).

6.2.3 Sequencing of RT-PCR products

Non-radioactive RT-PCR products were electrophoresed through low melting point agarose (Gibco, USA). Bands were excised and DNA extracted and sequenced according to methods described in chapter 2 (page 51).

6.3 RESULTS

6.3.1 Results of SSCP analysis using 7 different RT-PCRs

Figure 6.2 shows the SSCP patterns for 16 AML samples and 1 control using 3 different gel conditions. Five samples showed abnormal SSCP patterns using the first gel running condition (Figure 6.2, RT-PCR1A). But only 3 of these 5 samples showed abnormalities in the other two conditions (Figure 6.2, RT-PCR1B and 1C). Hence, the most sensitive gel condition for SSCP analysis of the p16 PCR fragments was the one without glycerol run at room temperature.

The HL60 cells showed an abnormal SSCP pattern (Figure 6.2, lane marked H). This concurred with the protein analysis data where no p16 protein was detectable for the cell line (chapter 7). TF1 cells showed a normal SSCP pattern and was subsequently used as the positive control (Figure 6.3A, lane marked T).
Figure 6.2: RT-PCR-SSCP analysis of exon 2 p16 gene using 3 different gel running conditions

(A) Without glycerol, room temperature, 15W for 5 hours

(B) With 10% glycerol, room temperature, 17mA for 16 hours

(C) Without glycerol, room temperature, 10mA for 16 hours
All 25 AML cases consistently showed a normal SSCP pattern for exon 1 (data not shown). For exon 2, seven repeat SSCP analysis were performed using different RT-PCR products each time. The reason for the multiple analysis was two-fold. One was to confirm the abnormal SSCP patterns found in the first analysis and secondly, to investigate for inconsistencies of abnormal results. The results for the 7 SSCP analyses involving 16 of the 25 patients are shown in Table 6.1. The other 9 had demonstrated normal SSCP patterns in two repeat analysis.

Table 6.1: Results of SSCP analysis of exon 2-3 of the p16 gene using 7 different RT-PCR reactions (Gei condition: without glycerol, run at room temperature, 15W for 5 hours)

<table>
<thead>
<tr>
<th>RT-PCR reaction number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>SEQ.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Abn</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Abn</td>
<td>Abn</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Abn</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>4</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Abn</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Abn</td>
<td>Abn</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>6</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Abn</td>
<td>Abn</td>
<td>Abn</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Abn</td>
<td>Abn</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Abn</td>
<td>Abn</td>
<td>Abn</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>N</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>N</td>
<td>Abn</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>Abn</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations:
N: Normal results
Abn: Abnormal SSCP pattern
(-): Failed RT-PCR
SEQ: Sequencing
The corresponding autoradiographs for the 16 samples are shown in Figures 6.2 (RT-PCR1), 6.3A and 6.3B. The most striking observation from the table of results is the failure to reproduce the abnormal SSCP patterns consistently using different RT-PCR reactions.

For the 7th RT-PCR reaction, 5 samples with at least two abnormal SSCP patterns were specifically chosen. Three different gel conditions were used for this final analysis. None of the samples showed any abnormal SSCP pattern in any of the conditions.

6.3.2 Results of sequencing of samples showing at least two abnormal SSCP patterns

To be certain that the failure to consistently reproduce the abnormal results was due to technical variability rather than a true mutation, a total of five RT-PCR products from samples showing at least two abnormal SSCP patterns were sequenced. All showed the normal coding sequence for exon 2. Four samples which showed consistently normal SSCP results were also sequenced and were found to be completely normal.

6.4 DISCUSSION

In this study, SSCP analysis of the \textit{p16} gene was undertaken to screen for mutations and polymorphisms in 25 AML cases with high blast counts. It was intended that for those showing abnormal SSCP patterns, the PCR products would be sequenced to characterise the mutations/polymorphisms.

The first analysis showed 5 samples with abnormal SSCP patterns (Figure 6.2, RT-PCR1). Three of these patterns were similar, suggesting that they could be due to a common polymorphism. The second analysis revealed abnormalities in 7 samples including all five from the first analysis (Figure 6.3A, RT-PCR2). However, the pattern of the mobility shifts was not consistent. It was then decided to perform 5 further repeat analyses using different RT-PCR reactions. As shown in Table 6.1, there is considerable variability in the abnormalities obtained from subsequent analysis. Most alarmingly, there was also a marked heterogeneity in the SSCP patterns seen on different analyses confirming the unpredictable nature of the mobility shifts in this particular study.
Figure 6.3A: SSCP analysis of exon 2 \(p16\) gene in 16 AML patients: showing variability in the mobility in 6 different RT-PCR reactions.
Figure 6.3B: SSCP analysis of exon 2 p16 gene in 16 AML patients: showing variability in the mobility in 6 different RT-PCR reactions
The intriguing question here was whether the abnormalities were real or not. If they were real, it could be possible that the experimental conditions were not identically simulated in succeeding analysis. Although all of the analyses represented in this table were performed at room temperature, which was the most sensitive of the three conditions, there was a high variability of the actual ambient temperature of the room concerned which was beyond the author's control. Hence, unnoticed fluctuations in the ambient temperature could have caused this puzzling variability in the results. In retrospect, an apparatus with a temperature probe for the gel could have been used to help maintain a constant running temperature.

The other factor which could have contributed to such high variability in the SSCP patterns was in the sequence of the PCR products themselves which was obtained from exonic primers. Stability of intrastrand bonds vary from one fragment to another. For the p16 PCR fragments, although the bonds might be sufficient enough to affect conformational changes, they may have been too weak to withstand changes in temperature hence giving rise to abnormal patterns only within a very narrow window of gel temperature. It would be interesting to compare the changes in sensitivity of the SSCP analysis using intronic primers for p16 as done by some studies (Hussussian et al., 1994; Okamoto et al., 1994).

However, the eventual sequencing of the RT-PCR products confirmed that all the samples which showed abnormal SSCP patterns had no mutations. The possibility of Taq induced errors giving rise to the abnormal SSCP patterns is remote since four of the samples had their abnormal SSCP pattern reproduced in two separate analysis. Furthermore, for Taq errors to occur and give rise to sufficient signals, they must happen early on in the PCR which is very improbable. It is most likely that the abnormal SSCP patterns observed are technical artefacts. What is most difficult to explain is that the abnormal patterns of some samples were found on the same gels that had normal patterns, suggesting that subtle changes in sample concentration, salt or protein contamination could also influence the mobility patterns. It should also be noted that SSCP analyses performed in the same laboratory over the same period for GM-CSF receptor α and β chains showed no such unexplained variability. The message again here is the importance of reproducibility of results and confirming abnormal data by other available means.
The final data showed that none of the 25 samples had point mutations/polymorphisms of the p16 gene. The rarity of deletions, rearrangements (chapter 5) and point mutations in AML again concurred with other studies (Ogawa et al. 1994; Quesnel et al., 1995; Sill et al., 1995). It is interesting as well to note that Otsuki et al (1995) reported similar findings in a study involving primary lymphoid tumours. None of the 117 cases analysed by the SSCP method had any mutations although homozygous deletions were found in 9 of them, mainly in ALL.

There is accumulating evidence from many studies which showed that the frequency of homozygous deletions of p16 is far greater than mutations. This interesting phenomenon is similar to that found in the globin gene where the α-globin gene is more frequently deleted than mutated in contrast to its β-globin counterpart (Weatherall, 1991). The speculation for p16 was that homozygous deletion was the major mechanism for inactivation in most primary tumours. This pattern also suggests the possibility that other tumour suppressor genes may be present within the 9p21 locus and may be deleted together with p16 in the evolution of certain malignancies. The p15 gene is one of the likely candidates for this since deletions of this gene have been found to accompany p16 deletions in several malignancies (Okamoto et al., 1995) although the concordance is not total.

In conclusion, the data presented here, together with the results of Southern blot analysis provide firm evidence that gross deletions and point mutations are very rare in AML. This however does not exclude the presence of mutations in the promoter region as well as in the introns or of trans acting factors which could give rise to changes in the expression of the p16 protein. The status of the p16 protein in the AML cases is presented in the next chapter.
CHAPTER SEVEN

THE STATUS OF p16 PROTEIN EXPRESSION IN AML PATIENTS AND THE CORRELATION BETWEEN pRB AND p16 DATA

7.1 INTRODUCTION

The discovery of the role of p16 as a CDK inhibitor links a putative oncogene (cyclin D1) and two tumour suppressor genes (RB1 and p16) via regulatory kinases (CDK4 and CDK6). Both pRB and p16 are negative regulators whilst CDKs and cyclins are positive regulators of cell growth. In a normal cell, the level of expression, the integrity and the timing of activation/inactivation of each of these components is crucial to ensure correct cell cycle transitions and coupling them to further progression through the cell cycle. An obvious prediction would be that deregulation of any of these components could result in disorder.

There is accumulating evidence that the disruption of each of these components independently or otherwise, can be potentially carcinogenic. For example, amplification of cyclin D1 resulting in the overexpression of the protein occurs in several tumours. This increasing body of evidence implicating cyclin D1 as a putative oncogene is reviewed in chapter 1. The inactivation of pRB by deletions or point mutations contributes to carcinogenesis (reviewed in chapter 4). Deletions of the p16 gene have been shown to be common at least in a subset of malignancies (reviewed in chapter 5). Finally, CDK4 amplification has been shown to occur in a subset of glioma cell lines with intact p16 genes (He et al., 1994). Overexpression of CDK4 has also been found in other tumour cell lines (Tam et al., 1994) and in some primary tumours (Khatib et al., 1993). True to the multi-step model of carcinogenesis, there is indirect evidence which show that dysfunction of these components can occur in succession to result in the malignant transformation of a cell. For example, both cyclin D1 overexpression and inactivation of pRB has been postulated to be crucial events in the development of parathyroid carcinoma from the benign adenoma stage (Cryns et al., 1995). What is most fascinating is that the common denominator for the events described above is the eventual inactivation of pRB.

The p16 protein was identified by its association with human CDK4 in a yeast two-hybrid protein interaction screen. It was found to inhibit specifically CDK4-
cyclin D kinase activity \textit{in vitro} (Serrano \textit{et al}., 1993) and later shown to inhibit CDK6 as well (Hannon and Beach, 1994; Parry \textit{et al}., 1995). The discovery of the frequent deletions found in cell lines provided \textit{in vivo} evidence of its importance (reviewed in chapter 5). In humans, the \textit{p16} gene is adjacent to a gene encoding a very similar protein, now called p15 (Hannon and Beach, 1994). The sequences of p16 and p15 proteins are 44% identical in the first 50 amino acids and 97% identical in the following 81 amino acids. Both bind and inhibit only CDK4 and CDK6 among the known CDKs. Unlike p16, the levels of p15 mRNA are induced more than 30-fold after TGFβ treatment of cells, indicating that it is likely to be responsible for the G\textsubscript{i} arrest of these cells (Hannon and Beach, 1994). The role of \textit{p15} gene in primary tumours has not been extensively investigated, in contrast to the \textit{p16} gene, although it is an excellent candidate tumour suppressor gene based on its growth arresting properties and the observation that deletions of the gene frequently accompany \textit{p16} deletions both in cell lines (Kamb \textit{et al}., 1994) and primary tumours (Okamoto \textit{et al}., 1995; Rasool \textit{et al}., 1995).

Merlo \textit{et al} (1995) recently reported that methylation of the 5' CpG island within exon 1 can inhibit the transcription of the \textit{p16} gene with a resultant absence of protein expression. The group found presence of methylation in 20% of the various tumours analysed. Similar CpG islands have been described in other genes including \textit{RB1} (Sakai \textit{et al}., 1991) and the \textit{VHL} (Von Hippel Lindau syndrome) tumour suppressor genes (Herman \textit{et al}., 1994). The CpG islands are located around the promoter region of housekeeping genes and methylation of cytosine to 5-methylcytosine is thought to play a role in the regulation of transcription (Bird, 1992). Methylation of the CpG islands of the \textit{RB1} has been described in retinoblastoma tumours (Sakai \textit{et al}., 1991).

The Southern blotting and the SSCP data (chapter 5 and chapter 6) showed that homozygous deletions and point mutations of the \textit{p16} gene are uncommon in AML. However, Serrano \textit{et al} (1994) proposed that physiological inactivation of pRB during G\textsubscript{i} leads to increased p16 protein expression in order to limit CDK4 activity. In other words p16 possibly prevents inappropriate phosphorylation of pRB. By extension, in cell lines lacking in pRB, p16 protein was shown to be overexpressed in a futile attempt to dampen uncontrolled cell proliferation (Tam \textit{et al}., 1994, Parry \textit{et al}., 1995). This negative feedback model predicts that tumours deficient in pRB would have increased expression of p16. The data from the analysis of pRB in the AML cases (see chapter 4) as well as from other studies (Weide \textit{et al}. 1993;
Zhu et al. 1994; Kornblau et al., 1994) have shown that lack of pRB expression is frequent in AML and so the prediction from studies with cell lines (Parry et al., 1995) was that increased p16 expression would also occur with the same frequency. Hence, this study was designed to test whether this inverse relationship was true for primary tumours. This chapter reports on the expression of p16 in AML and the correlation between p16 and pRB expression in primary tumours.

7.2 MATERIALS AND METHODS

Protein samples (prepared using methods described in chapter 2, page 45) from a total of 60 patients with AML and 9 haematologically normal controls were analysed by western blotting.

7.2.1 p16 western blotting

The western blotting technique is described in detail in chapter 2 (page 46). For the p16 westerns, 15% polyacrylamide gels were used. Membranes were probed with p16 antibody as described and later reprobed with an actin antibody. Two of the 3 primary antibodies against p16 used were the p16-N20 which recognises the amino-acid residues 4-23 at the N-terminus and the p16-C20 which recognises residues 128-147 at the C-terminal end of the protein (both are rabbit anti-peptide antibodies from Santa Cruz Biotechnology Inc. USA). These two antibodies were used at a concentration of 0.2μg/ml. The other antibody, a polyclonal antiserum raised against the whole protein (Pharmingen CA), was used at a concentration of 0.1μg/ml. Anti-tubulin (Boehringer Mannheim, Germany) and anti-actin (Cambridge Bioscience, UK) antibodies were used at 0.1μg/ml concentration as controls for protein loading and integrity. The secondary antibodies were a 1:2500 dilution of peroxidase conjugated anti-mouse or anti-rabbit immunoglobulins (Dakopatts, Denmark) as appropriate. Detection was by ECL (Amersham, UK).

7.2.2 CDK4 western blotting

For the analysis of CDK4, membranes for the p16 western blots were re-incubated with anti-CDK4 antibody (Santa Cruz, USA). This mouse monoclonal antibody was used at a concentration of 0.2μg/ml.
7.2.3 Analysis of p16 expression in different cell lines and during differentiation

In order to establish whether any changes in the expression of p16 could be due to normal variation which occurs during the cell cycle or the stage of myeloid differentiation, the abundance of p16 was determined by analysing several cell lines. This was crucial for the interpretation of levels of p16 protein when the AML samples are screened. The J6 cell line which lacks the p16 gene (chapter 5) was used as the negative control.

All cell lines were cultured in RPMI 1640 (Sigma, UK) with 10% (v/v) fetal calf serum (Gibco-BRL, Life Technologies Ltd., Scotland) in a humidified atmosphere containing 5% CO\textsubscript{2}. U937 cells (Harris et al., 1995) were cultured for 14 days with and without γ-interferon (Macintyre et al., 1988; Roberts et al., 1992). HL60 cells (Harris et al., 1995) were cultured for up to 7 days with or without the differentiating agents DMSO, vitamin D\textsubscript{3} or retinoic acid as described previously (Devalia et al., 1992). For both cell lines maturation by each agent was determined by the reduction of nitroblue tetrazolium (NBT). Cell numbers were determined on a daily basis by counting viable cells which exclude trypan blue and cell cycle profiles were determined as described previously (Burke et al., 1992). In each case the maturation agents caused cell cycle arrest of HL60 cells in G\textsubscript{1} whereas U937 cells continued to divide and mature in response to γ-interferon (data not shown). J6 cells (Parry et al., 1995) were grown as for U937. Total protein lysates were made according to methods described in chapter 2.

7.2.4 The specificity of p16 by western blotting

7.2.4.1 Peptide inhibition experiment

In this experiment, the specific peptide (10x in molar excess) was incubated with the anti-peptide p16 antibody for 2 hours at room temperature before being used. Two duplicate p16 western blot membranes were prepared: one was incubated with the p16 primary antibody and the other incubated with the mixture of p16 antibody and its specific peptide. The result showed that the peptide inhibited the antibody completely (Figure 7.1A) and the 16kDa bands did not appear. On the other hand, the 16kDa bands were prominent in the membrane incubated with the primary antibody alone (Figure 7.1B). This experiment confirms that the p16 antibody used binds specifically to a 16kDa
Figure 7.1: Specificity of p16 western blotting

7.1A: p16 peptide inhibition

- p16
- 14.3kDa marker

Without peptide

AML patients (n=7)

With peptide inhibition

AML patients (n=7)

7.1B: *in-vitro* translation of *p16* cDNA followed by immunoprecipitation and western blotting

- Lanes:
  - a Daudi cells extract
  - b Extract from IVT of p16 cDNA
  - c IVT using luciferase control
protein. However, the experiment does not prove that the 16kDa protein is p16.

7.2.4.2 *In vitro* translation of *p16* cDNA followed by immunoprecipitation

In order to determine whether the 16kDa protein is truly p16, p16 was transcribed and translated from the *p16* cDNA (using the TNT™ Coupled Reticulocyte Lysate Systems, Promega, UK), immunoprecipitated and then detected by western blotting.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amount in µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNT lysate</td>
<td>25</td>
</tr>
<tr>
<td>TNT Reaction Buffer</td>
<td>2</td>
</tr>
<tr>
<td>TNT RNA Polymerase</td>
<td>1</td>
</tr>
<tr>
<td>Amino acid mixture minus methionine, 1mM</td>
<td>1</td>
</tr>
<tr>
<td>35S-methionine (1000Ci/mmol) at 10µCi/mol</td>
<td>4</td>
</tr>
<tr>
<td>RNAsin Ribonuclease Inhibitor, 40U/ml</td>
<td>1</td>
</tr>
<tr>
<td><em>p16</em> cDNA (250ng/ml)</td>
<td>2</td>
</tr>
</tbody>
</table>

The *p16* cDNA-containing plasmid was a gift from Dr. David Beach, USA, and a Luciferase cDNA was used as the negative control. Transcription/translation was according to the manufacturer's instructions and the incubation was carried out for 2 hours to maximise the amount of protein synthesised. At the end of the incubation, p16-immunoprecipitation was done on the lysates using methods described in chapter 2 (page 48). A Daudi cell lysate was used as a positive control. Western immunoblotting was then performed. Figure 7.1B shows the result of this experiment. The 16kDa bands detected was identical for the Daudi cells and the lysate from the *in vitro* translation. The luciferase control was totally negative. This result verified that the 16kDa bands obtained from the western blotting correspond to p16 protein.

7.2.4.3 Analysing the methylation status of the 5' CpG island of *p16* gene in samples with reduced p16 expression

Ten µg of DNA was digested with 20U of *EcoR*I overnight at 37°C. This was then divided into two aliquots of 5µg each. One of them was then digested with 10U of *Sma*I, a methylation sensitive restriction enzyme, for a further four hours at room temperature. Southern blotting was then performed according to methods described in chapter 2 (page 45). The probe used was a 340bp
7.3 RESULTS

7.3.1 p16 protein expression in AML patients

Protein samples from 60 patients with AML and 9 haematologically normal controls were analysed by western blotting with two anti-p16 antibodies, one directed to the N-terminus and the other to the C-terminus. All the samples had some detectable p16 protein. However, six samples (10%) analysed with the N20 antibody showed a marked reduction in the p16 levels (represented by lanes b-e, middle panel, Figure 7.2). Using a polyclonal anti-serum (Pharmingen, USA) these six samples had undetectable p16 (represented by lanes b-e, lower panel, Figure 7.2). This indicated that the N20 antibody was more sensitive than the polyclonal antiserum.

A marked increase in the abundance of p16 was detected with both antibodies in a further six samples (10%). Two of these cases are represented in Figure 7.2, middle and lower panels, lane f and g. In all cases the blots were re-probed with antibodies to tubulin or actin to correct for loading and transfer differences. The p16 signals were scanned with a flatbed scanner (Howtek) and quantified using the Whole Band Analyzer software (Millipore). The range of signal intensity of the p16 band from 9 normal samples were measured and this was used as the reference range to compare against the signals from the AML patients. The six samples with p16 overexpression, when corrected for actin, had intensities 5 times greater than the average for normal samples while those with markedly reduced expression had signals 4 times less than normal. These differences were statistically significant (P value = 0.02). The Pharmingen anti-p16 antibody and the N20 (Santa Cruz) also detect p15 and in no case did the abundance of p15 change.

7.3.2 p16 protein in cell lines

Cell lines were used in order to determine whether alterations in p16 levels observed above were not due to changes in cell cycle progression or cell differentiation. When HL60 cells are treated with DMSO, retinoic acid or
Figure 7.2: Variable expression of p16 in AML patients

lanes:
a Normal control
b-d Patients with reduced levels of p16
e-f Patients with increased levels of p16
Vitamin D3, they arrest in the G1 phase of the cell cycle and differentiate. In contrast, γ-interferon induces differentiation without also causing cell cycle arrest. No significant change in the level of p16 was observed in either the HL60 or the U937 cell lines under any of the conditions studied (Figure 7.3). It should be noted that a second batch of HL60 cells in our laboratory has no detectable p16 in line with the report from Otterson et al (1994) (Figure 7.4, lane b). The WERI-1 cell line which lacks pRB has intact p16 (Figure 7.4, lane d) while the J6 cell line has no detectable p16 (Figure 7.4, lane c).

7.3.3 pRB levels in AML patients with altered p16 expression

The pRB data (chapter 4) was correlated with the results of the p16 protein studies. pRB was found to be abnormal (2 lacking pRB and 2 with truncated protein) in four of the six samples with elevated p16 levels. The other 2 samples had normal pRB. Ten other AML samples with no detectable pRB had normal p16. In the 6 cases with reduced p16, the pRB was normal. The correlation between the p16 and pRB data is shown in Table 7.1. The proportion of AML patients with abnormal pRB showing overexpression of p16 is statistically significant (P value = 0.023).

Table 7.1: Correlation between p16 overexpression and pRB expression in 60 AML cases

<table>
<thead>
<tr>
<th></th>
<th>Total number of cases</th>
<th>Cases with increased p16</th>
<th>Frequency of p16 overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal pRB</td>
<td>14</td>
<td>4</td>
<td>29%</td>
</tr>
<tr>
<td>Normal pRB</td>
<td>46</td>
<td>2</td>
<td>4%</td>
</tr>
</tbody>
</table>

7.3.4 CDK4 protein levels in AML patients with altered p16 expression

Since p16 exerts its actions in the cell cycle by competitive binding to CDK4, 9 of the 12 patients with abnormal p16 expression were analysed for CDK4 levels by western blotting. A representative blot is shown in Figure 7.5. Three out of the six cases with reduced levels of p16 had apparent high levels of CDK4 when corrected for actin (Figure 7.5, lanes f-h). The cases with high p16 levels however showed no reciprocal reduction in CDK4 levels. (Figure 7.5, lanes b-d).
Figure 7.3: p16 expression in cell lines with and without differentiating agents

Lanes:
- a  U937 cells
- b  U937 cells cultured with gamma-interferon
- c  HL60 cells
- d  HL60 cells cultured with DMSO
- e  HL60 cells cultured with retinoic acid
- f  HL60 cells cultured with Vit D3
Figure 7.4: Absence of p16 expression in J6 (T-cell) and HL60 cell lines

lanes:
a  Daudi cells
b  HL60 cells
c  J6 cells
d  WERI-1 cells
Figure 7.5: CDK4 expression in 8 samples with abnormal p16 expression

Lanes:

b-d  Patients with increased levels of p16
a, e-i Patients with reduced levels of p16
7.3.5 Methylation status of the 5' CpG island in cases with reduced p16 expression

The methylation status of the 5' CpG island was analysed in the six samples with reduced expression of p16. A representative Southern blot is shown in Figure 7.6. Digestion with EcoRI alone resulted in a 4.2kb band in all the cases. Digestion with both EcoRI and Smal gave rise to two bands: a 0.4kb band and a 0.65kb band. Methylation of the 5' CpG island would have protected this site from digestion with Smal resulting in an intact 4.2kb band. Hence, the result indicate that there was no methylation of the CpG island in the six cases analysed.

7.4 DISCUSSION

The data in this chapter shows that p16 is expressed in all the AML cases analysed but that the level of p16 protein varied over an arbitrary 9-fold range. Six of the 60 samples (10%) had reduced levels p16 protein. This reduction is probably not due to changes in cell cycle progression or differentiation as the studies with HL60 and U937 cell models showed that the levels of p16 expression do not change during differentiation or cell cycle arrest in G1. It is possible that the low levels of p16 found in certain AML samples represent 'contamination' by non-leukaemic cells and that the leukaemic cells are totally deficient in p16. Although the current study was carried out using protein samples prepared from mononuclear cells which would predominantly contain leukaemic blast cells, the presence of non-leukaemic cells could not be excluded.

The underlying cause for reduced expression of p16 in these six samples was further investigated by analysing the methylation status of the 5' CpG island within exon 1. Methylation of this site was reported to be a mechanism for the transcriptional silencing of the p16 gene (Merlo et al., 1995). However none of the six samples analysed had methylation of the 5' CpG island indicating that this mechanism was not responsible for the reduced expression of p16 protein.

It is of interest that in two batches of HL60 cells that were studied, p16 was absent in one batch but not in the other, suggesting that the loss of p16 might be a secondary event arising during in vitro culture. This observation could explain the high frequency of deletions in cell lines and it also serves to
Figure 7.6: Southern blot showing methylation status of CpG island in 6 AML cases with reduced p16 expression.
illustrate the importance of studying primary cells. There has been speculation on the possibility that the \textit{p16} gene could be selectively deleted for the establishment of cell lines (Okamoto \textit{et al.}, 1994). If this is true, then it is understandable that Kamb \textit{et al.} (1994) found a high frequency of deletions in cell lines since the absence of \textit{p16} has could have contributed to the immortalisation of these cell lines. Against this idea however is the common occurrence of deletions in oesophageal carcinomas (Mori \textit{et al.}, 1994) and pancreatic adenocarcinoma (Caldas \textit{et al.}, 1994). It is most likely that inactivation of \textit{p16} contributes to the genesis of only a subset of malignancies rather than the wider role speculated earlier by Kamb \textit{et al.} (1994).

The most important observation in this study was the correlation shown between the levels of \textit{p16} protein and the status of \textit{pRB} expression especially in the six cases in which the \textit{p16} protein was overexpressed. Four of these samples had abnormal \textit{pRB} expression (2 non-detectable, 2 altered bands). Statistical analysis on the frequencies of \textit{p16} overexpression in cases with normal \textit{pRB} against those with abnormal \textit{pRB} showed that this correlation between \textit{p16} overexpression and abnormal \textit{pRB} expression is significant (P value = 0.023).

It has been shown \textit{in vitro} that \textit{p16} competes with cyclin D1 for binding to CDK4 and thereby inhibits CDK4 activity (Nobori \textit{et al.}, 1994). It has been postulated that \textit{p16} acts in a feedback loop to down-regulate CDK4 once \textit{pRB} has been inactivated by phosphorylation and that in cell line lacking \textit{pRB}, \textit{p16} expression is increased with the aim of inhibiting CDK4 (Serrano \textit{et al.}, 1994). This loop is represented in Figure 7.7. However, if the only substrate for CDK4 is \textit{pRB} then this feedback loop is futile in these cells as they lack functional \textit{pRB}. This overexpression of \textit{p16} in response to a defective \textit{pRB} has been shown to occur universally in cell lines (Parry \textit{et al.}, 1995). They also demonstrated that in cells lacking \textit{pRB}, both CDK4 and CDK6 form binary complexes with the highly expressed \textit{p16} at the expense of the D type cyclins. This reciprocal modulation between \textit{pRB} and \textit{p16} was also demonstrated recently in 4 out of 5 cases of small cell lung carcinoma (SCLC) (Shapiro \textit{et al.}, 1995).

Our data on the four cases with abnormal \textit{pRB} showing overexpressed \textit{p16}, are consistent with a negative feedback response occurring \textit{in vivo} in human primary myeloid cells. However this situation does not occur in all cases with abnormal \textit{pRB}, which is consistent with the data for SCLC. Ten other AML
Figure 7.7: Feedback loop between pRB and p16 and the resultant overexpression of p16 in cells lacking pRB

Cells with normal pRB

Cells lacking pRB
cases with no detectable pRB had normal p16 protein levels. This is particularly important as these data show that the feedback loop does not cause overexpression in all cases. It is possible that these samples may have mutations in other genes which dampen the p16 feedback loop or that the overproduction of p16 reported by others only occurs in certain specific instances. The abundance of the related p15 protein did not vary in our samples indicating that a similar loop probably does not occur for p15. Other studies have found p15 gene deletions in a subset of tumours with p16 deletions (Jen et al., 1994; Okamoto et al., 1995; Rasool et al., 1995).

Our data on CDK4 levels in cases with abnormal p16 protein again highlights the fact that although the network of interactions between pRB, p16 and CDK4 has been shown in vitro, it is difficult to prove any direct correlation between the protein levels in vivo in primary tumor cells. He et al (1994) recently showed that CDK4 amplification occurs in a subset of glioma cell lines with intact p16 genes, suggesting that overexpression of the CDK4 gene could be an alternative mechanism for abrogating the growth-regulatory effects of p16. None of their cases with p16 deletions had amplified CDK4. Our data instead shows that in three of six cases with low levels of p16 protein the CDK4 levels were increased. Therefore it appears that there is considerable heterogeneity in the feedback mechanisms affecting the abundance of these cellular proteins, but it is probable that inactivation of pRB is central to these changes. Further studies are required to determine the clinical significance of these protein interactions and their contribution to the pathogenesis of leukaemia.

In summary, this chapter confirmed the rarity of p16 inactivation in AML which concurred with the data from the Southern blotting. The reciprocal interaction between pRB and p16 provides evidence that the postulated feedback loop does exist in primary cells. This observation also suggests that malignant transformation of a cell can produce changes in the modulation of pRB, CDK4 and p16. However, the fact that it was only seen in a subset of patients with abnormal pRB in contrast to the findings found in cell lines (Parry et al., 1995) underlines the differences between observations using cell lines and events occurring in vivo in primary cells. Further studies are required to define how the relative levels of p16, pRB, CDK4 and cyclin D are balanced in normal cycling cells so as to provide some understanding of the modulatory changes which are observed in primary tumours.
CHAPTER EIGHT

CONCLUSIONS

Acute myeloid leukaemia is a clonal proliferative disorder whereby the balance between self-renewal and differentiation of a myeloid progenitor stem cell has been disrupted by multiple genetic events. These events could involve the activation of oncogenes and/or inactivation of tumour suppressor genes. These genes are vital players in three main growth control pathways: (1) the signal transduction pathway, (2) the pathway which involves p53, and (3) the intrinsic cell cycle pathway which involves pRB. Every known oncogene and tumour suppressor gene can be placed in at least one of these pathways. The third pathway was the main theme of this thesis and two of its components \textit{RB1} and \textit{p16} were the principal targets of analysis in an attempt to define their contribution to the pathogenesis of AML. The proteins encoded by these two genes, together with CDK4 and cyclin D1, regulate the G\textsubscript{1} checkpoint control in the cell cycle. The function of one component is as crucial as the others, whereas the deregulation of any one of them is likely to be sufficient to disrupt the pathway.

Analysis at DNA and protein levels was performed. The data from the individual chapters provide the frequencies of gene rearrangements and protein inactivation in AML. More importantly, the correlation between the pRB and p16 protein data provided the opportunity to examine the interaction between these two proteins and postulate on the modulatory changes which may accompany malignant transformation of a cell.

The DNA analysis of \textit{RB1} in chapter 3 is by far the largest study involving AML to date. Gross rearrangements in the retinoblastoma gene were found to be rare. In fact, none of the 106 cases analysed using 4 different restriction enzyme digests showed any true rearrangements. Analysis of \textit{RB1} at the DNA level has been made difficult by the complexity in the structure and organisation of the gene, much like the proverbial 'finding the needle in a haystack'. It was in view of this that Lee \textit{et al} (1993) in a review chapter on the retinoblastoma gene, forewarned against the misinterpretation of abnormal \textit{RB1} Southern blotting results. The only comparable studies at the DNA level available for AML were by Ahuja \textit{et al} (1991) which reported a 13\% frequency of rearrangements in 54 cases and by Chen \textit{et al} (1991) which showed no abnormalities in all 69 cases analysed. The former study further described
that abnormalities were commoner in the M4 and M5 subtypes. I have attributed the marked contradiction between the results of this study and my own to the difference in interpretation of Southern blots. Thirteen of the cases analysed in my study had 'abnormal' bands which were eventually proven to be due to either polymorphisms or artefacts. These conclusions were reached after repeated analyses as well as using other RB1 probes. The analysis of 18 normal controls substantiated the fact that polymorphic bands appear as novel bands and may look as abnormal as true rearrangements. At the end of it all, the warning about 'misinterpretation' in the paper by Lee et al had been prophetic indeed. The significance of the results would have been misleading, had the alleged 'common' novel bands been accepted without question as true rearrangements. This chapter underscored the importance of repeating and confirming abnormal looking results to avoid spurious interpretation of data.

The protein analysis in chapter 4 revealed that 31% of the samples had abnormal expression of pRB. This frequency was within the range reported by previous studies. Another interpretative issue was highlighted in this chapter, which was whether reduced expression of pRB could just reflect the quiescent state of the majority of the cells analysed. This issue is currently still being debated and until a reasonable criteria for reduced expression of pRB is established, I believe that it should not be interpreted as abnormal. The discordance between the lack of rearrangements at the DNA level and the relatively high frequency of abnormal pRB suggests that the mechanism for inactivation of pRB in the 31% of cases is likely to be point mutations, minor deletions or mutations in the promoter region of the gene. This seem to be the case for most solid tumours, where point mutations in the RB1 are more common than gross rearrangements. Other possible mechanisms include methylation of CpG sites in the promoter region resulting in the repression of transcription, and disruption of other genes which may play a role in controlling RB1 transcription.

The fact that none of the AML samples showed rearrangements despite having protein abnormalities, also highlights the difference between handling leukaemic samples and solid tumour tissues. Whereas a reasonably 'clean' excision of solid tumours can be achieved for the purpose of DNA extraction and analysis, those analysing leukaemic samples have to cope and deal with the inevitable 'contamination' by non-leukaemic cells. Researchers must
always take this into account until better methods to purify leukaemic samples are available.

The fact that there is no preponderance of pRB inactivation in the M4 and M5 FAB types refutes the initial postulation of lineage specificity by Ahuja et al (1991) although it must be stressed here that the study by Ahuja et al involved mainly DNA analysis which is much less sensitive than western immunoblotting. In fact, Kornblau et al (1994) also found no selectivity of protein inactivation of pRB for these FAB types in their study. In fact, both Kornblau's and my studies showed a higher frequency of inactivation in M2 and M3 cases, but statistical analysis of this apparent preponderance revealed that these differences were not significant. The message here is that even in a relatively large study, differences in frequencies of involvement in different FAB types may just be a chance occurrence rather than reflecting a lineage specificity. The practice of making postulations based on small scale studies is dangerous and should be avoided.

The question remained as to where pRB inactivation sits in the leukaemogenesis pathway. Although there are only two possibilities, either initiation or progression of leukaemia, assigning which one of these applies to AML is not easy and cannot be made conclusively from the studies here. For the 69% of cases without abnormality, pRB obviously did not have a role at all. On the other hand for the 31% of the cases with abnormal pRB, this inactivation could have been an early or a late event. The fact that only a subset of AML had abnormal pRB suggests that other genetic events could have occurred to initiate the leukaemia in all the cases, and that inactivation of pRB was actually a later event in a subset of patients. Other indirect evidence comes from the study by Preudhomme et al (1994) which showed that inactivation of pRB is rare in myelodysplasia which is considered to be a pre-leukaemic condition. This postulation is consistent with the multi-step model of carcinogenesis and the current belief that initiation and progression of disease are two separate processes. To further confirm this, analysis at single cell level must be attempted in future using immunostaining methods or flow cytometry for the protein, and the elegant in-situ PCR or in-situ RT-PCR for DNA and RNA studies respectively. Such analysis is not easy to optimise but will certainly be more precise.

The current data on solid tumours generally show that pRB inactivation is more common in high grade as compared to low grade tumours, and in
metastatic tumours as compared to non-metastatic tumours. Whether this can be translated to the fact that pRB inactivation is a bad prognostic marker for cancer remains to be fully established. Studies involving solid tumours have shown conflicting data. Univariate and multivariate analyses done to relate pRB inactivation and breast cancer survival (Pietilainen et al., 1995) showed that pRB expression had no prognostic value whatsoever, even though 36.6% of cases had inactivation of pRB. Studies by Kornblau et al. (1994) suggest that AML patients with low levels of pRB tend to have a poor treatment response and survival rates. My data also showed a similar pattern but the differences obtained were not statistically significant.

The second half of this thesis dealt with the p16 gene. The great excitement that initially greeted the arrival of this CDK4 inhibitor was basically aroused by the discovery that a large proportion of cell lines had homozygous deletions of this gene (Kamb et al., 1994). It also triggered a multitude of studies on primary tumours including the study in this thesis. The p16 study presented here capitalised on the fact that pRB inactivation had been detected in a significant number of AML patients. Hence, the analysis of the p16 status in these patients was of considerable interest.

The analysis of homozygous deletions of the p16 gene in chapter 5 was made difficult by the issue of 'contamination' of the leukaemic samples with non-leukaemic cells. This problem will be encountered by any study involving leukaemic samples and is amplified further by the use of powerful techniques such as the PCR. I attempted to address this issue and with quantification and the use of a proper internal control, the study has shown that accurate interpretation of results can be achieved with the less sensitive Southern blotting analysis. Although I believe that the PCR approach could have been utilised in the same manner, the amplification factor in the technique itself which could amplify significantly non-leukaemic cells in the samples, was the major overriding factor in the conversion to Southern blotting as the mode of analysis. The conclusion of the screening analysis was that homozygous deletions of the p16 gene were rare in AML. The results concurred with at least 3 other studies (Ogawa et al., 1994; Quesnel et al., 1995; Sill et al., 1995).

The search for point mutations in the p16 gene was then pursued using the RT-PCR-SSCP analysis. The data presented in chapter 6 showed that none of the 25 samples screened had consistent mutations in the gene. It can be
inferred here that SSCP analysis is very much sequence dependent. For a small gene as in the case of \( p16 \), direct sequencing of PCR products is probably more efficient. The conclusion from this study was that point mutations and polymorphisms of the \( p16 \) gene were rare in AML.

Within the same time period there were studies reporting on the frequency of \( p16 \) deletions in acute lymphoblastic leukaemia (ALL). At least five studies (Ogawa et al., 1994; Hebert et al., 1994; Quesnel et al., 1995; Okuda et al., 1995; Rasool et al., 1995) showed that deletions of the \( p16 \) gene were commoner in ALL, especially those of T-cell lineage. This preponderance for the T-cell lineage as compared to the non-T cell lineage has been shown to be significant in one of the studies (\( P = 0.0171 \)) (Rasool et al., 1995). It is still premature to conclude that there is a lineage specificity until more studies are done. It is also known that inactivation of pRB is infrequent in ALL, as opposed to AML. This fascinating paradox mirrored the findings in lung cancer where \( RB1 \) deletions are more common in small cell lung cancer (SCLC) compared to non-small cell lung cancer (NSCLC) whereas the reverse is true for \( p16 \) deletions (Okamoto et al., 1995). There have been no reported findings of simultaneous deletions of \( RB1 \) and \( p16 \) in the same tumour. The current impression is that either of these deletions or inactivations is sufficient for cancer formation. If this hypothesis is true, one might expect that for a particular malignancy, there would be an equal proportion with \( RB1 \) deletions and another with \( p16 \) deletions, but this does not seem to be the case. The implication of this paradox is even more interesting for haemopoietic malignancies, given the fact that the lymphoid and myeloid lineages arise from different committed progenitor cells. The inevitable speculation is that there is a lineage specificity for \( p16 \) and pRB inactivation in the pathogenesis of leukaemia. It can be further proposed that \( p16 \) deletion selects for the expansion of a lymphoid leukaemic blast, possibly T-cell lineage, whereas an \( RB1 \) deletion selects for a myeloblast expansion. Further studies to confirm this hypothesis are needed.

The analysis of \( p16 \) protein expression in chapter 7 confirmed the rarity of \( p16 \) gene deletions in AML. None of the six cases with reduced expression of \( p16 \) protein had methylation of the 5' CpG island suggesting that other mechanisms are involved. The more interesting observation here was that overexpression of \( p16 \) protein occurred in a significant subset of patients with abnormal pRB (\( P \) value = 0.023). The regulation of pRB by \( p16 \) and CDK4 predicts this occurrence, assuming that there is a negative feedback loop.
operating between pRB and p16. This reciprocal interaction has been confirmed to be present in cell lines (Parry et al., 1995; Tam et al., 1994). The correlation of data from the pRB and p16 studies in this thesis however showed that this inverse relationship does not occur universally, suggesting that this feedback loop may be affected by other proteins as well, either directly or indirectly. It also highlighted the danger of over-interpreting in vitro observations and extrapolating this to explain in vivo events.

The rarity of p16 gene deletions found in this study has added AML to a significant list of primary tumours with similar findings. It is now likely that p16 gene deletions are restricted to only a subset of human tumours and that other tumour suppressor genes may be located within the 9p21 locus. Although the excitement over its wide-ranging tumour suppressive power has substantially abated, the role of p16 and the other CDK inhibitors within the cell cycle remains unchallenged and within a short period of time has remarkably changed the understanding of the pathways involved.

In the final analysis, inactivation of pRB is common in AML. On the other hand p16 deletions and its inactivation in AML are rare. Inactivation of pRB seem to be the convergence point for deletion of p16 and overexpression of CDK4 or cyclin D1. It is fascinating that the genesis of a significant proportion of malignancies, in addition to other genetic events, appears to involve the deregulation of only one of these components emphasising the crucial importance of the pRB pathway in tumorigenesis. The G1 checkpoint control and the main players operating it are without doubt destined to dominate cancer research for years to come.

My suggestions for further studies include the following:

(1) To analyse leukaemic blasts at a single cell level using flowcytometric methods, or in situ PCR or RT-PCR analyses for DNA and RNA respectively, to ascertain the presence of sub-populations of blast cell with different expression of pRB, to confirm further the role of pRB in progression rather than initiation of leukaemia.

(2) To analyse the 'pocket' region of the RB1 gene for mutations using direct sequencing of PCR products to establish whether the frequent inactivation seen in AML could be due to mutations within this location.
(3) To perform tests of pRB function such as E2F band shifts or immunoprecipitation studies using properly prepared protein samples for the analysis of the interaction of pRB with other proteins. This would allow a more specific and more accurate interpretation than immunoblotting.

(4) To analyse T lymphocytes, B lymphocytes, CD34+ve cells and other progenitor cells of both the myeloid and lymphoid lineages for differential expression of either pRB or p16. This could help us to understand the reason for the apparent preferences of p16 gene deletions for ALL and pRB inactivation for AML.

(5) To analyse CDK4 and cyclin D1 at both the DNA and protein level to define further the role of the G1 check-point control in leukaemogenesis.
REFERENCES


Liu, Q., Neuhausen, S., McClure, M., Frye, C., Weaver-Feldhaus, J., Gruis, N., Eddington, K., Allalunis-Turner, M., Skolnick, M., Fujimura, F. and Kamb,


detection of point mutations and DNA polymorphisms using the polymerase
chain reaction. Genomics 5, 874-879.

Detection of polymorphisms of human DNA by gel electrophoresis as single-

Involvement of CDKN2 (p16/MTS1) and p15/MTS2 in human leukaemias and

p16 protein is restricted to the subset of lung cancer lines that retains wildtype
RB. Oncogene 9, 3375-3378.

the retinoblastoma gene product recognize different DNA motifs related to the
E2F binding site. Oncogene 7, 1075-1081.

proliferation. Proc Natl Acad Sci USA 71, 1286-1290.

Ann Rev Biochem 47, 715-750.

complexes in Rb-negative cells correlates with high levels of p16 tumour
suppressor gene product. EMBO J 14, 503-511.


Cell Biol 6, 853-858.

Pietilainen, T., Lipponen, P., Aaltomaa, S., Eskelinen, M., Kosma, V. and
cancer as related to established prognostic factors and survival. Eur J Cancer
31, 329-333.


Wiggs, J., Nordenskjold, M., Yandell, D., Rapaport, J., Grondin, V., Janson, M., Werelius, B., Petersen, R., Craft, A., Riedel, K., Liberfarb, R., Walton, D.,


ABSTRACTS PUBLISHED


PAPERS SUBMITTED

Jamal, R., Thomas, N.S.B., Gale, R.E. and Linch, D.C. (1995). Variable expression of p16 protein in patients with Acute Myeloid Leukaemia without gross rearrangements at the DNA level. (Accepted by "Leukaemia" subject to 1st. revision)