EVALUATION AND APPLICATION OF SCREENING STRATEGIES FOR POINT MUTATIONS IN THE RETINOBLASTOMA GENE

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To My Wife Gökçen

DECLARATION

I CERTIFY THAT THIS THESIS DOES NOT CONTAIN MATERIAL PREVIOUSLY PUBLISHED OR WRITTEN BY ANY OTHER PERSON, EXCEPT WHERE REFERRED TO IN THE TEXT AND THAT THE RESULTS IN THIS THESIS HAVE NOT BEEN SUBMITTED FOR ANY OTHER DEGREE OR DIPLOMA.

MUNIS DÜNDAR

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A	adenine
aa	amino acid
AIP	acute intermittent porphyria
AFP	alphaphetoprotein
AMD	amplification and mismatch detection
APS	ammonium persulphate
ARMS	amplification refractory mutation system
ASO	allele specific oligonucleotide
ATP	adenosine tri-phosphate
bp	base pairs
C	cytocine
cDNA	complementary copy DNA
CI	confidence interval
cm	centimetre(s)
cpm	count per minute
dATP	2'deoxyadenosine 5'-triphosphate
dCTP	2'deoxycytidine 5'-triphosphate
ddNTP	dideoxyribonucleoside triphosphate
DEPC	diethyl pyrocarbonate
DGGE	denaturing gradient gel electrophoresis
dGTP	2'deoxyguanidine 5'-triphosphate
dH_2O	distilled water
dITP	2'deoxyinosine 5'triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease

dNTP	deoxyribonucleoside triphosphate
dsDNA	double strand DNA
DTT	dithiothreitol
dTTP	2'deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
et al	et alia
g	gravitational (centrifugal) force
G	guanine
g	gram
hr	hour(s)
kb	kilobase
l	litre
M	molarity
MCAD	medium-chain acyl-CoA dehydrogenase
mg	miligram
min	minute
ml	millilitre(s)
mM	millimolar
MOPS	3-N-morphino-propanesulphonic acid
mRNA	messenger ribonucleic acid
n	nano
nm	nanometre
OD	Optical density
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

pmol	pico mole
poly(A)$^+$	polyadenylated
RB	retinoblastoma gene locus
Rb	retinoblastoma
rev/m	revolutions per minute
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNases	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
sarcosyl	sodium salt of N-lauryl sarcosine
SDS	sodium dodecyl sulphate
sec	second
SCLC	small cell lung carcinoma
SIDS	sudden infant death syndrome
SSC	standard saline citrate
SSCP	single strand confirmation polymorphism
ssDNA	single strand DNA
T	thymidine
Taq	*Thermus aquaticus*
TBE	Tris-borate-ethylenediaminetetraacetic acid
TE	Tris-ethylenediaminetetraacetic acid
TEMED	N,N,N,N'-tetramethylethylenediamine
Tm	melting temperature
Tris	tris(hydroxymethyl)aminoethane

tRNA	transfer ribonucleic acid
u	unit(s)
μg	microgram(me)
UV	ultra violet
Xp	short arm of the X chromosome

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CHAPTER ONE: SUMMARY.

1. SUMMARY

The present study aimed to evaluate two screening strategies, single strand conformation polymorphism (SSCP) and amplification mismatch detection (AMD) analysis, for the detection of point mutations in the retinoblastoma gene (RB). SSCP was optimised and applied to exons 12-22 of the RB gene which constitute the most important functional domain. Leukocyte DNA from 20 patients with bilateral retinoblastoma (Rb), tumour DNA from 40 patients with bladder carcinoma and tumour DNA from 39 patients with breast carcinoma were subjected to SSCP analysis. SSCP band shifts were found in 4 of 20, 1 of 40 and none of 39 patients respectively. AMD was optimised and applied to exons 12-16 of the RB gene and also to reverse-transcriptase PCR in the 20 patients with bilateral Rb. Cleavage was found in 2 patients: one was found in a cDNA segment and the other was found in genomic DNA. Neither of these patients corresponded to the 4 with SSCP band shifts. Thus in total, 6 patients with Rb and one with bladder carcinoma had mutations detected and proof was sought by sequencing.

Amplification of segment C of the cDNA of patients with bilateral Rb has revealed that patient EAS showed an additional band indicating either a deletion or a splice mutation. Analysis of exon 17 and the flanking intron of the same patient with AMD showed a cleavage with hydroxylamine. Sequencing of the exon revealed that the mutation is a C substitution of the A at position -2 of the acceptor site of intron 16, impairing normal splicing of the RNA. The mutation results in skipping of exon 17, because the short transcript band on the agarose gel was approximately 196 bp shorter than the original band and exon 17 was 196 bp in size. This leads in turn to the production of a truncated RB protein. By analogy to other published mutations, this aberrant, destabilised protein might not be able to bind the E1A oncoprotein. In addition, the mutant RB protein may fail to complex with SV40 large T antigen.

Analysis of segment C of the cDNA from patient PC with bilateral Rb showed a cleavage with hydroxylamine reaction. Sequencing of the segment revealed the mutation to be a T →G transversion at nucleotide position 1587 within exon 16 causing a substitution of histidine to glycine. The missense mutation may or may not have a functional effect. However, this residue lies within an RB domain (aminoacids 393-572) identified recently by in vitro deletion mutants to be required for oncoprotein binding. This mutation creates a restriction site for *Nde* I.

Sequencing of exon 21 from patient MH who had an SSCP band shift, revealed that an insertion of a G at nucleotide position 2251 within exon 21 resulted in a novel stop codon (TAA) at codon 719 (nucleotide position 2295) within exon 21 thus deleting the domain interacting with the SV40 T antigen. The translated protein is most probably too short to be functional. To confirm whether the observed SSCP pattern in the region of exon 21 of the RB gene was a new germ line mutation or inherited from one of the parents, heteroduplex analysis of the parents revealed either the mutation was de novo or one of the parents had germ line mosaicism. In addition, the change creates a restriction site for the restriction enzyme *Fok*I.

Another mutation detected (from patient AR) by SSCP analysis was an A to C transversion at position 1636 in exon 16 causing AGA to CGA codon change, both coding for the same amino acid: Arginine, this mutation abolishes a restriction site for the restriction enzyme *Cvij*I. The mutation site was located in the last base of the exon 16, although it has not been shown in this study in mRNA, it could affect splicing of the mRNA of RB gene.

Two mutations found were considered to be silent mutations, because they do not cause any amino acid change. Their RNA transcripts were found to be normal. These mutations

were a T to C transition at position 1617 in exon 16 (Patient GM) which alters the codon GTT (GUU) to GTC (GUC) both coding for the same amino acid: Valine and an A→G transition in intron 19 (patient EAS) which abolishes a restriction site for the restriction enzyme *Tsp* 509 I which may be useful in tracking this mutation in affected family members. Analysis of 100 samples (patients with bilateral Rb and other tumours) for this restriction site revealed that none of them has same change.

The mutation found from the patient with bladder carcinoma was a G to C transversion at position +1 of the donor site of intron 12, probably impairing normal splicing of the RNA. The second mutation was assumed to lie in a part of the retinoblastoma gene that was not analysed, since in somatic cases two hits in the RB gene are expected. Intact RNA could not always be recovered from the clinical material used in this study, therefore the diagnostic strategy for bladder carcinoma was chosen not to be based on the analysis of RNA transcript. The change creates a recognition site for the restriction enzymes *Mae*II, *Bpu*101, *Dde*I.

The seven mutations detected in this study were all novel and emphasise the heterogeneity of the molecular pathology in this gene. The screening approach each contributed to the identification of the region of interest, but there was no overlap between the two. This suggests that a single approach would fail to detect all mutations and that a combination of the two would be more effective. The failure to detect molecular pathology in 14 of 20 patients with bilateral retinoblastoma was also surprising and reflects the growing experience from other centres. This might be intrinsic to this gene given the success of screening strategies in other disorders, or it may be that mutations are present in areas of the gene not studied for instance promoter sequence.

CHAPTER TWO: INTRODUCTION.

2. INTRODUCTION

2.1 Molecular pathology of single gene disorders.

The inheritance of single gene defects can be divided into autosomal dominant, autosomal recessive, sex linked and mitochondrial which are inherited as Mendelian traits (McKusick, 1990). Mutations in DNA sequence may occur by a variety of mechanism. The category of mutations that includes deletions and insertions accounts for 5% to 10% of all known mutations (Cummings, 1994) but for most genes, point mutations appear to be of primary importance.

Point mutations may affect the transcription of the gene into mRNA or the processing of mRNA during the production of its mature form or the translation of mRNA into protein. On the basis of the amino acid or codon changes, point mutations have been classified into missense, nonsense and sense. Missense mutation is a single nucleotide change that causes the substitution of one amino acid for another in a protein. This substitution may or may not affect the function of the gene product. For example a single nucleotide substitution in codon 6 from GAG (glu)→GUG (val) of beta globin results in sickle cell anaemia, an autosomal recessive disorder leading to abnormal red cells and a potentially lethal phenotype (Orkin and Kazazian, 1984). Another nucleotide substitution in the same codon from GAG (glu)→AAG (lys) results in a condition known as HbC (Haemoglobin C), associated with mild clinical symptoms. In a third beta globin variant called HB Makassar, the codon at the sixth position is changed from GAG (glu)→GCG (ala), causes no symptoms and is regarded as a harmless haemoglobin variant. Nonsense mutations cause a change from one of the 61 codons that specify an amino acid to one of the three termination codons (UGA, UAA, UAG). Since there are only three stop codons, therefore most of the point mutations are likely to be missense rather than the nonsense. Nonsense mutation usually lead to premature termination of translation and the formation of shortened polypeptide

chains. In thalassemia, a change at codon 39 from CAA (gln)→UAA (termination) produces a nonfunctional shortened polypeptide only 38 amino acids long. In individuals homozygous for this mutation, no beta globin is produced, resulting in a condition known as beta-zero-thalassemia (Cummings, 1994). In the beta globin variant McKees Rock, the last two amino acids are missing and the protein is only 143 amino acids long. The change in codon 144 UAU (tyr)→UAA (termination) results in a beta chain that is shorter by two amino acids. This change has little effect on the function of the beta globin molecule as a carrier of oxygen (Cummings, 1994). Nonsense mutations may also arise from insertion or deletion of one or more bases leading to a premature introduction of a stop. Sense mutations produce longer than normal proteins by changing a termination codon into one that codes for amino acids. In Hb Constant Spring-1, the alpha chain termination codon UAA at position 142 is changed to CAA (gln), and as a result, 31 additional amino acids are added to the alpha chain before another termination codon is encountered in the mRNA (Weatherall, 1991).

Nucleotide substitutions have so far been the most common type of mutation in coding DNA sequence. Transitions, substitution of a pyrimidine by a pyrimidine, or a purine by a purine, are more frequent than transversions (substitution of a pyrimidine by a purine or vice versa). The excess of transitions is partly due to evolutionary instability of methylated cytosine residues. Cytosines which occur in the dinucleotide CpG are often methylated in human and vertebrate DNA to give 5-methylcytosine. Spontaneous deamination of 5-methylcytosine occurs over an evolutionary time-scale to generate thymine (Bird, 1986). Because the latter is a natural base in DNA, however it may not be recognised by DNA repair systems as being the product of an aberrant process (Strachan, 1992). The CpG sequence is effectively replaced in this process by TpG and CpA on the complementary DNA strand following DNA replication. Since the observed frequency of CpG in human genomic DNA is about 20% of the expected

frequency, the CpG dinucleotide appears to be a mutational hot spot and contributes significantly to the molecular pathology of many disorders. For example, in a group of nearly 400 patients with haemophilia B showed that 50% of the mutations found in multiple unrelated patients were due to mutation of a CpG dinucleotide in over 70% of cases (Cooper and Krawsak, 1990).

Sequence variation of human gene products reflects alterations of sequences in the nuclear DNA and at the level of gene expression, notably RNA processing. At the level of the genome differences between allelic sequences at a single chromosomal locus and also differences between related non-allelic sequences at different loci can contribute to sequence diversity. In the former case allelic sequence variation is traditionally described as polymorphism if more than one variant (allele) at a locus occurs in a human population with a frequency greater than 0.01. Although each individual possesses a maximum of only two different alleles at any one locus, a population survey of many individuals may reveal several different alleles at that locus, especially in the case of highly polymorphic loci. The small minority of DNA polymorphisms which lead to amino acid differences contribute to polymorphism at the protein level, as do differential transcription and RNA processing events (Strachan, 1992). For example the human dystrophin gene has two different promoters which are activated in different tissues; a brain specific promoter activates transcription at a location which is more than 90 kb upstream of the muscle-specific promoter (Strachan, 1992).

Translation begins at the 5' end of the mRNA with AUG (met). There is another sequence just upstream of the AUG codon that controls the rate at which translation occurs. In alpha thalassemia, a nucleotide substitution in this region reduces the rate of translation of mRNA (Weatherall, 1991). Genes which are very actively transcribed, either at a specific stage in the cell cycle (e.g. histones) or in specific cell types (e.g. β-

globin), always have a TATA box in their promoter. This element, often TATAAA or a variant, normally occurs at a position about 30 bp upstream from the transcriptional start site. However, TATA boxes are absent from the promoters of many other genes, including housekeeping genes. Instead, the latter often have GC-rich sequence elements, especially variants of the consensus sequence GGGCGG. Other common promoter elements include the CAAT box, usually at about -80, which is often the strongest determinant of promoter efficiency. Several forms of β thalassaemia have been described in which point mutations were found upstream from the β globin gene either within or adjacent to the promoter boxes (Orkin et al., 1983; Weatherall, 1991). These mutations are associated with a variable reduction in output from the adjacent β globin chain loci. The activity of many promoters is modulated by an enhancer, which is a regulatory sequence that stimulates transcription. This is located on the same DNA molecule and can be situated, either upstream or downstream from the promoter region (Connor and Ferguson-Smith, 1991).

Correct processing of pre-mRNA molecules in the nucleus requires precise alignment of the coding regions. Nucleotide substitutions at intron-exon boundaries can interfere with normal splicing, resulting in an mRNA that retains an intron or is missing an exon. Single base substitutions within introns may result in preferential alternative splicing of the mRNA molecules at the site of the mutation. A common form of β thalassaemia that occurs in the Mediterranean population results from a single nucleotide substitution, G→A, at position 110 of the first intervening sequence (IVS1) of the β globin gene (Weatherall, 1991). This change produces an AG sequence that happens to be preceded by a stretch of pyrimidines and thus forms a functional 3' acceptor consensus sequence. About 80% of the processed mRNA is the result of splicing into this site rather than the normal 3'IVS1 AG. The mRNA produced as the result of the abnormal splicing contains intron sequences and is therefore useless as a template for globin chain synthesis. Because this site is used

preferentially, more abnormal than normal mRNA is produced and therefore there is a severe deficiency of β chain production (Weatherall, 1991).

2.2 Retinoblastoma gene structure and function.

The human retinoblastoma susceptibility gene is located within human chromosome 13, band q14 (Sparkes et al 1980; 1983). Using DNA probe H3-8 which is within 25 kb of a cloned genomic sequence, Dryja et al.(1986) detected deletions involving this locus in three of 37 retinoblastomas. One probe from this region was found to contain a transcribed sequence and was used to successfully isolate a 4.7-kb human cDNA segment corresponding to the mRNA of the retinoblastoma gene (Friend et al., 1986). Other research groups also cloned similar cDNA fragments, using the H3-8 clone as a starting point (Fung et al., 1987; Lee et al., 1987a). The RB transcript is encoded in 27 exons dispersed over approximately 200 kilobases of genomic DNA. The length of individual exons ranges from 31 to 1889 base pairs. The largest intron spans more than 60 kb and the smallest has only 80 bp. The cDNA sequence contains 4724 nucleotides with an open reading frame (ORF) that codes for a protein consisting of 928 amino acids (Friend et al., 1987; Lee et al., 1987ab).

The 5' end of the gene, which is directed toward the centromere, lacks a typical TATA box, but contains an unmethylated CpG-rich DNA sequence (CpG-island), which is characteristic of "house-keeping genes" (Friend et al., 1987). The RB gene codes for a nuclear phosphoprotein of 110 kd ($p110^{RB}$). which is present throughout the cell cycle (Chen et al., 1989; Buchovich et al., 1989; DeCaprio et al., 1989)). In G1, this protein lacks phosphate groups and this appears to block progression to S phase by binding transcription factors. When $p110^{RB}$ is phosphorylated by cell cycle kinase, it releases transcription factors, allowing them to activate the gene transcription necessary for progression through the cell cycle. In the absence of intact

$p110^{RB}$ cells are not blocked in G1 and unscheduled cell proliferation occurs. At this stage, the cells are not malignant.

Three biochemical activities of the RB protein have been discovered (Goodrich et al., 1991). First, the carboxy-terminal half of $p110^{RB}$ is capable of binding to DNA, though no sequence specificity for this binding has been demonstrated (Lee et al., 1987b; Wang et al., 1990). Secondly, the transforming proteins of several DNA tumour viruses, including SV40 T antigen adenovirus E1A, can bind $p110^{RB}$ (DeCaprio et al., 1988; Dyson et al., 1989; Whyte et al., 1988). Two regions, also within the carboxy-terminal half of the protein, are required for T antigen and E1A binding (Hu et al., 1990; Huang et al., 1990). Mutational analysis of the transforming proteins has demonstrated a correlation between their ability to transform cell binding $p110^{RB}$ (Cherington et al., 1988; DeCaprio et al., 1988; Lillie et al., 1987; Moran, 1988; Moran et al., 1986; Smith and Ziff, 1988; Whyte et al., 1989), although some transformation-related properties are not dependent on binding $p110^{RB}$ (Thompson et al., 1990). Mutant RB proteins found in naturally occurring tumours fail to bind T antigen, and the mutations map to the regions required for binding in vitro (Hu et al., 1990; Huang et al., 1990). Thirdly, the transcription factor as well as several unidentified cellular proteins bind RB protein, apparently within the same binding domain used by T antigen (Bagchi et al., 1991; Bandara and La Thangue, 1991; Bandara et al., 1991; Chellappan et al., 1991; Chittenden et al., 1991; Defeo-Jones et al., 1991; Huang et al., 1991; Kaelin et al., 1991). These observations suggest that the antigen-binding domain is critical to function of the protein. Although provocative, these biochemical properties have not yet provided a satisfactory explanation for the physiological effects of loss of RB function. The genetic events leading to malignant development appear to occur much latter. Certain viral oncoproteins such as adenovirus E1A and SV40 large T antigen can bind to unphophorylated $p110^{RB}$ and displace transcription factors, allowing these factors to

active gene transcription. This mechanism appears to be an important step in the transformation of cells by DNA tumour viruses. Qian et al. (1992) identified two regions of pRb that are required for E2F binding and for hyperphosphorylation. E1A binding domains partially overlap but are distinct from both of these other two regions. Biological function of pRb is dependent on retention of the integrity of both of these biochemically defined domains. They speculated that these data support the model that pRb is a transducer of afferent signals (via the kinase that phosphorylates it) and efferent signals (through transcription factor binding), using distinct structural elements.

The genetic predisposition to retinoblastoma is transmitted by mutant alleles of the retinoblastoma susceptibility gene (Cavenee et al., 1983; Knudson, 1973). Inactivation of RB is both necessary and sufficient to form clonal proliferations of retinal cells (Friend, 1989). Few, if any, other cancers have such an apparently simple aetiology. Much more frequently tumour development is dependent on a number of genetic alterations in both oncogenes and tumour-suppressor genes. Of the genes involved in the development of cancer, many oncogenes have been identified but few tumour-suppressor genes, although may have been postulated (Stanbridge, 1990). RB (like p53) has been implicated in a tumour-suppressing role in a wide range of tumours including retinoblastoma, osteosarcoma (Friend et al., 1986; Fung et al., 1987; Reissmann et al., 1989; Toguchida et al., 1988; Yokota et al., 1988; Horowitz et al., 1989; Kaye et al., 1990; Mori et al., 1990), breast cancer (Lee et al., 1988; T'Ang et al., 1988; Varley et al., 1989), bladder carcinoma (Dunn et al., 1989; Horowitz et al., 1989) and leukaemia (Liu et al., 1992). Patients successfully treated for retinoblastoma have a higher incidence of certain second-site primary tumours, such as osteosarcoma and soft-tissue sarcomas (Abramson et al., 1984). Mutations of RB that ablate normal gene expression have been found in these tumours as well as in carcinomas of the breast, prostate, bladder, and small cell lung, as well as leukaemia

(Bookstein et al., 1990; Liu et al., 1992; Friend et al., 1987; Harbour et al., 1988; Hensel et al., 1990; Horowitz et al., 1990; Lee et al., 1988; Shew et al., 1989,1990; Toguchida et al., 1988: Ahuja et al., 1991). Expression of normal RB protein in prostate carcinoma, osteosarcoma, breast carcinoma, and bladder carcinoma cells also suppresses their tumourigenicity in nude mice (Bookstein et al., 1990; Huang et al., 1988). The risk of osteogenic sarcoma is increased 500-fold in patients with bilateral retinoblastoma, the bone malignancy being at sites removed from those exposed to radiation treatment for the eye tumour (Abramson et al., 1976). Francosis (1977) concluded that there is a special predisposition to osteogenic sarcoma, both radiogenic and nonradiogenic, in retinoblastoma patients and possibly in their relatives Expression of normal RB protein (Horowitz et al., 1990; Lee et al., 1987b) or mRNA (Dunn et al., 1988; Friend et al., 1987; Fung et al., 1987; Lee et al., 1987a) is lacking in all retinoblastomas examined to date. Replacement of the normal RB gene in cultured retinoblastoma cells by retrovirus-mediated gene transfer consistently suppresses their tumourigenic potential in nude mice (Huang et al., 1988; Sumegi et al., 1990).

2.3 Molecular pathology of the retinoblastoma gene.

The tumour exists in both hereditary and sporadic forms (Vogel, 1979). In the hereditary form one allele is inherited in a mutated form and the other is altered somatically, whereas in the sporadic form changes to both alleles occur somatically (Murphree and Benedict, 1984) (Figure 1). Hereditary predisposition to retinoblastoma is caused by a germline mutation at the retinoblastoma gene locus (RB) (RB1) and is transmitted as an autosomal dominant trait with 90% penetrance (Vogel et al., 1979). Approximately 40% of patients carry a germline mutation at this locus in all cells (Vogel 1979) (Table 1). Three quarters of these alterations represent de novo mutations. Most patients with hereditary retinoblastoma have bilateral

disease. The average number of tumour foci in these patients is three to five. 10% of carriers develop no tumour (reduced penetrance)(Horsthemke, 1992). In 60% of all retinoblastoma patients, both alleles are inactivated by somatic mutations in a single cell (Vogel, 1979) (Table 1). These patients develop only a single tumour focus, and there is no risk to their offspring. However, 15% of patients with single tumours will have a germline mutation. Risk estimates for relatives of isolated cases of retinoblastoma are given in Table 2.

In 1971, from studies of age/incidence curves, Knudson (1971) postulated that the disease arose from two sequential events. In the hereditary form of the disease, one mutation is inherited in the germ-line and is phenotypically harmless. A second 'hit' occurring in the retinal cell causes the tumour. As there are a large number of retinoblasts in the eye (over 10^7), which are all at risk because they already carry one mutation, a second 'hit' will occur frequently enough to cause a high proportion of tumours in at least one eye and often in both. In the sporadic form of the disease, both mutations occurring in the somatic tissue. The probability of two mutations occurring in the same cell is low, therefore the disease is both rare and unilateral (Macdonald and Ford, 1991). This 'two-hit' hypothesis has subsequently been confirmed by identification of mutations or deletions of the gene and more recently by analysis of the cloned retinoblastoma gene itself. Benedich et al. (1982) and Cavenee et al. (1983) obtained evidence that the two mutations necessary for tumour formation-one germline mutation and one somatic mutation in hereditary retinoblastoma or two somatic mutations in non-hereditary retinoblastoma-affect the two alleles of the same gene. Thus, homozygous loss of function of the RB gene initiates retinoblastoma tumour formation and the RB gene is termed a tumour suppressor gene.

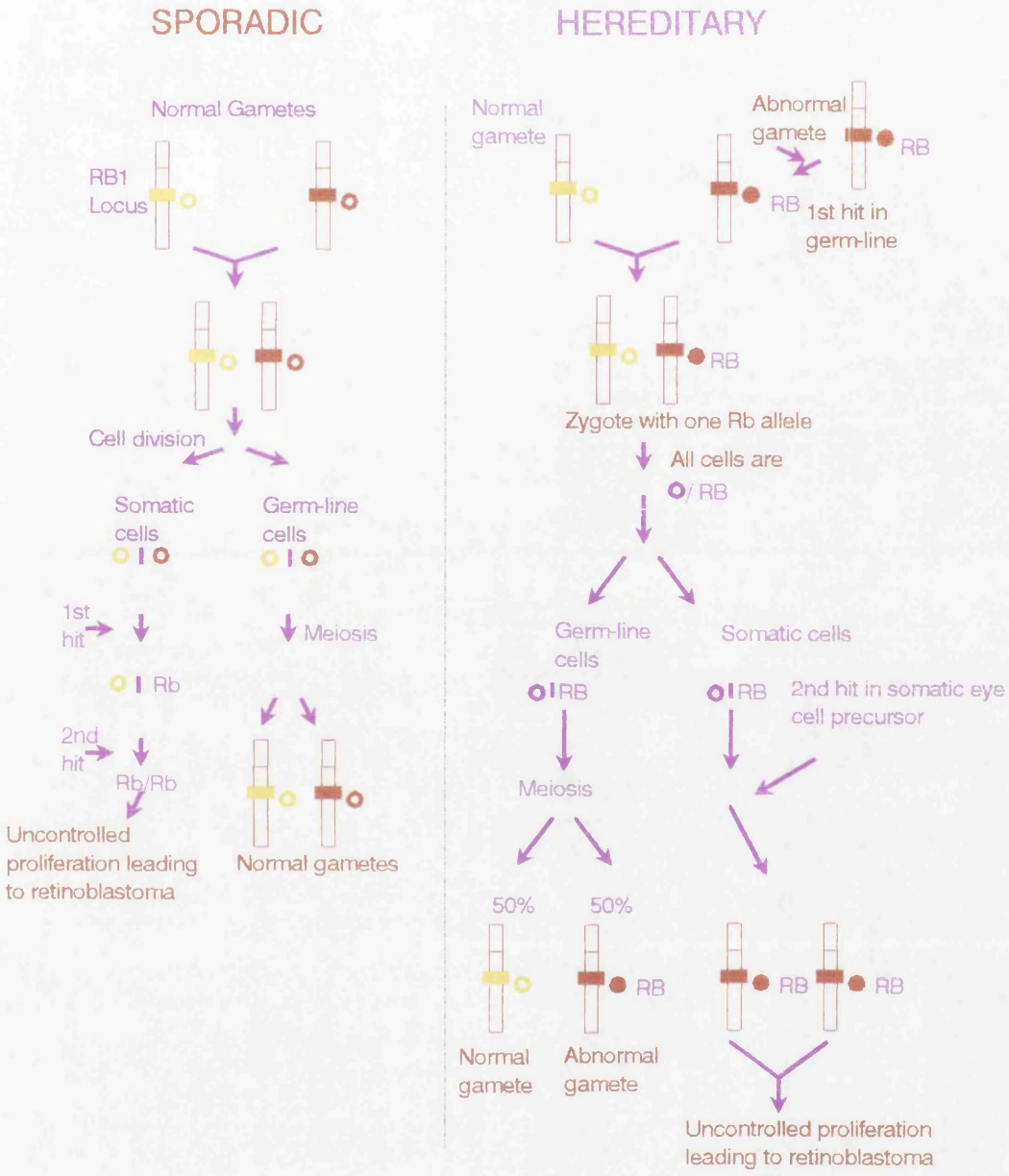


Figure 1. Origin of hereditary and sporadic retinoblastoma.

Genetic form	Type of mutation	Frequency (%)	Type of disease
Hereditary retinoblastoma	transmitted germline mutation	10	Bilateral
	De novo germline mutation	30	Bilateral
Nonhereditary retinoblastoma	somatic mutations only	60	Unilateral

Table 1: Genetic forms of retinoblastoma.

RB in proband	Relation to proband	Risk of carrying RB mutation
Bilateral	Offspring	50%
Bilateral	Sibling or dizygotic twin	5%
Bilateral	Offspring of unaffected sibling	0.5%
Bilateral	First cousin	0.05%
Bilateral	Monozygotic twin	100%
Unilateral	Offspring	7.5%
Unilateral	Sibling or dizygotic twin	0.8%
Unilateral	Offspring of unaffected sibling	0.08%
Unilateral	First cousin	0.008%
Unilateral	Monozygotic twin	10%

Table 2. Risk of retinoblastoma in relatives of a child with retinoblastoma and no family history (Hodgson and Maher, 1993).

In families with hereditary retinoblastoma, close linkage was demonstrated between esterase D alleles and retinoblastoma, suggesting that the two genes had to be close. In non-hereditary cases, approximately 20% of the retinoblastomas were shown to have an abnormality; usually absence or a deletion in one copy of chromosome 13, reduced levels of esterase D were detected in the tumours (Benedict et al., 1982). Patients with two detectable variants of esterase D in somatic tissues had only one variant present in their tumours. These studies suggested that in tumours there was loss or deletion of part of chromosome 13 and it was assumed that there was a mutation in the RB gene on the remaining copy of chromosome 13. These changes were confirmed at the molecular level by Cavanee and colleagues (Cavanee et al., 1983) using the loss of heterozygosity test which has been widely used for detection of tumour suppresser genes. The loss of heterozygosity test depends on differences in the lengths of DNA fragments generated by digestion of genomic DNA with restriction enzymes. These restriction fragment length polymorphisms (RFLPs) present within the population can be detected by DNA probes specific for the DNA fragment of interest. In the case of retinoblastoma. the probes selected for use were located on 13q. Patients suitable for study were those who had different sized fragments of the DNA (alleles) on each of the two chromosome 13s in their somatic tissue, that is, they were heterozygous. When tumours from the same patients were analysed, only one of the two alleles was present. This loss of heterozygosity can occur by a number of possible mechanisms, including loss of the normal chromosome possibly followed by reduplication of the abnormal one, an interstitial deletion of the normal chromosome, or a recombination event resulting in two copies of the deficient allele. The tumour only occurs when both copies of the RB gene are altered or lost, therefore the tumour phenotype is recessive. However, the tumour is inherited as if it were dominantly expressed, because, the likelihood of the second mutation in the retinal cells is close to 100%.

Since retinoblastoma results from the homozygous or hemizygous state of a gene at 13q14, retinoblastoma could either be as a recessive disease or the susceptibility could be is dominant. It is of interest that 90% of de novo mutations occur in the paternal germline (Dryja et al., 1989; Zhu et al., 1989); this may be because far more cell divisions occur between embryonic development and meiosis in males than in females. Ejima et al. (1988) showed that cytogenetically visible deletions resulting in a retinoblastoma are usually in the paternally derived chromosome. Such a bias would not be expected for sporadic retinoblastoma where both mutations occur in somatic tissue, but there had been some indication of a bias toward initial somatic mutation in the paternally derived gene on chromosome 11 in sporadic Wilms tumour. There was a growing body of data indicating a difference in behaviour of maternally and paternally derived autosomal genes. Germinal imprinting may be mediated by some epigenetic process such as de novo DNA methylation and carried over to postzygotic stages. Dryja et al. (1989) and Zhu et al. (1989) found that in bilateral retinoblastoma there is a preferential retention of the paternal chromosome in the process of loss of heterozygosity (LOH). They indicated that either mutation of RB is more common during spermatogenesis than oogenesis as a result of differences between male and female meiosis, DNA methylation or environmental exposure; or the paternal chromosome in the early embryo is more at risk for mutation, or deficient in DNA repair.

A few patients are mosaic for an RB gene mutation (Ribeiro et al., 1988; Greger et al., 1990). In these patients, the mutation occurred during early embryonic development. If the mutation arises before the germline is partitioned off from other cell lines, it may be transmitted to the offspring. One such case was analysed by Greger et al. (1990). A single mutation event during early embryonic development gave rise to two cell lines with related but different deletions. A different deletion was transmitted to each of the two children.

Deletions and point mutations have also been observed as the result of somatic mutations. Local mutations at the RB locus occur in approximately 30% of patients. Most frequently, the second RB allele is lost by chromosomal mechanisms (Cavenee et al., 1983). These mechanisms can be detected by comparing the patient's constitutional genotype with the tumour genotype. Such comparisons are performed with the help of polymorphic DNA markers along chromosome 13. In approximately 50% of patients, the second allele is lost by mitotic nondisjunction with or without duplication of chromosome 13 (Horsthemke. 1992). In these cases, tumour cells are either hemizygous or homozygous for alleles of the chromosome 13 that carries the first RB gene mutation. In approximately 20% of patients the second allele is lost by mitotic crossover. In these patients, tumour cells are heterozygous at loci proximal to the RB gene and homozygous at the RB locus and loci distal to it.

Early data, derived mostly from Southern blot analysis identified structural abnormalities of RB in 10%-40% of hereditary and sporadic Rb tumours (Cowell and Hogg, 1992). The vast majority of these abnormalities included deletions of all or part of the gene (Friend et al., 1986; Fung et al., 1987). It appears that deletion breakpoints can occur throughout the length of the gene (Canning and Dryja, 1989; Kloss et al., 1991). Canning and Dryja, (1989) found that 2 out of 12 deletions had breakpoints in the region containing exons 13-17, and 7 out of 12 deletions included that region. Others groups also found this region was involved in deletions and rearrangements (Fung et al., 1987) possibly indicating the location of a breakpoint cluster region. It is not known whether this is related to the chromosome abnormalities, but these DNA sequence can form stem and loop structures that possibly promote rearrangements (Cowell and Hogg, 1992). Canning and Dryja, (1989) reported that there was a direct repeat at the breakpoint site and one of these was always lost in 6 of 8 deletions. Cowell and Hogg (1992) also found short direct repeat associated with small deletions within RB. This observation is consistent with

a slipped mispairing during DNA replication. In this model, pairing on one DNA strand occurs with the downstream sequence on the other strand creating a loop. When the replication loop is resolved, one copy of the repeat, plus the intervening sequence, is deleted.

Sakai et al. (1991a) investigated the methylation pattern at the 5' end of the retinoblastoma gene, including its promoter region and exon 1, in DNA purified from 56 primary retinoblastomas. They found five tumors with evidence for hypermethylation, all from unilateral, simplex patients. No methylation abnormalities were detected in DNA purified from the leukocytes from these patients. They speculated that erroneous hypermethylation without alteration of nucleotide sequence occasionally plays a role in the genesis of this cancer. Cytosine methylation of CpG sites in the promoter region of eucaryotic genes is involved in the inactivation of expression of certain genes. Given that methylation can lead to reduced transcription, it is possible that expression of tumor-suppressor genes is also inactivated by hypermethylation, thereby contributing to the etiology of cancer. Recently OhtaniFujita et al. (1993) found five sporadic retinoblastoma tumors (16% of all unilateral cases) with hypermethylation of the 5' end of the retinoblastoma gene without detecting any structural abnormalities. However, it is unclear whether the promoter of the retinoblastoma gene is actually inactivated by its hypermethylation. They showed that specific hypermethylation in the promoter region of the retinoblastoma gene reduces its expression to only 8% of the unmethylated control. Furthermore, they have found that two transcription factors important for the promoter activity, an activating transcription factor (ATF)-like factor and the retinoblastoma binding factor 1, do not bind when their recognition sequences are GpG methylated.

Some patients have a somatic 13q- mosaicism (Ribeiro et al., 1988). Insertion and translocations involving band 13q14 have also been observed (Turleau et al., 1985), but are much less frequent than deletions. Most patients with a deletion of the entire band of 13ql4 are mental and physically retarded: cytogenetic analysis is indicated only in patients with such stigmata. To date, the gene for esterase D is the only gene known to be close to the RB gene, it maps at least 200 (kb) proximal to the RB gene (Sparkes et al., 1980). Deletion of the esterase D gene, however, has no clinical phenotype. Deletions of 13ql4 are rare in retinoblastoma tumour cells. In some tumours monosomy 13 is observed. In contrast, tetrasomy 6p and trisomy 1q are frequent findings (Kusnetsova et al., 1982; Benedict et al., 1983; Squire et al., 1984). In tetrasomy 6p, two additional chromosomes are often present in the form of an isochromosome of 6p, which is rare in other tumours (Hoersthemke, 1992). DNA polymorphism studies have indicated that mitotic nondisjunction leading to trisomy 6 precedes isochromosome formation. The isochromosome is then formed by transverse division of centromere or intrachromosomal chromatid exchange (Horsthemke et al., 1989). The functional role of tetrasomy 6p and trisomy 1q is unknown.

Anecdotal reports suggested that bladder carcinomas appear more frequently than expected in retinoblastoma families (Tarkanen and Karjalainen, 1984) and subsequent, larger studies have confirmed this observation (DerKinderen et al., 1988; Sanders et al., 1989). Where age at diagnosis was reported, this was markedly lower than the mean age of onset in the general population. Higher incidence and lower age of onset of a cancer in a particular subgroup points to a genetic predisposition and suggests that germline mutations in the RB gene may predispose to bladder cancer (Cairns et al., 1991). Horowitz et al. (1989) reported another mechanism of RB gene inactivation, namely, abnormal splicing due to a point mutation in the consensus sequence of the exon-intron junction in a bladder cancer cell line; the short RB transcript in this cell line does not contain the sequence of exon 21. A similar example

was also reported by another group (Dunn et al., 1989). Murakami et al. (1991) found a novel RB gene transcript that does not contain the sequence corresponding to exon 2 of the gene in human lung carcinoma cell line suggesting the involvement of alternative splicing.

Evidence that RB may also be involved in the development of sporadic bladder cancer has come from the finding that a number of bladder carcinoma cell lines possess an altered RB protein or show no detectable RB expression (Horowitz et al., 1989; 1990). Cairns et al. (1991) have examined 162 bladder tumours for evidence of structural alterations to the RB gene. Ninety-four patients were informative with one or more intragenic RB probes, 28 of these (29%) showed loss of heterozygosity (LOH). Of these, two tumours showed homozygous deletions with the 5' intragenic probe p123M1.8. The 28 tumours with Rb LOH were screened with the RB cDNA probes pR3.8 and pR0.9 which revealed two homozygous deletion and one rearrangement. This is similar to the frequency at which abnormalities of the RB protein product have been detected in bladder carcinoma cell lines (33%) by Horowitz et al. (1990). Southern analysis of LOH is a relatively crude method of detecting gene inactivation and will not identify small internal deletions or point mutations. Indeed only 10-20% of retinoblastomas have obvious structural alterations of an RB allele which are detectable by Southern analysis (Yandell and Dryja, 1989b) and only 70% show loss of the other allele (Zhu et al., 1989), yet loss of function of RB is thought to occur in 100% of retinoblastomas.

More recently, loss of function of the RB gene has been associated with at least two different types of primary cancer. Structural abnormalities were detected in 13% of primary small cell lung carcinoma (SCLC), and in 18% of SCLC cell lines (Harbour et al., 1988). More significantly, loss of RB mRNA was seen in 60% of the SCLC lines. SCLC, like retinoblastoma, is a tumour of neuroendocrine origin, and Harbour

et al. (1988) suggested that the involvement of the RB gene in two tumour types displaying phenotypic properties of neuroendocrine differentiation may be of significance. There seems to be no evidence for familial clustering of SCLC, nor for any association of SCLC with retinoblastoma or any of the mesenchymal tumours which also exhibit deletion of RB. Frequent aberration to the RB gene have also been detected in primary carcinoma of the breast (Lee et al., 1988; T'Ang et al., 1988). Breast carcinomas are not, however, of neuroendocrine origin, but they can show a strong familial trend, with some families showing a very strong genetic component (Sattin et al., 1985; Ottman et al., 1986; Newman et al., 1988). Whilst there is evidence that mothers of children with osteosarcoma and soft tissue sarcoma show a higher rate of breast cancer, there is no evidence of any association between breast cancer and retinoblastoma. Varley et al. (1989) have analysed the organisation of the retinoblastoma gene in 77 primary breast carcinomas using cDNA probes 3.8 and 0.9. They found deletions or rearrangements of the RB locus in 15 cases out of 77 carcinoma samples indicating that the loss of RB function is important in the progression of human breast carcinoma. In 1988 T'Ang et al. reported that 25% of breast cancer cell lines and 7% of primary tumour samples showed a deletion or rearrangement of the RB gene. Varley et al. (1989) have shown that changes to the RB gene leading to loss of expression of both alleles occurred in 19% of primary breast tumours. Hovig et al. (1992) have examined RB exon 21 from 78 tumours included 11 breast carcinomas, 30 non small cell lung carcinomas, 6 colon carcinomas, and 31 sarcomas by constant denaturant gel electrophoresis (CDGE). They did not detect any mutations in the examined region in any of the tumours.

DeCaprio et al. (1989), Buchkovich et al. (1989), and Chen et al. (1989) demonstrated that the RB gene product has the properties of a cell cycle regulatory element and that its function is modulated by a phosphorylation/dephosphorylation mechanism during cell proliferation and differentiation. Goodrich et al. (1991) found

that injection into cells of either full-length or a truncated form of the RB protein containing the T antigen-binding region inhibited progression from G1 into S phase. Coinjection of anti-RB antibodies antagonised this affect. The results indicated that RB regulates cell proliferation by restricting cell cycle progression at a specific point in G1 and established a biologic assay for RB activity. Coinjection of RB with a T antigen peptide or injection into cells expressing T antigen was accompanied with no inhibition of progression into S phase. This was interpreted as indicating that the transforming proteins of some DNA tumour viruses, including SV40 T antigen and adenovirus E1A, may promote cell growth, at least in part, by binding and inactivating RB. The RB gene has been shown to be inactivated in various way, such as by loss of part or all of the gene (Lee et al., 1987a; Fung et al., 1987; Friend et al., 1987; Lee et al., 1988), disorders gene expression (Lee et al., 1987a; Fung et al., 1987; Harbour et al., 1988) or dysfunction of the RB protein (Horowitz et al., 1989; Shew et al., 1990). Amino acid substitutions or creation of stop codons due to point mutations or shifts of the reading frame (Dunn et al., 1989) results in activation of the RB protein. Hu et al. (1990) generated a series of deletion mutants of the RB cDNA sequence that have been tested for their ability to interact with two viral oncoproteins, the adonevirus E1A and SV40 large T antigen. Two separate regions of RB are essential for association with E1A or large T antigen. The two regions include a fragment of 180 amino acid residues (393-572) and one of 127 residues (646-772) which fall in exons 12-22 inclusive (Hu et al., 1990; Huang et al., 1990). A comparison of these binding sites on RB with the sites of naturally occurring mutations reveals that all of the RB mutations identified to date contain deletions either in one or both of the binding regions (Hu et al., 1990). This suggest that the DNA tumour virus transforming proteins are predicted to bind to and inactive normal RB function, thus removing a barrier to cell proliferation.

2.4 Available methods used in the detection of point mutations.

2.4.1 Polymerase chain reaction (PCR).

The polymerase chain reaction (PCR) is a technique for the *in vitro* amplification of specific DNA or RNA sequences. The PCR method was devised and named by Mullis and colleagues at the Cetus Corporation (Mullis et al., 1987), although the principle had been described in detail by Khorana and colleagues over a decade earlier (Taylor, 1993). The development of the PCR has led to a variety of new approaches for sequencing human genes.

The principle behind PCR is illustrated in Figure 2. The polymerase chain reaction (PCR) uses two oligonucleotide primers to direct the synthesis of specific sequences of DNA. One primer anneals to the coding strand of DNA and other to the noncoding strand. These primers are used to direct the enzyme DNA polymerase to copy each strand in opposite directions. The primers are added in great excess to the source DNA, in the presence of buffer, enzyme, and free nucleotides. The source DNA is denatured at 95^{o}C and then cooled to 40-65^{o}C. The requirements of the reaction are simple: deoxynucleotides to provide both the energy and nucleosides for the synthesis of DNA, DNA polymerase, primer, template, and buffer containing magnesium. Initially synthesis will go beyond the sequence complementary to the other primer, but with each cycle of heating and cooling, the amount of DNA in the region flanked by each primer will increase almost exponentially, whilst longer sequences will only accumulate in linear fashion, provided that the amount of starting DNA is present in limiting quantities. Thus, after several cycles the predominant reaction product will be that fragment of DNA which is flanked by the primers, and will include the primers themselves.

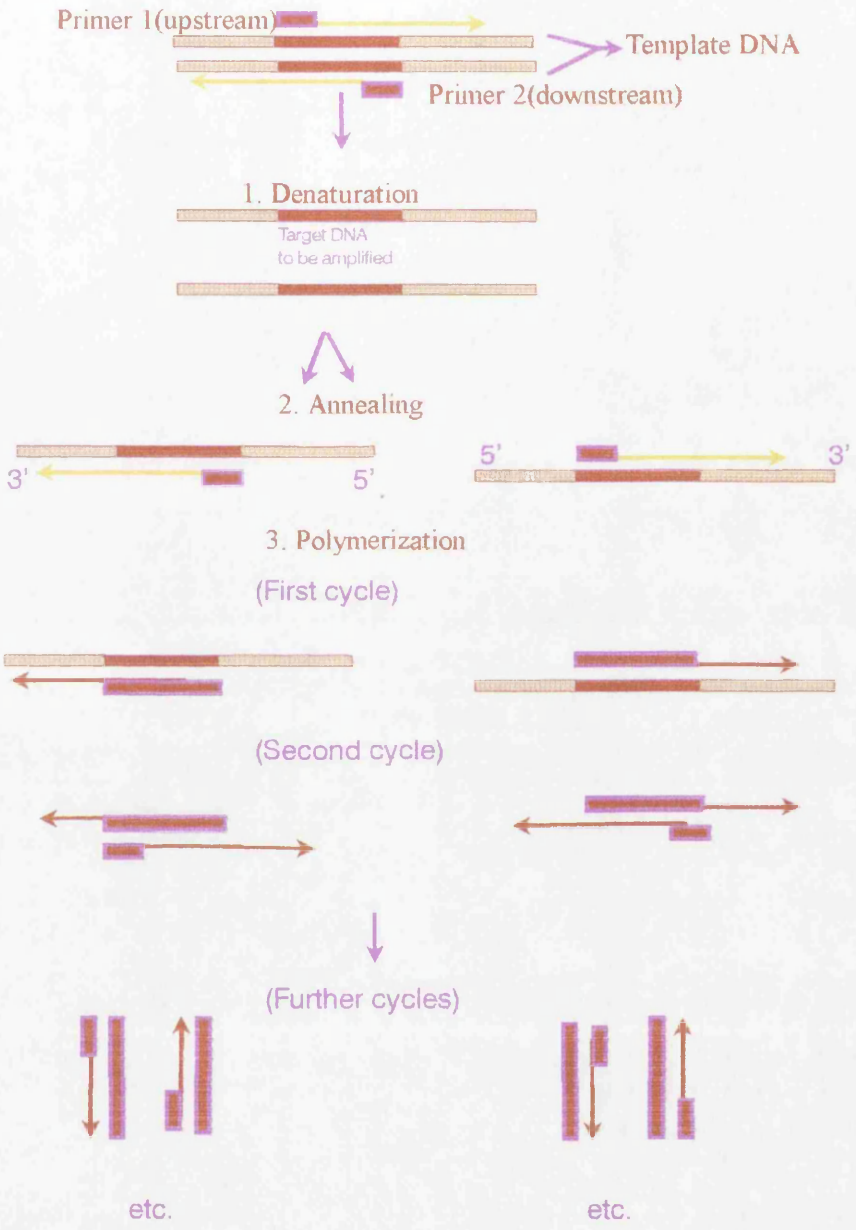


Figure 2.The principle of polymerase chain reaction. In the first cycle, a fragment larger than the targeted DNA is synthesised. However, after, two amplification cycles targeted DNA fragment is produced and eventually it becomes the predominant product.

The heating and cooling cycles can be repeated and DNA will continue to accumulate exponentially until one of the reaction products is exhausted or the enzyme is unable to synthesis new DNA quickly enough. At high DNA concentrations, the DNA may also begin to prime itself and result in the synthesis of non specific products. Thus, it either stops the amplification or produces non-specific products. The number of cycles required for optimum amplification varies depending on the amount of starting material and the efficiency of each amplification step. After about 30 cycles of successive steps of denaturation, annealing of primers and DNA synthesis, the products of the PCR will include, in addition to the starting DNA, about 10^5-10^6 copies of the specific target sequence, an amount which is easily visualised as a discrete band of a specific size on agarose gel electrophoresis. Repeated cycles of enzymatic amplification increase the quantity of the targeted DNA region more than 2000-fold. Generally, 25 to 35 cycles should be sufficient to produce 100 ng-1μg of DNA of single-copy human sequence from 50 ng of genomic DNA. A final incubation step at the extension temperature (usually 72^0C) results in fully double-stranded molecules from all nascent products. The primers should be similar in length and composition, so that their predicted melting temperatures (T_m), the temperature at which 50% of the strands are separated) are within 5^oC. For calculation of the approximate T_m of the oligonucleotide primers, a simple formula such as T_m = 2(A+T) + 4(G+C) in ^{o}C can be used (Thein and Wallace, 1986). Furthermore, GC content should be similar to the GC content of the template and of the other primer, ideally 50-60% GC. They should not have self-complementarity, secondary structures or be complementary to each other. Computer programs are available to help identify such complementarity. Primers should also have no runs of three or more Gs or Cs at their 3' ends. If mismatches between primer and template are known or likely to occur, these should be minimised at the 3' end of the primer, i.e., where the DNA polymerase binds. Highly degenerate primers may work under non-stringent reaction conditions, provided that at least three bases match at the 3' end of the primer

(McMahon et al., 1988). Restriction sites can be included in the primer to help in efficient and directional cloning of the amplified product. Lengths between 100 and 2000 bp can, however, often be amplified efficiently.

There are several PCR based methods for detection of mutations. These are restriction fragment length polymorphism analysis (Saiki et al., 1985; Kogan et al., 1987), single-stranded conformation polymorphism analysis (Orita et. al., 1989), denaturing gradient gel electrophoresis (Cariello et al., 1988; Traystman et al., 1990), electrophoresis of heteroduplex DNA (White et al., 1992), RNase cleavage analysis (Myers et al., 1988), chemical cleavage of mismatch (Cotton et al., 1988; Grompe et al., 1991), allele specific oligonucleotide hybridisation (Embury et al., 1987; Lo et al., 1988) and direct genomic sequencing (Wrishchnik et al., 1987; Wong et al., 1987; Newton et al., 1988).

2.4.2 Amplification and mismatch detection (AMD) analysis.

AMD analysis is a combination of PCR and the chemical mismatch method (Cotton et al., 1988). To screen for mutation sites within the amplified PCR products, the AMD method described by Cotton et al. (1988) and Dahl et al. (1989) is used. In this technique, mutant DNA is allowed to form a duplex with a radiolabelled probe of control DNA. Where mutations are present, the mutant strand does not exactly match the control and fails to anneal correctly. The resulting mismatch is chemically more reactive than the surrounding DNA, allowing chemical cleavage at this site and detection of different-sized radioactive fragments. Because cytosine and thymine react specifically with hydroxylamine and osmium tetroxide (OsO_4), respectively, the nature of the reactivity also gives information about the base change. Since only mismatched cytosines and thymines show marked reactivity, probes in both sense and antisense strands

must be used to ensure detection of the complimentary guanine and adenine mismatches (Howells et al., 1990) (Figure 3).

Sites modified by hydroxylamine and osmium tetroxide are more susceptible to cleavage by piperidine than unmodified base pairs. Piperidine is therefore used to cleave DNA fragments at the site of modified bases. Cleavage occurs on one strand of helix only. Products are resolved by denaturing electrophoresis to allow the identification and location of mutation sites. Chemical cleavage has been shown to be an excellent method for the detection and location of mutations, particularly because it is possible to easily scan up to 2 kb segments of DNA at a time and additionally, it can be used on either DNA or RNA templates (Cotton et al., 1988: Dahl et al., 1989). Improved resolution of larger cleavage products is possible by the use of ^{35}S-labelled dATP rather than [^{32}P] dCTP-labelled probes (Saleeba and Cotton, 1991). Mutation detection by chemical cleavage has been applied to the analysis of gene structure in human systems and in other organisms such as *Caenorhabditis elegans* (Han and Sternberg, 1990), *Escherichia coli* (Grompe et al., 1991), and dengue virus (Cotton and Wright, 1989). Example of human diseases investigated by this method include ß-thalassaemia (Dianzani et al., 1991), osteogenesis imperfecta (Bateman et al., 1989; Lamande et al., 1989), dihydropteridine reductase deficiency (Howells et al., 1990), ornithine transcarbamylase deficiency (Grompe et al., 1989), pyruvate dehydrogenase deficiency (Dahl et al., 1990), colorectal cancer (Rodrigues et al., 1990), and phenylketonuria (Forrest et al., 1991).

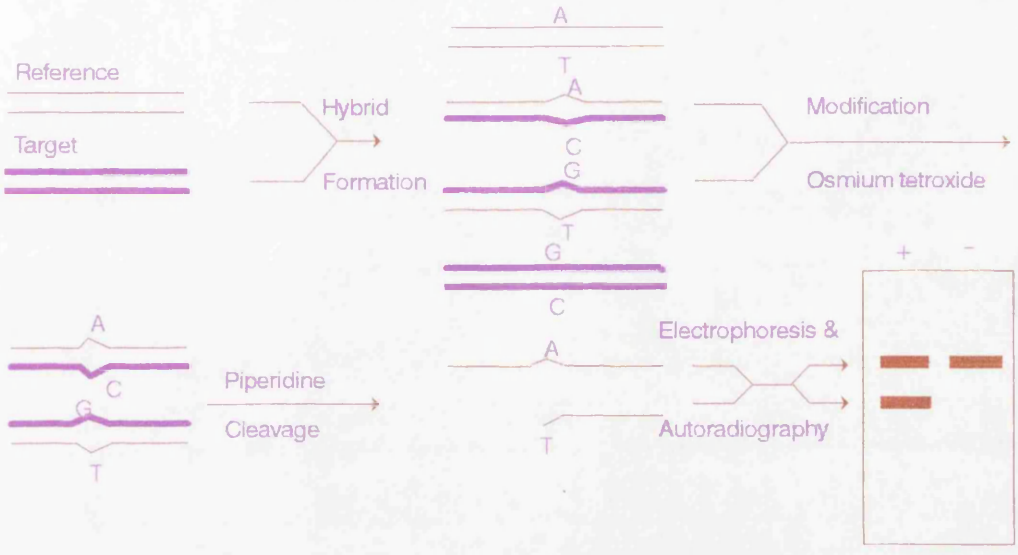


Figure 3. Analysis of sequence variation by AMD.

2.4.3 Single stranded conformation polymorphism (SSCP) analysis.

In PCR-SSCP analysis, a DNA sequence of interest (or a cDNA that has been reverse transcribed from mRNA) is first amplified by PCR. Single-stranded DNA has a tendency to fold up and form complex structure stabilised by weak intramolecular bonds, notably base-pairing hydrogen bonds. The electrophoretic mobilities of such structures on non-denaturing gels will depend not only on their chain lengths but also on their conformations, which are dictated by the DNA sequence (Orita et al., 1989). Single strand conformation polymorphism (SSCP) analysis is most conveniently performed by PCR amplification of the desired region of genomic DNA to produce a labelled product (either by using end-labelled PCR primers, or by incorporation of labelled nucleotides during the PCR reaction) (Hayashi et al., 1989; Makino et al., 1992). Amplified DNA samples are denatured and loaded on a non-denaturing polyacrylamide gel electrophoresis (PAGE) (Orita et al., 1989). A test DNA sample that differs by a single base from a standard DNA sample can be identified by a comparative mobility shift during electrophoresis. The base change results in a change of conformation.

2.4.4 Heteroduplex DNA analysis.

The PCR products of normal and mutant alleles can be separated from each other using hydrolink (non-denaturing) gel electrophoresis (Keen et al., 1991). Heteroduplex DNA is generated during PCR amplification when two homologous DNA segments, or alleles, which have sequence differences are amplified. Double stranded DNA is formed not only from identical complementary strands (homoduplexes) but also from the annealing of complementary strands from the 2 different amplified segments (heteroduplex). Heterodublex DNA can also be formed by mixing two homologous DNA fragments together, heating to 95^{o}C, and slowly

cooling to room temperature. Although the effective size range for resolution of DNA containing single base pair mismatches is up to 1300 bp (Boyd et al., 1993), size from 100-400 base pairs of DNA are optimal for effective resolution of heteroduplex. Separation of DNA fragments based on conformational differences induced by single base mismatches.

2.4.5 Denaturing gradient gel electrophoresis (DGGE).

DGGE analysis has been applied to screening of genes involved in several genetic diseases (Traystman et al., 1990; Sheffield et al., 1989). Denaturing gradient gel electrophoresis (DGGE) detects DNA sequence differences. Thus, it can be used to screen for point mutations or other types of mutations prior to DNA sequencing. The technique first described by Fischer and Lerman, (1979) entails electrophoresis of DNA fragments at high temperature in an acrylamide gel that contains a gradient of denaturant such as formamide and urea.

Denaturing gradient gel electrophoresis allows the separation of DNA molecules differing by as little as a single base change (Fischer and Lerman 1983: Myers et al., 1985ab). The separation is based on the melting properties of DNA in solution. DNA molecules melt in discrete segments, called melting domains, when the temperature or denaturant concentration is raised. Melting domains vary from about 25 base pairs to several hundred base pairs in length, each melting cooperatively at a distinct temperature called Tm. Due to the considerable contribution of stacking interactions between adjacent bases on a DNA strand to double helical stability, the Tm of a melting domain is highly dependent on its nucleotide sequence. The Tms of DNA fragments differing by even very small changes, such as a single base substitution, can differ by as much as 1.5^{o}C. In the DGGE system, DNA fragments are electrophoresed through a polyacrylamide gel that contains a linear gradient, from top

to bottom, of increasing DNA denaturant concentration. DNA fragments enter the concentration of denaturant where its lowest temperature melting domain melts (equivalent to the Tm of the domain), the molecule forms a branched structure that has a retarded mobility in the gel matrix. If the gradient conditions are chosen properly, DNA fragments differing by single base changes begin branching, and hence slowing down, at different positions in the gel, resulting in the separation of the fragments at the end of the electrophoretic run.

2.4.6 Temperature gradient gel electrophoresis (TGGE).

The TGGE is based on the same principles as the DGGE (Meyer et al., 1991). Using a thermal instead of a chemical denaturing gradient, the preparation of gradient gels is avoided. The horizontal acrylamide gels fixed on a solid gel support make the handling of the gel very convenient. In contrast to DDGE analysis, only one gel condition is sufficient for TGGE. Similar to DGGE the TGGE results are easy to interpret because of the two heteroduplex bands are reproducibly observed when tumour tissue or peripheral blood harbouring a germline mutation is analysed. In contrast, interpretation of SSCP gels is often impaired by the presence of only three or more than four single-strand bands. Like all screening techniques, the detection of mutants by TGGE does not discriminate between mutations resulting in an amino acid change and silent mutations or natural polymorphisms.

The TGGE allows the separation of molecules depending on their different melting behaviour in temperature gradients and has already been applied to separation of HLA alleles (Meyer et al., 1991). Mixed wild-type and homozygous mutated fragments are denaturated and renaturated in the presence of 4M urea to get two kinds of heterodimers in addition to the wild-type and mutant homodimers. One of those samples is separated on a 5 % polyacrylamide gel (in 4 M urea, 20 mM MOPS, and I

mM EDTA, pH 8.0 for 2 h at 250 V within a test temperature gradient (30°C--70°C) perpendicularly orientated to the electrical field in the TGGE. Due to the formation of two homodimers and two heterodimers, 4 melting profiles are observed. From these the temperature range of effective separation can be determined. This interval is defined by the highest temperature where all duplices remain double stranded and by the melting temperature of the most stable homoduplex. Then the optimal temperature gradient which overlaps effective separation is selected and orientated parallel to the electrical field in the following electrophoresis. The approximate running time of the parallel TGGE in order to get effective separation is also calculated. Aliquots of the control samples are loaded serially at different times at 20°C resulting in different 'run-in' times. Then the parallel electrophoresis proceeds within the selected ideal temperature gradient. After that the optimal running time for effective separation of all kinds of duplices is selected for subsequent parallel TGGE experiments.

2.4.7 Restriction fragment length polymorphism (RFLP).

One of the most commonly used tools for following the inheritance of genes or gene markers is that of the linked restriction fragment length polymorphism (RFLP). Traditionally, this technique involved the digestion of approximately 5μg of genomic DNA, followed by electrophoresis, Southern transfer, radioactive (or, less commonly, non-radioactive) probing, followed by the exposure of the filters to X-ray film (Maniatis, Fritsch, Sambrook, 1982). The use of PCR has made it possible to amplify a short region of DNA surrounding the restriction site of interest which is then exposed to the relevant restriction enzyme. The amplified DNA is then visualised directly on an appropriate gel matrix. This method has many advantages over the traditional Southern analysis, not least of which is the great saving in time (Williams, 1988).

In the case of retinoblastoma, the probes selected for use are located on 13q. Patients suitable for study are those who have different sized fragments of DNA (alleles) on each of the two chromosome 13s in their somatic tissue, that is, they are heterozygous. When tumours from the same patients are analysed, only one of the two alleles is present (Figure 4). This loss of heterozygosity can occur by a number of possible mechanisms, including loss of the normal chromosome possibly followed by reduplication of the abnormal one, an interstitial deletion of the normal chromosome, or a recombination event resulting in two copies of the deficient allele.

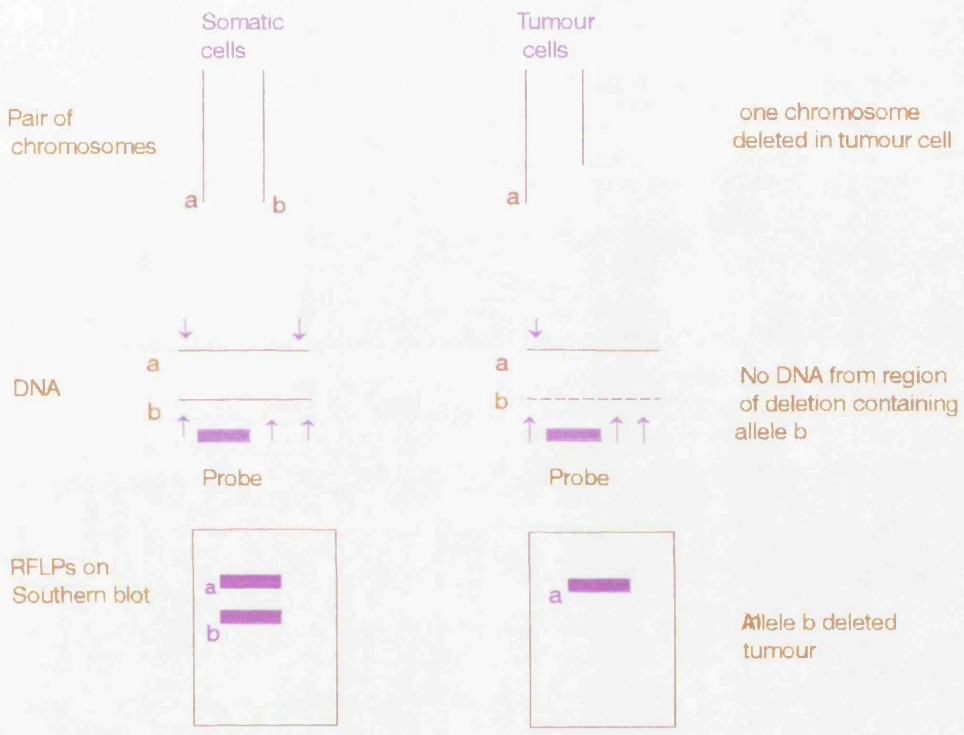


Figure 4: RFLP analysis as a means of detecting loss of heterozygosity (LOH).

2.4.8 The amplification refractory mutation system (ARMS).

The amplification refractory mutation system (ARMS) is a simple and rapid method of detecting point mutations, restriction fragment length polymorphisms (RFLP), and small nucleotide insertions or deletions. The method was first described by Newton (1989a) for analysing single DNA base differences in patients with α-antitrypsin deficiency and has since been applied to prenatal diagnosis and carrier detection of cystic fibrosis (Newton et al., 1989b). The technique is based on allele-specific priming of the polymerase chain reaction. To diagnose a specific mutation, two oligonucleotide primers are required that are identical in sequence except for the terminal 3' nucleotide. The normal primer has the 3' terminal nucleotide sequence complementary to the normal DNA sequence. Under the right conditions a primer will act as a template for DNA polymerase only when the terminal 5' nucleotide is perfectly matched to the target DNA sequence. Thus, the normal primer when hybridised to the mutant genomic DNA and, conversely, the mutant primer when hybridised to the normal DNA, will not function properly as a template for DNA amplification and no amplified product is observed at the end of the PCR process. To screen for a particular mutation, only the mutant ARMS primer is required (in combination with three others- a common primer and two control primers). However, for prenatal diagnosis of individuals homozygous for a particular mutation, both normal and mutant primers will be required. The design of the ARMS primers is very important. The technique is a combination of a PCR assay and examination of the amplification products by agarose gel electrophoresis and ethidium bromide staining. In this technique two polymerase chain reactions involving four primers in the one reaction mixture is required. Two of the primers are control primers that amplify a segment of the DNA some distance away from the site of the mutation so they do not interfere with the amplification of the DNA fragment produced by the ARMS primers and common primer. The control fragment indicates that the PCR reaction was set up

properly. When it is not observed in an ARMS analysis, the result obtained with ARMS primers must be discounted and the analysis repeated. The other two primers are the ARMS primers that are allele-specific to either the mutant DNA sequence or normal DNA sequence as required, and a common primer, which matches the same sequence in both normal and mutant DNA. These two primers combine to produce the diagnostic ARMS DNA fragment in the amplification reaction. The success of the ARMS technique is dependent on the ARMS primers working specifically.

2.4.9 RNase cleavage analysis.

Single base changes in DNA can be detected by cleavage of mismatches in RNA:DNA duplexes with the enzyme RNase (Myers et al., 1988; Winter et al., 1985; Gibbs and Caskey 1987). A uniformly labelled single-stranded RNA probe is synthesised. The ssRNA probe is synthesised as run-off transcripts from a cloned DNA template. The probe is mixed with double-stranded test DNA, which can be cloned, genomic DNA or PCR-amplified DNA fragment, and the mixture is heated to separate the DNA strands. This is then followed by an annealing reaction whereby the labelled RNA probe is allowed to anneal with its complimentary strand in the test DNA fragment forming an RNA:DNA duplex. The presence of any base changes in the test DNA will cause base mismatches in the RNA:DNA duplex. The annealed mixture is then treated with the enzyme RNase A to cleave the RNA strand at the mismatched sites. After the cleavage reaction, the duplex is treated with denaturants to separate the strands and then run on a denaturing gel to separate the RNA fragments by size. If the test DNA had no mutation, a single band on the autoradiograph, corresponding in length to the full-length protected test fragment, is observed. If a mutation was present in the test DNA, two bands will be observed on the autoradiograph.

By using this procedure, approximately 30 to 40% of all possible mismatches in the RNA:DNA duplexes can be efficiently detected. The detection rate can however, be improved to detect up to 60 to 70% of the possible base changes, by testing a DNA fragment with each of its two corresponding labelled RNA probes in separate cleavage reactions (Myers et al., 1985c). This method is most suitable for screening DNA fragments between 100 and 1000 bp long. Fragments longer than 1000 bp are difficult to screen because of random cleavages which occur even in perfectly matched bases and may be numerous enough to interfere with the results. Moreover, analysis of such long RNA fragments requires the use of denaturing agarose gels (Myers et al., 1988). Therefore, the most obvious limitation of this method is that it cannot detect all possible mismatches. Further more, the method requires extra cloning for probe production and the screening is limited to DNA fragments of up to 1000 bp only.

2.4.10 Allele specific oligonucleotide (ASO) hybridisation.

A point mutation which does not produce a restriction site change may be detected using allele-specific oligonucleotide (ASO) probes (Studencki and Wallace, 1984). ASO probes are typically 15-20 nucleotides long and normally employed under hybridisation conditions at which the DNA duplex between probe and target is only stable if there is perfect base complimentary between them. A single mismatch between probe and target sequence is sufficient to render the short heteroduplex unstable. Oligonucleotide probes can therefore be designed to hybridise to specific alleles of a gene which differ by a single nucleotide at a diagnostic site. Although ASOs can be used in conventional Southern blot hybridisation, it is more convenient to use them in dot-blot assays.

2.4.11 Direct genomic sequencing.

There are two popular methods for sequencing. One of them is the chemical method described by Maxam and Gilbert (1977), the other one is the enzymatic method of Sanger et al. (1977). Of the two methods, Sanger's method, known as dideoxy sequencing or the chain termination method of sequencing, is most widely used. In the Maxam and Gilbert method, the DNA is cleaved into fragments which are then radioactively labelled at one end, subsequently divided up into four batches, each of which is treated differently by chemicals to modify a particular base or bases resulting in very small DNA fragments, some of which will be end-labelled. The fragments are then resolved in denaturing polyacrylamide gels resulting in base specific bands on autoradiography.

The enzymatic method of sequencing involves the synthesise of a DNA strand by a DNA polymerase using a single-stranded DNA template. An universal oligonucleotide primer is annealed to the template and this followed by a period of extension where dTTP, dGTP, dCTP, and radioactively labelled dATP are incorporated into the growing strand. Reactions are terminated by addition of dideoxynucleotides into the strands in four separate reactions, one each for ddGTP, ddTTP, ddCTP, and ddATP (Dideoxynucleotides lack a 3' hydroxyl group and cannot form phosphodiester bonds). This results in the production of a family of DNA fragments of different lengths depending on the site of incorporation of dideoxynucleotide. High resolution gel electrophoresis separates the fragments, and because of the incorporation of radioactive dATP they can be visualised by autoradiograph. If the four termination reactions are run next to each other on the gel, then the sequence can be read directly from the order of the ladder of the bands (Watson et al., 1987).

2.5 Aims of the present study.

The overall aim of this project was to compare different strategies in screening for molecular pathology using the RB gene as a model system.

Specifically this involved:

2.5.1. Optimisation of PCR, RT-PCR of retinoblastoma cDNA, single-stranded conformation polymorphism (SSCP) and amplification and mismatch detection (AMD) analysis.

2.5.2. Characterisation of mutations with direct genomic sequencing.

2.5.3. Application to breast and bladder carcinomas.

CHAPTER THREE: METHODOLOGY.

3. Material and Methods.

3.1 Patients.

The Patients recruited were those with clinically confirmed bilateral retinoblastoma referred to the Duncan Guthrie Institute of Medical Genetics, Glasgow. Blood samples were collected and immortalised lymphoblastoid cell line established by Mair Crouch from The Duncan Guthrie Institute of Medical Genetics, Glasgow. Controls were randomly selected from individuals who presented with problems other than retinoblastoma. 20 patients with bilateral retinoblastoma and 3 of whom were related and 6 control subjects were included in this study. As well as patients with bilateral retinoblastoma, 40 patients with bladder carcinoma and 39 patients with breast carcinoma were screened for RB gene mutations. Tumour tissues from patients with bladder carcinoma were provided from Pathology Department of Western Infirmary Hospital Glasgow and obtained by transurethral resection. Tumour tissues from patients with breast carcinoma were provided from The Duncan Guthrie Institute of Medical Genetics, Glasgow. Additionally, to screen MCAD (Medium-Chain Acyl-CoA Dehydrogenase) deficiency in Scottish population, 552 DNA from clotted bloods obtained from The Duncan Guthrie Institute of Medical Genetics Alphafetoprotein Screening Laboratory and postmortem livers of 233 SIDS (Sudden Infant Death Syndrome) patients (representing most patients in the past 10 years in Scotland) obtained from Pathology Department of the Royal Hospital for Sick Children in Glasgow.

3.2 Preparation of genomic DNA.

Genomic DNA was extracted from blood, tumour tissue, blood clots, liver tissue and Guthrie Cards. Extraction of DNA from blood was modified from methods described by

Kunkel et al. (1977) whereas extraction of DNA from tissue was done with a method according to Hogan et al. (1986). Small scale DNA extraction method was used for the DNAs extraction from fresh blood clots (Williams et al., 1988).

3.2.1 Extraction of DNA from blood.

DNA from patients with bilateral retinoblastoma was isolated from peripheral blood lymphocytes on the protocol by Kunkel et al. (1977). 20 ml of ice cold lysis buffer* was added to 5 ml blood and mixed gently. This was incubated on ice for 10 minutes, centrifuged at 2800 g for 10 minutes, and then the supernatant was discarded. The pellet was resuspended in 3 ml nucleic lysis buffer** and the following solutions were added to the tube; 200µl 10% SDS and 100µl proteinase K. The solution was then incubated at 37°C overnight. 1 ml of 6 M NaCI was then added to overnight incubation and the sample was centrifuged at 2.5 K for 15 minutes. The top layer was transferred into a 10 ml tube. After this, the samples were extracted twice with phenol/chloroform, followed by a chloroform extraction to remove any traces of phenol. DNA was precipitated by two volume of absolute ethanol.

DNA was spooled out using sealed pasteur pipettes, washed in 70% ethanol, air dried and dissolved in 200-500 µl of TE buffer. The samples were stored at 4°C until required. Final DNA concentration was estimated by measuring the optical density at 260 nm in a Perkin Elmer 6000 spectrophotometer.

*Lysis buffer: 0.32 M Sucrose, 10 mM Tris-HCI pH 7.5, 5 mM $MgCI_2$, 1% Tritonx100.

**Nucleic lysis buffer: 10mM Tris-HCI pH 7.5, 0.44 M NaCI, 2 mM EDTA pH 7.2.

3.2.2 Extraction of DNA from tissue.

To screening MCAD deficiency, extraction of DNA from postmortem liver tissue of 233 SIDS was based on methods of Hogan et al. (1986). Same method was used for extraction of DNA from tumour tissue (breast carcinoma and bladder carcinoma) to screen RB gene mutations.

After thawing, the tissues (approximately the size of a pea) were minced with fine-pointed sterile scissors The following reagents were added; 3 ml lysis mix , 200µl 10% SDS, 100µl proteinase K (10 mg/ml). Then they were incubated at 55°C for 4-16 hours. After incubation, 1 ml 6M NaCl was added and sample vigorously shaken. The samples were then centrifuged at 3000g for 10 min. The supernatant was transferred to another tube and an equal volume of phenol-chloroform was added to each tube (5 ml of each). After centrifugation at 3000 g for 10 min the supernatant was transferred to a fresh tube. DNA was precipitated by adding 2 volumes of ethanol, 1/10th volume 3M sodium acetate. DNA was spooled out using sealed glass pipettes. The DNA was rinsed in clean 70% ethanol, air dried and resuspended in an appropriate small volume of TE buffer (about 500µl).

3.2.3 Small scale DNA extraction.

Small scale DNA extraction method was used for the DNA extraction from fresh blood clots which were obtained from pregnant western Scottish women undergoing alphafetoprotein (AFP) analysis for foetal abnormality. Two methods of small scale DNA extraction were performed to determine which was the most amenable to PCR amplification and which was also cost and time effective. The majority of the DNAs were

extracted by the Proteinase K method of Williams et al. (1988), who have shown that the quantity and purity of the DNA is sufficient for the subsequent PCR amplification. The remainder of the DNAs were extracted by the alkali method of Ivinson and Taylor (1993), which is a much quicker and simpler procedure.

3.2.3.1 Proteinase K method.

Batches of 10-20 DNAs were prepared simultaneously. For each blood sample, a clot (approximately the size of a pea) was placed into a 1.5 ml eppendorf tube and minced with scissors. The following reagents were then added to each eppendorf; 375 µl Nucleic lysis mix ,25 µl Proteinase K (10 mg/ml). The reagents were mixed well by inverting each tube for approximately 30 sec. The reaction components were then incubated at 55°C or at 37°C for at least 4 hrs with mixing each hour or overnight. Phenol/chloroform extraction was performed three times on the resultant DNA mixture, followed by a single chloroform extraction (The upper layer in each extraction contained DNA). The DNA was then precipitated out of solution by addition of roughly 1 ml of ice-cold ethanol to the DNA solutions in each 1.5 ml eppendorf tube. If visible, the DNA was spooled out on a glass pipette, washed in ethanol and left to air dry. The DNA was subsequently redissolved in 200 µl of TE buffer. If the DNA was not visible on ethanol precipitation, the DNA/ethanol mixture was microfuged at 10.000 rpm (IEC Centra-4x). The ethanol supernatant was poured off and the pellet allowed to air-dry. The DNA pellet was then redissolved in solution, as above. The DNA solutions were stored at 4°C until required. The optical density (OD) of the DNA solutions was read at 260 nm using quartz cuvettes. The DNA concentration (µl/ml) was calculated by multiplying the OD reading by the dilution factor of the DNA solution in the cuvette (1/100) and also by multiplying by 50 as 1 OD unit is equivalent to 50 µg of DNA. One µg of DNA was used in the PCR.

3.2.3.2 Alkali method.

DNA blood clots were prepared as in the proteinase K method. To release the DNA into the solution, 0.1M NaOH (100 µl) was added to the minced clot and mixed thoroughly by vortexing for roughly 15 sec. The mixture was then boiled for 6 min on a preheated Dri-Block (techne DB-1) and afterwards microfuged (IEC Centra-4X) at 10,000 rpm for 3 min. The supernatant was immediately removed from the underlying cell debris and stored at 4°C unless the PCR was performed immediately. Depending on the volume of supernatant recovered, 5-10 µl of the DNA solution were used for the PCR.

3.3 Total cellular RNA extraction.

Total cellular RNA was extracted from peripheral blood lymphocytes and lymphoblastoid cell lines. Extraction of RNA from lymphocytes was modified from methods described by Lench et al. (1988) and Sherman et al. (1989), whereas extraction of total cellular RNA from lymphoblastoid cells was based on the methods of Chomczynski and Nicoletta,

(1987). The use of high-quality RNA is critical for the success of RT-PCR analysis. The RNA must not be degraded by ribonucleases, as determined by the intactness of ribosomal (rRNA) bands, and contaminating genomic DNA must be removed. Therefore, plastic used for the preparation and storage of RNA were treated in 0.1% diethyl pyrocarbonate (DEPC) in water for 14-16 hours before being autoclaved to avoid contamination with RNases. All plastic-ware for RNA use were kept aside from general use. Isolated RNAs were stored as an ethanol precipitate at -20°C or in aqueous solution at -70°C or below up to a year without appreciable deterioration. Repeated freeze and thaw cycles were avoided.

3.3.1 Extraction of RNA from lymphocytes.

10 ml of venous blood was collected in heparinised bottles The diluted blood was carefully layered over 10 ml of histopaque and centrifuged at 1400 rpm for 30 min at room temperature. After centrifugation the opaque interface mononuclear cells, including lymphocytes, was carefully removed with a pasteur pipette into a fresh tube and washed in 20 ml of cold PBS (phosphate buffered saline), then pelleted by centrifugation at 1400 rpm. for 20 min. The supernatant was discarded in chloros and the cell pellet resuspended in phosphate buffered glucose (pH 7.4). Approximately four million cells were then removed and washed in PBS and pelleted in a microcentrifuge by centrifugation at 1400 rpm for 10 min. The cell pellet was resuspended in 500 µl of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M 2-Mercaptoethanol). [To minimise handing of guanidinium thiocyanate (hazardous) a stock solution was prepared as follows: 250 g guanidium thiocyanate (Fluka) was dissolved in the manufacturer's bottle (without weighing) with 293 ml water, 17.6 ml 0.75 M sodium citrate, pH 7, and 26.4 ml 10% sarcosyl at 65°C. This stock solution can be stored for at

least 3 months at room temperature (Chomczynski and Nicoletta, 1987)]. The resultant mixture was transferred to an autoclaved and DEPC treated 1.5 ml eppendorf tube and the following solutions; 50µl of 3M Sodium Acetate, 500µl of phenol, 100µl of chloroform-isoamylalcohol (49:1 ratio) were added and vortexed for 20 seconds. The tubes were placed on ice for 15 minutes. Then the tubes were centrifuged for 20 minutes at 10000 rpm. The aqueous phases of the mixtures were transferred into 1.5 ml eppendorf tubes. Then 1000 µl of isopropanol was added to the tubes, and mixed well. They were kept at -20°C for 1 hour. The tubes were then centrifuged, and the supernatant was discarded. 300 µl of solution D was added to the pellets and the pellets were dissolved. 300 µl of isopropanol was added to each tube and then the solution quickly chilled at -20°C. After an 1 hour, the tubes were centrifuged for 10 min. The pellet was washed in 250 µl of 75% ethanol, then dried. Finally the pellet was dissolved in DEPC treated water.

3.3.2 Extraction of total cellular RNA from lymphoblastoid cell line.

The most common and consistently successful method for isolating pure, intact total RNA are modifications of the original guanidinium thiocyanate method of Chirgwin et al. (1979). To each cell pellet in a 30 ml Sorvall tube, 3 ml of Solution D was added. 2-mercaptoethanol was added to a final concentration of 1% (0.14 M) only just before use. Homogenisation was done by carefully vortex mixing the cell pellet in the homogenisation buffer and then passing the homogenate several times through a 23-gauge needle until the cell lysate was no longer viscous. After homogenisation, the protocol was carried out as described above.

3.3.3 Determination of RNA concentration.

RNA quantitation was carried out by spectrophotometry. The optical density (OD) of the RNA was determined both at 260 and 280 nm. To carry out determination of the optical density (OD) of RNA, 10 µl of RNA solution was diluted in 990 µl of TE buffer in a 1 ml cuvette. An OD of 1 at 260 nm corresponds to approximately 40 µg/ml of RNA. The ratio of 260 nm and 280 nm was used to determine the purity of the RNA solution. The value of the ratio was expected to be 2 to determine good RNA (Gurr and McPherson, 1993).

3.3.4 Qualitative assessment of RNA.

The quality of RNA was evaluated by agarose gel electrophoresis. To prepare the gel, 0.45 g agarose was weighed out in 22 ml of water in a flask . The flask was weighed and boiled until the agarose had dissolved. The flask was then re-weighed and the evaporated water replaced. The gel was cooled to 55°C. After cooling, in a fume hood, the following reagents were added; 5 ml formaldehyde, 3 ml 10xMOPS buffer [3-(*N*-morpholino) ethanesulphonic acid] and the gel quickly poured before setting. 1 µl of RNA solution was mixed with 5 µl of formamide, 1.65 µl of formaldehyde, 1 µl of 10X MOPS and 1.3 µl of dHO_2. The mixture was heated to 55°C for 10 min, quenched on ice and 2 µl of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% sucrose in water) was added. The samples were then immediately loaded with 1µg *Escherichia coli* ribosomal RNA (Boehringer) as markers and electrophoresed at 75 V until the bromophenol blue reached the bottom of the gel. 1xMOPS was used as a running buffer. After running the samples, the gel was soaked in water for 1 hour to wash out the formaldehyde. The gel was stained for 5 min in a 1/1000 solution of ethidium bromide,

then destained for 2 hr or overnight in H_2O and viewed under U.V. light to check for the integrity of ribosomal bands and to estimate the concentration.

3.4 Polymerase chain reaction.

3.4.1 Amplification of DNA from retinoblastoma gene.

Primers flanking exons 12-22 of the retinoblastoma gene, designed by Thompson and his co-workers (1991) (Table 3), were used in this study for the amplification of DNA from the retinoblastoma gene. The synthetic oligonucleotide primers were prepared by on a Applied Biosystems DNA synthesiser. Amplification was performed by adding 1 µl of DNA to a 15 µl reaction mixture containing 50 mM KCl, 10 mM Tris.HCl (pH 8.4), 1.5 mM $MgCl_2$, 100 µg/ml gelatine, 10 µM of each dNTP (dATP, dCTP, dGTP, dTTP)(Boehringer Mannheim) and 0.5 µM of each primer. The mixture was then put in an U.V. box (Amplirad II, high intensity shortwave cabinet with 4xG875 germicidal lamps, 1.8 watts each, 254 mm wavelength) for 5 min. After U.V. treatment DNA was added and thoroughly mixed by vortexing. To this 1.25 units of *Taq* DNA Polymerase was added, the mixture overlaid with 15 µl of light mineral oil and amplified for 30 cycles. Denaturation was carried out at 94°C for 45 sec, annealing was performed at 52-57°C (Table 5 in the results section) for 2 min and extension at 72°C for 1.5 min except for the last extension which was carried out for 10 min. At the end of the PCR, samples were briefly centrifuged and 5 µl of each product was taken below oil and electrophoresed on a 1% agarose gel in 1X TBE buffer. Electrophoresis was carried out at 10-15 V/cm until the bromophenol blue in the loading mixture migrated two thirds of the distance of the gel. The gel was stained by soaking for 30 min at room temperature in

the running buffer containing 0.5 μg/ml of ethidium bromide. When necessary the gel was destained for 20 min before visualisation under U.V. light.

3.4.2 Amplification of DNA from medium-chain Acyl-CoA dehydrogenase (MCAD) gene.

A 0.5 ml sterilised tube was used for the PCR reaction. The reaction mix, which was made up to a total volume of 100 μl with double distilled water, was set up using 10μl of 10xPCR reaction buffer (50 mM KCl, 10 mM Tris (pH 8.4), 1.5 mM $MgCl_2$, 100 mg/ml gelatine), 10 μl dNTP's mix containing 50μM of each nucleotide and 50 pmol of each oligonucleotide primer (Gregersen et al., 1991). The concentration of each oligonucleotide primer was calculated according to the method described by Thein and Wallace, (1986). All reagents were kept at -20°C, vortexed briefly after thawing and spun down before pipeting with positive displacement pipettes (Lab systems). The mix was irradiated for 5 minutes in an Amplirad before the addition of 1μg of template. The samples were than heated at 95°C for 5 minute, 60°C for 5 minutes, and 2 units of *Taq* DNA polymerase (Boehringer Mannheim, 5 U/μl) were added. Each PCR reaction mix was overlaid with 100μl of light mineral oil (Sigma), and centrifuged for 3 seconds before it was placed in Techne PHC 1 automatic thermocycler. Amplification conditions consisted of 30 cycles of the following: denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and primer extension at 72°C for 2 min. The PCR cycles were followed by a final 10 min extension step at 72°C. The products were directly digested with NcoI and analysed in 8% polyacrylamide gels.

Primer set	Sequence
12A	5'-GAT ACA TTT AAC TTG GGA GA-3
12B	5'-CAT GTT AGA TAG GAG ATT AG-3'
13A	5'-GAT TAC ACA GTA TCC TCG AC-3'
13B	5'-TAC AA CTG GA AGA TGC TG-3'
14A	5'-AAA CAG TGA GAC TCC ATC TC-3'
14B	5-'TGG CCA GGA TGA TCT TGA TG-3'
15A	5'-TAA GGT TTC AAT TAA ACA AC-3'
16B	5'-CTT TAA ATT GAA CAA AAG TG-3'
17A	5'-TAC CTA GCT CAA GGG TTA AT-3'
17B	5'-TAG CCA TAT GCA CAT GAA TG-3'
18A	5'-AAT TAT GCT TAC TAA TGT GG-3'
18B	5'-ATT TGC AGT TTG ATG GTC AA-3'
19A	5'-TGT GAT TCT TAG CCA ACT TG-3'
19B	5'-TCA GAG TCC ATG CTC TTG AA-3'
20A	5'-AGA GGT TTC TGT TAA AAT GC-3'
20B	5'-AGAAGG TGA AGT GCT TGA TT-3'
21A	5'-ATT CTG ACT ACT TTT ACA TC-3'
21B	5'-ATG AGA TCA AAT GAA TTA CC-3'
22A	5'-TTT ACT GTT CTT CCT CAG-3'
22B	5'-GTG GAC CCA TTA CAT AG-3'

Table 3. Sequence of PCR primers for the exons studied (Thompson et al., 1991).

3.4.3 PCR primer design.

Primers for RT-PCR were designed using computer program OLIGO^{TM}(MEDPROBE). Segments were designed which overlapped each other and almost covered the entire cDNA. Oligonucleotide primers were generally synthesised in the range 21-22 bases. Primers were stored in the ammonia eluant used for deprotection which stays liquid at -20^{o}C, enabling the dispensing of primers without repeated freeze-thawing. Before use, 20-30 µl of this stock was heated to 95^{o}C for 3 minutes to drive off the ammonia. Total cellular RNA from samples was used to synthesise cDNA using oligonucleotide primers with nucleotide sequences complementary to various regions of the RB mRNA. The single-stranded cDNA segments carrying portions of the RB mRNA thus obtained were then amplified by PCR to double-stranded DNA segments using sets of two primers one of which was the same oligonucleotide as that used for reverse transcription. The regions thus amplified, designated as segments A to E (Table 4), with nucleotide lengths of 302 bp to 1433 bp, are shown in Figure 5. These 5 segments overlap each other and cover almost the whole coding sequence of the RB cDNA of 3938 kb. Amplified products were then subjected to AMD analysis.

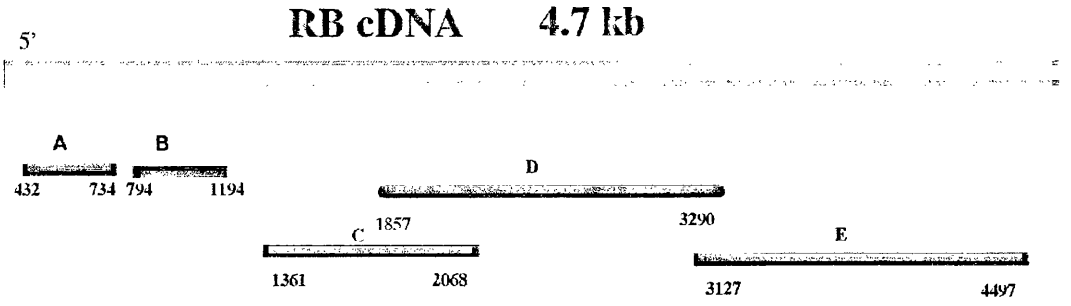


Figure 5. The strategy for amplification of RB cDNA, and the positions of the segments used for RT-PCR in the gene is shown.

POSITION	SYMBOL	POSITION AND LENGTH	SEQUENCE
Sense	**A5**	432 (21)	5'GTG GGG AAT CTG TAT CTT TAT-3'
Antisense	**A3**	734 (21)	5'-ATA CTT CCC CTT TAG CTA ATA-3'
Sense	**B5**	794 (21)	5'-TGC TAT GTG TCC TTG ACT ATT-3'
Antisense	**B3**	1199 (21)	5'-TAC TTT TTC GTG GTG TTC TCT-3'
Sense	**C5**	1361 (22)	5'-CAG TGA ATC CAA AAG AAA GTA T-3'
Antisense	**C3**	2068 (22)	5'-AAA ACA GTG AAA GAG AGG TAG A-3'
Sense	**D5**	1857 (21)	5'-TAA ACA ATC AAA GGA CCG AGA-3'
Antisense	**D3**	3290 (21)	5'-AAA AAC AAG AGC AAA CAT CAC-3'
Sense	**E5**	3127 (21)	5'-TTG AAA ATC TTG TGT AAA TCC-3'
Antisense	**E3**	4497 (21)	5'-CCC TTG ACC TAA AAA CTA ACT-3'

Table 4. Sequences of the oligonucleotide primers used in various amplifications and their positions in retinoblastoma cDNA are shown.

3.4.4 Asymmetric PCR amplification of retinoblastoma cDNA.

The PCR technique allows the rapid isolation of specific DNA targets for sequencing. Asymmetric PCR reaction was performed according to the protocol developed by Mgone (1992). By this method single-stranded DNA was generated during the PCR reaction. Primers were included in a ratio of 50:1 to 100:1, one primer at the usual concentration and one diluted. The best ratio was generally determined by trial and error. 5 µl of a 10 µ M and 0.1 µM dilution of each primer were used in a PCR reaction in a total volume 100 µl. Other components were the same as for the standard reaction. The reactions were checked for the production of single-stranded DNA on a 1% TBE agarose gel, and visualised by staining with ethidium bromide (10µg/ml).

3.4.5 Reverse-transcriptase PCR (RT-PCR).

Reverse transcription of RNA followed by the polymerase chain reaction (RT-PCR) is a sensitive method to detect specific mRNAs. RNA was first isolated from cells and then used as a template for reverse transcription to complimentary DNA (cDNA). The cDNA in turn was used as a template for PCR, using primers designed to amplify the selected cDNA region. Following PCR, the product was typically analysed by agarose gel electrophoresis. The amplified cDNA was identified by the size of the PCR product, which is predicted from knowledge of the cDNA nucleotide sequence.

3.4.6 Reverse-transcriptase PCR using downstream primers.

A 3' (antisense) gene-specific primer was annealed to the mRNA and extended with reverse transcriptase. thus generating a cDNA template for the 5' (sense) primer, allowing

PCR amplification to occur. To accomplish this, 0.5-1 μg of RNA was heated at 95 °C for 5 min, quenched on ice and then 20 pmol of antisense primer was added. To anneal the primer, the samples were heated at 65 °C for 10 minutes and quenched on ice again and then reverse transcribed in a 20 μl reaction mixture containing 20 Units Ribonuclease inhibitor (RNasin; Boehringer Mannheim Biochemica; 2,000U/50μl), 50 mM Tris-HCI, pH 8.3, 3 mM $MgCI_2$, 75 mM KCI, 10 mM dithiothereitol (DTT), 200μM each dNTP (dATP, dCTP, dGTP, dTTP), 200 units of Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT; Gibco BRL) for 1 hour at 42°C. At the end of the reverse transcription the mixture was heated to 95°C for 5 min and then quenched on ice. To this solution 30 pmol of the antisense primer and 50 pmol of the sense primer were added with a final concentration of 0.5 μM of each primer. The volume of the reaction mixture was adjusted to 100 μl and 2 units of *Taq* DNA polymerase added. The samples were then briefly vortex mixed and capped with 100 μl of light mineral oil. Amplification conditions consisted of 40 cycles of the following: denaturation at 94°C for 1 minute, annealing at 52°C for 2 minutes, and primer extension at 72°C for 2 minutes. The PCR cycles were followed by a final 10 minutes extension step at 72°C.

The samples were loaded onto 1% agarose gels and electrophoresed in TBE buffer. The products were then visualised under U.V. light by staining with ethidium bromide (10μ g/ml). The appropriate band was excised and the cDNA eluted by soaking for 24 hours in 100 μl of TE buffer at -20°C. The cDNA in solution was removed from the agarose by pipetting and stored at -20°C or used directly in asymmetric PCR or AMD analysis.

3.4.7 Reverse-transcriptase PCR using Oligo (dT).

mRNA molecules were first converted into cDNA by priming with 0.2 μg of oligo(dT) ($d(T)_{12-18}$; Pharmacia). At the end of the reverse transcription two gene-specific primers were added for amplification. The amplification was carried out as described above.

3.4.8 Purification of PCR products.

At the end of PCR, the amplified DNA or cDNA was purified by removing excess dNTPs, primers and salts. Various techniques were used. These included Geneclean purification (Vogelstein and Gillespie, 1979), and selective alcohol precipitation (Gyllensten and Ehrich, 1989; Brow, 1990).

3.4.8.1 Geneclean™ purification.

Chemical cleavage of PCR products requires elimination of primers, dNTP's, PCR buffer, and *Taq* polymerase, since these would interfere with the reaction. At the end of PCR amplification, the purification was carried out according to the manufacture's protocol (BIO 101). The paraffin oil was removed. 2-3 volumes of the supplied stock solution of NaI were added to the products giving a final concentration of at least 4 M and to this, 15 μl of the provided 'glassmilk' suspension was added, mixed and the mixture kept on ice for 15 min to bind the DNA molecules. The mixture was then centrifuged in a microcentrifuge for 5 seconds and the supernatant removed. The pellet was then resuspended in 300 μl of the New Wash provided, centrifuged for 5 seconds and the supernatant discarded. This washing was repeated three times and after the third wash the pellet was resuspended in 20 μl of TE buffer and incubated at 65°C for 10 min.

The mixture was then centrifuge for 3 minutes and the supernatant containing eluted DNA was transferred to a fresh microfuge tube.

3.4.8.2 Selective alcohol precipitation.

Precipitation was performed according to the protocol modified by Mgone (1992). The method used was based on the fact that 2.0-2.5 M ammonium acetate reduces the coprecipitation of dNTPs with DNA (Okayama and Berg, 1982). Mineral oil was removed. An equal volume of 4 M ammonium acetate (pH 5.2) was then added, mixed and to this 2 volumes of ethanol added and again mixed. The mixture was left at room temperature for 10 min, then centrifuged at 12,000 rpm for 10 min. The DNA pellet was washed in 70% alcohol, dried in air and dissolved in 14 µl of TE buffer (pH 7.4).

3.5 Single-stranded conformation polymorphism (SSCP) analysis.

SSCP analysis was applied to screen functionally important RB gene regions. Exons 12 to 22 (Table 3) of the RB gene were screened for sequence change with PCR-SSCP analysis. A 30 cycle PCR was performed on the target DNA sequences using 100 ng of genomic DNA. 20 pmol ^{32}P dCTP (60 µCi) was included in the reaction mixture to label the PCR products. After thermal cycling, the amplified product was diluted 1:7 in gel loading buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol). The sample was heated at 95^{o}C for 2 minutes to denature the DNA. The denatured DNA was then placed directly onto ice for several minutes. Then, the samples were loaded on to 0.5XMDE nondenaturing gel (20cmx40cmx0.4cm) containing 5% glycerol. Electrophoresis was carried out at 5 W for 14-24 hours, with a fan heater, set on cold, directed at the gel as a cooling device. The gels were dried on a gel dryer

(Biorad model 543). After drying, the gels were placed on a sheet of Kodak XAR-5 film in an autoradiographic cassette with "Dupont lightning plus" intensifying screens (Hoeffer) and exposed for 3-16 hours at -70°C.

3.6 Heteroduplex analysis of the retinoblastoma gene.

After the final thermal cycle of PCR was completed, the reaction was inactivated by adding EDTA to 5 mM (1µl of 0.5 M EDTA per 100 µl reaction). Amplified DNA fragments were heated to 95°C for 3 minutes, and cooled slowly (20-30 minutes) to room temperature to form heteroduplexes (Keen et al., 1991). A sequencing plate was set up with 0.4 mm spacers and filled with gel solution including 25 ml of MDE gel, 69 ml of dH_2O, 6 ml of 10X TBE buffer, 0.4 ml of 10% APS, 0.04 ml of TEMED. Appropriate DNA size markers were also included on each gel as well as control homologduplex DNA. Approximately 100-200 ng of DNA was loaded per lane. Before loading the samples, 1 µl of gel loading buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol) for each sample was added and mixed well. Electrophoresis was done at a maximum constant voltage of 20 Volts per cm of the gel. So, for 40 cm gel, 800 Volts was used.

3.7 Amplification and mismatch detection (AMD) analysis.

This technique was developed to screen for point mutations, but deletions and insertions too small to be recognised by gel electrophoretic techniques are also detected (Howells et al., 1990). In this method, a reference DNA probe was mixed with excess test DNA or RNA. The mixture was then heated to denature the double strands of the DNA, and followed by cooling to allow reannealing and, thus, heteroduplex formation occurred with

mismatched or unmatched C and T bases detected by reactions with hydroxylamine and osmium tetroxide, respectively. Cleaved by piperidine treatment was followed. The samples were then run on a 8% polyacrylamide/urea denaturing gel. 5 segments shown in Figure 5 of the RB gene and some of exons of the RB gene from patients with bilateral Rb were subjected to AMD analysis.

3.7.1 Probe preparation.

The purified PCR product chosen as the probe was end labelled or internally labelled. End labelling was done either by 3'-end labelling or 5'-end labelling. Approximately 5ng of probe DNA per target sample per modification reaction was used. Radiolabelling of the probe was done by using either T4 polynucleotide kinase (5' end labelling) or the Klenow fragment of DNA polymerase I (3'-end).

3.7.1.1 ^{32}P Internal labelling and probe preparation.

Probes for each of the PCR products were prepared by PCR amplification between the sense and antisense oligonucleotides using an unaffected sample in the presence of 20 pmol of ^{32}P dCTP (60 μCi). To allow efficient incorporation of label, the final concentration of non radioactive dCTP in the reaction was reduced from 200 μM to 6 μM. To prevent misincorporation of alternate dNTPs as [dCTP] fell, only 25 PCR cycles were performed. The probe was then isolated from an agarose gel and resuspended in 1xTE (10mM Tris-HCl, 1mM EDTA, pH 7.4) buffer at 1,000cpm/μl.

3.7.1.2 5'-end-labelling and probe preparation.

10 µl of Geneclean PCR product of unaffected control were mixed with 0.5µl of (γ-^{32}P)-ATP (3000 Ci/mmol; 10 Ci/ml), 5µl of 10X kinase buffer (freshly made; 500mM Tris-HCI pH 7.6, 100mM DTT, 100mM magnesium chloride), 1µl of 10 units/µl T4 polynucleotide kinase and 33.5µl of TE buffer (10 mM Tris-HCI, 0.1 mM EDTA, pH 7.4). This mixture was incubated at 37^{o}C for 30 minutes. After that, it was placed on ice or stored at -20^{o}C.

3.7.1.3 Probe preparation using 5'end-labelling primers.

Either the sense or the antisense strand of the wild-type cDNA was labelled by incorporation of radiolabelled oligonucleotide primer during the PCR reaction. 20-50 pmol of primer was labelled as follows: To a final volume of 50µl of TE buffer; 5µl of 5X kinase buffer, 5µl of diluted primer, 50-70 µCi (γ-^{32}P)ATP (6000 Ci/mmol), and 30 units of T4 polynucleotide kinase were added. Then it was incubated at 37^{o}C for 45 minutes. End-labelled primers were used in the PCR reaction. After that, the reaction mixture was purified using Geneclean and used as a probe.

3.7.1.4 3'-end-labelling and probe preparation.

3'-end labelling can be used to selectively label each strand of the probe separately, but this requires that the appropriate restriction sites are present in the oligonucleotide primers. 6.5 µl volume probe DNA was mixed with 1µl of (α-^{32}P)-dNTP(s) (3000 Ci/mmol; 10 Ci/ml), 1 µl of TM (100 mM Tris-HCI pH 8.3, 50 mM Magnesium chloride), 1 µl of 0.1 M dithiothereitol (DTT), 0.5 µl of 5 units/µl Klenow fragment

(Boehringer-Mannheim) to a final volume of 10 µl of $T_{0.1}E$. Then it was incubated at room temperature for 15 minutes and placed on ice or stored at -2°C.

3.7.2 Duplex formation.

By means of siliconized eppendorf tubes, homoduplexes and heteroduplexes were prepared with 28,000 cpm ^{32}P control DNA (approximately 10.5ng) and a 10-fold excess of the unlabeled test DNA (105ng) in 162µl of TE buffer. 18µl of 10X hybridisation buffer (3M NaCI, 1M Tris-HCI, pH 8.0) was added, and the tubes were boiled for 5 minutes and transferred to 65°C. After 5-16h incubation, the tubes were removed and the DNA duplex was precipitated in 750µl of stop solution (63mM sodium acetate, 20µM EDTA, 80% ethanol) as well as 4µl of 20 mg/ml mussel glycogen (Boehringer-Mannheim). After mixing well, the samples were chilled on dry ice for 10 minutes. The samples were then spun at 14000g in a microcenrifuge for 10 minutes and the supernatant was discarded. The pellets were then washed with 70% v/v ethanol, and resuspended in 14µl of TE buffer. Two reactions were carried out for each duplex.

3.7.3 Hydroxylamine reaction.

20 µl hydroxylamine solution (1.39 g hydroxylamine (BDH) in 1.6 ml distilled water, adjusted to pH 6.0 by addition of approximately 1.6 ml diethylamine), was added to 7 µl of the duplex solution and the mixtures incubated for 10 and 60 min, respectively, at 37°C. After incubation, 750 µl "stop solution" was added, and the DNA precipitated on dry-ice for 10 min. After centrifugation the DNA pellets were rinsed with 70% ethanol, and all liquid was removed after brief centrifugation. Finally the pellets were dried.

3.7.4 Osmium tetroxide (OsO_4) reaction.

18 µl of osmium tetroxide solution (pyridine 6.75 µl (BDH), 4% w/v osmium tetroxide (Aldrich; 4% solution), 154 µl of $T_{0.1}E$) was added to 7 µl of the duplex solution and the tubes mixed and incubated at 37°C for 2 hours. After incubation the reactions were stopped by the addition of 750 µl stop solution, and the DNA precipitated and was dried as for the hydroxylamine reactions. Osmium tetroxide irritates mucous membranes, therefore it should be used in a fume hood.

3.7.5 Piperidine cleavage of mismatches.

Fifty microliters of 10% piperidine (Fluka chemica) solution was added to each of the dried pellets and vortexed for 10 seconds, and the tubes then incubated at 90°C for 30 min and 300 µl of stop solutions were added and the DNA was precipitated on dry-ice for 10 minutes and centrifuged, washed with 70% ethanol, dried, and resuspended with 7 µl formamide dye (10 mg bromophenol blue, 10 mg xylene cyanol FF, and 0.2 ml of 0.5 mM EDTA dissolved in 10 ml deionized formamide). The samples were boiled for 2 min and cooled on ice, and then the sample was subjected to electrophoresis on a 0.4-mm-thick 8% polyacrylamide/urea denaturing gel run at 35 W until the bromophenol blue marker had run 30 cm.

3.7.6 Radioactive labelling of 1 kb ladder.

The DNA fragments of the 1 kb ladder (BRL) were labelled by filling in of the 3' recessed ends with Klenow enzyme using the dATP, dGTP and dTTP nucleotides included in the Random Primed DNA labelling Kit (Boehringer Mannheim). 1µg of 1 kb ladder was mixed with 1 µl of each dNTP, 2µl React 3 buffer (BRL), 2 µl of ^{32}P dCTP (Amersham

Life Science, 10 mCi/ml), 2 units of Klenow enzyme and made up to a final volume of 20 µl with distilled water. After then, the sample was incubated at 37 °C for 45 minutes. Then the labelled fragments were separated from other radioactive material on a pre-packed Nick Column (Pharmacia), containing Sephadex G-50, eluted in 200 µl 1x SSC buffer. The radioactively labelled 1 kb ladder was with loading mix before being applied on a gel.

3.8 Genomic sequencing.

The analysis of genetic variation in sequences amplified by PCR was carried out by sequencing the PCR product. The PCR amplified DNA and cDNA were directly sequenced using single-stranded templates (Mgone et al., 1992). Prior to sequencing, the PCR products were purified by removing excess deoxyribonucleoside triphosphates (dNTPs), salts and primers as described above. 7 µl of purified PCR product was mixed with 2 µl 5 X sequencing buffer giving a final concentration of 40 mM Tris-HCI (pH 7.5), 20 mM $MgCI_2$ and 50 mM NaCI; 1 pmol of sequencing primer, either limiting or nested and complementary to synthesised single-stranded cDNA or DNA, was used. The template-primer mix was annealed by heating to 65°C for 2 minutes and then allowed to cool slowly, to less than 35°C over a period of 30 minutes. The mixture was then placed on ice and 1 µl of 0.1 M DTT, 2 µl of a 1/5 dilution of labelling mix (7.5 µM of each dNTP except dATP), 0.5 µl α^{35}S-thio dATP (1000 Ci/mmol: 10 µCi/µl) and 2 µl (1.5 u) of a 1/8 dilution of T7 DNA polymerase (Sequenase; version 2.0, protocol no. 70770) were added and incubated at room temperature for 5 minutes. After incubation, 3.5 µl of the mix was added to 2.5 µl of each termination mix prewarmed to 37°C, and incubated for another 5 minutes. The termination mixes contained 80 µM of each dNTP and 8 µ M of the appropriate dideoxyribonucleoside triphosphate (ddNTP). The reactions were

terminated by the addition of 4 µl formamide-dye stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and denatured at 75°C for 2 minutes; 1.5 µl of each reaction mixture was immediately loaded onto an 8% polyacrylamide gel containing 7 M urea. The gels were run at a temperature of 50°C for 2-4 hours, then fixed in 10% methanol/10% acetic acid, dried and exposed for 16-18 hours to Kodak X-Omat AR film using intensifying screens .

CHAPTER FOUR: RESULTS.

4. Results

4.1 Amplification of DNA from postmortem liver tissue and blood clots.

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, is an autosomal recessive inborn error of metabolism associated with sudden unexplained death in young children. The DNAs from clotted bloods and postmortem livers of patients dying of sudden infant death syndrome (SIDS) were subjected to a modified PCR (Gregersen et al. 1991), whereby DNA fragments of 199 bp, including position 985 were synthesised. The sense and antisense primers used were (5'-TTT ATG CTG GCT GAA ATG GCC ATG-3')(bp 961 to 984 of the cDNA) and (5'-CAG GAT ATT CTG TAT TAA ATC CAT GGC CTC-3')(bp 1130 to 1159 of the cDNA) respectively (Gregersen et al. 1991). The sense primer contains a C:T mismatch at position 981, thus introducing a *Nco*I restriction site (CCATGG) when the mutant sequence is amplified. The antisense primer introduces a G:A mismatch at position 1135, creating another *Nco*I site. A *Nco*I site is not created and therefore cleavage does not occur when the PCR product is derived from the A985 allele. The other *Nco*I site is a control site, which is cleaved in both cases. G985 can therefore be distinguished from A985 because *Nco*I digestion of the PCR product results in a 158 bp band (G985) instead of a 178 bp band (A985) (Kolvraa et al., 1991) (Figure 6). 552 DNAs extracted from fresh blood clots of women from the West of Scotland were analysed two heterozygotes and no homozygotes were found. In addition, there were 3 G985 heterozygotes in a sample of 233 Scottish patients dying from SIDS from same geographical area and no homozygotes found. This means that the heterozygous frequency of the G985 mutation is 1/276 (95% CI: 1/76-1/2279) in the Scottish population and 1/74 (95% CI: 1/27-1/377) in Scottish patients with SIDS (Daly et al., 1991).

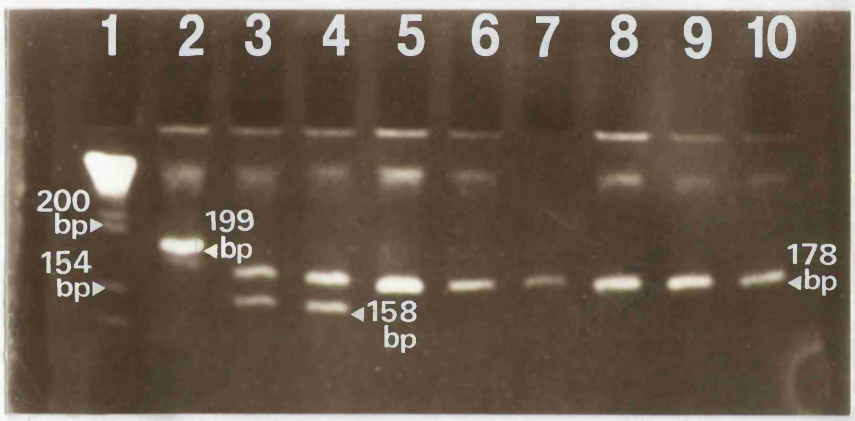


Figure 6. Amplification of DNA from tissues and blood clots.

Genomic DNA fragments (199 bp) of the MCAD gene were amplified and digested with *Nco*I and subjected to electrophoresis in an 8% polyacrylamide gel. Lane 1; size marker (1 kb ladder), lane 2; control (undigested), lane 3; heterozygous control, lane 4; heterozygous individual, lanes 5-10; normal individuals.

4.2 Application of PCR-SSCP analysis in regions of the retinoblastoma gene.

In PCR-SSCP analysis, a DNA sequence of interest was amplified by PCR. For efficient detection of the product, isotopic label was coupled with the PCR primers or was incorporated into the PCR product itself (Hayashi et al., 1989; Makino et al., 1992). Generally in 100 μl of PCR reaction for SSCP analysis, 2 μl of α-^{32}PdATP (3000Ci/mmol) and 2-3 unit of *Taq* polymerase are used. In order to save *Taq* polymerase and radioactive isotope PCR amplification was carried out in 15 μl of reaction solution. PCR in the small amount of reaction solution resulted in more efficient amplification and low expose radioactivity. On the other hand, when amounts of DNA from patients is limited for analysis, PCR can be carried out in small PCR reactions. In 100 μl of PCR reaction, the amount of DNA required is about 1 μ g, whereas using this approach, only 10-100 ng DNA used in the PCR reaction can yield about 1-1.5 μg of product which is more than enough for SSCP analysis. The optimal amount of DNA per lane for SSCP analysis was found to be 100 ng. The product was then heated to dissociate the strands, and analysed by non-denaturing MDE gel electrophoresis (Orita et al., 1989). In the present study non-denaturing MDE gels were used instead of polyacrylamide gels. The advantage of using MDE gels instead of polyacrylamide gels was that the separation of single strand of the DNA was better and clearer. This resulted in easier analysis of the samples. Under non-denaturing conditions, single stranded DNA fragments will fold into unique conformations determined by their primary sequence because the structure is stabilised by intramolecular interactions. This secondary structure is difficult to predict theoretically and is highly dependent on variables such as temperature and ionic concentrations. Experience in many laboratories has confirmed that even a single-base substitution in PCR fragment several hundred nucleotides in length can induced a conformational change that is detectable as altered mobility on non-

denaturing gel electrophoresis and that the technique requires relatively less labour and expense than most other approaches (Hayashi et al., 1993). The presence of low concentration of glycerol also affects folding and differences in the gel matrix can have a dramatic effect on mobility. In the present study 5% glycerol was used in the gel. Overloading sometimes results in abnormal migration of the bands and reduced resolution. For this reason, radioisotopic labelling of PCR products to a high specific activity can be recommended.

The full sequence of the coding region of RB gene, together with approximately 200 bp of the introns flanking each exon, has been reported by McGee et al. (1989). Since not all the mutations occur within exons, the sequences used for the oligonucleotide primers (Thompson et al., 1991) extend into the intron region. Details of primer sequences are given in Table 3 in the methodology section. The size of the flanking intron region included in the amplified product varied between 69 and 220 bp depending on the exon. The size of PCR products range from 182 to 300 bp. Details of annealing temperature of the primers are given in Table 5.

Following PCR amplification, the amplified DNA was analysed on a 1% agarose gel to check both the size and specificity of the product (Figure 7). The location of primers was important to get specific amplification. Single base pair mutations can be detected following SSCP analysis of PCR products up to 350 bp. However, subtle changes in nucleotide sequence are more readily detectable in smaller molecules (Orita et al., 1989). An additional factor which influenced the location of the primers was the GC-AT ratio. The higher the AT content, the lower the annealing temperature and the greater the likelihood of non-homologous pairing which results in background amplification. Generally, the higher the GC content of the primer, the higher the annealing temperature. The 3' primer for exon 20 is situated upstream of the highly polymorphic tandem repeat (VNTR) region

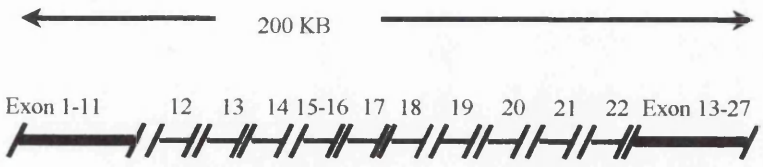
comprising variable numbers of CTTT(T) repeats (McGee et al., 1991). Since exons 15 and 16 are separated by only an 80-bp intron sequence, a single pair of primers was used to cover both exons (Thompson et al., 1991). All of the primers listed in table 3 were tested and their efficiency confirmed using a variety of DNAs from blood cells of normal individual and patients with bilateral Rb in whom a heterozygous mutation might be expected. After amplification, the samples were loaded on to 0.5XMDE gels containing 5% glycerol. Electrophoresis was carried out at 5 W for 14-24 hours, with a fan directed at the gel as a cooling device.

SSCP analysis was applied to screen functionally important RB gene regions. Exons 12 to 22 (Table 3) of the RB gene were screened for sequence change with PCR-SSCP analysis. This covers 1198 bp of the cDNA. Systematic screening was performed using DNA as a template from 20 patients with bilateral retinoblastoma, 40 patients with bladder carcinoma (primary bladder carcinoma) and 39 patients with breast carcinoma (primary breast carcinoma). The DNA fragments which presented a shift in the electrophoretic mobility were demonstrated in five patients; four patients with Rb and one patient with bladder carcinoma (Table 5). For those exons showing band shifts with SSCP analysis, direct sequencing of the PCR products was performed.

Primer set for exons and correspondence flanking intonic region	Size of amplified product (bp)	Temperature of PCR reaction (°C)	Conformation polymorphism detected
Exon 12	290	52	1
Exon 13	236	57	
Exon 14	276	57	
Exon 15 and 16	285	55	2
Exon 17	300	52	
Exon 18	206	55	
Exon 19	234	53	
Exon 20	295	57	1
Exon 21	216	55	1
Exon 22	182	52	

of amplified fragment

Table 5: Size and annealing temperature of the primers used for the amplification of the DNA fragments of the RB gene. First column: primer set used. Second column: size of the products. Third column: details of annealing temperature of the primers. Fourth column: results of PCR-SSCP analysis in regions of RB gene flanking exon 12 to 22.



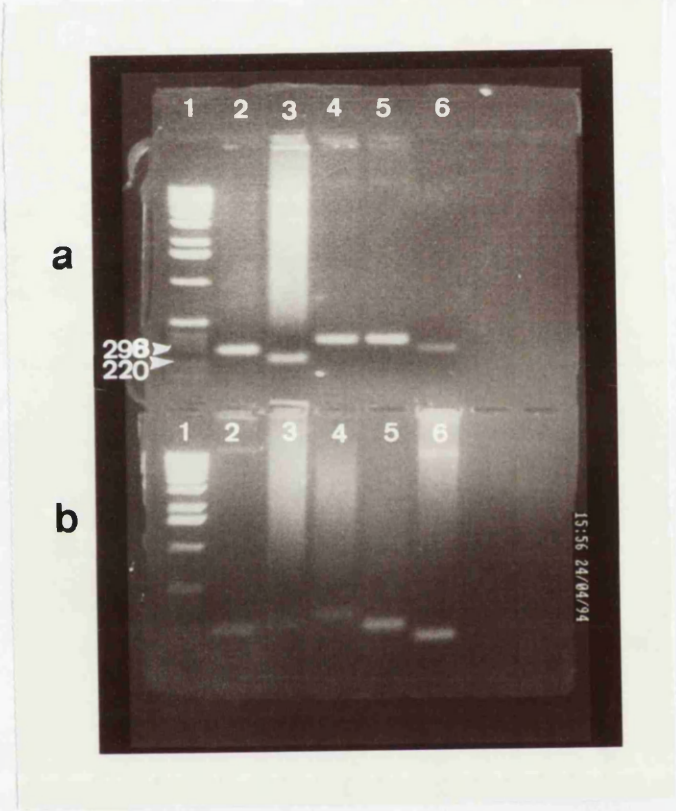


Figure 7: PCR amplification of exons 12-22 of the RB gene.
A schematic representations of exons studied in the upper part of the figure.
a) Lane 1: The DNA marker used is the 1 kb ladder (BRL). Lane 2: amplification of exon 12. Lane 3: exon 13. Lane 4: exon 14. Lane 5: exons 15-16. Lane 6: exon 17. b) Lane 1: 1 kb ladder. Lane 2: exon 18. Lane 3: exon 19. Lane 4: exon 20. Lane 5: exon 21. Lane 6: exon 22.

4.3 SSCP analysis of patients with retinoblastoma.

To investigate the nature of RB mutations in patients, an exon-by-exon analysis of DNA from samples from 20 unrelated individuals was performed. Exons 12-22 and flanking intron sequences were analysed in all cases. 4 different mutations from patients with bilateral Rb were detected by SSCP and confirmed by sequencing. Two of these were in exon 16, one was in exon 20 and one was in exon 21. A 285-bp fragment containing exon 15 and exon 16 was amplified using DNA purified from the blood of patients with Rb. One additional band was present in the amplified products from patient AR and patient GM. Both patients have a different SSCP pattern (Figure 8). Analysis of exon 20 showed an abnormal SSCP banding pattern (Figure 9) for patient E.A.S. When compared with DNA from a normal individual, E.A.S. had an additional band which migrated faster than the other normal bands. Since all 3 have two normal bands as well as an additional band, it can be predicted that these patients are heterozygous for the mutation. Analysis of exon 21 showed an abnormal pattern for patient M.H. When compared with DNA from others individuals M.H. showed an additional faster moving band below the two normal pattern bands (Figure 10). The results clearly indicate that only M.H. contains the mutated allele from exon 21.

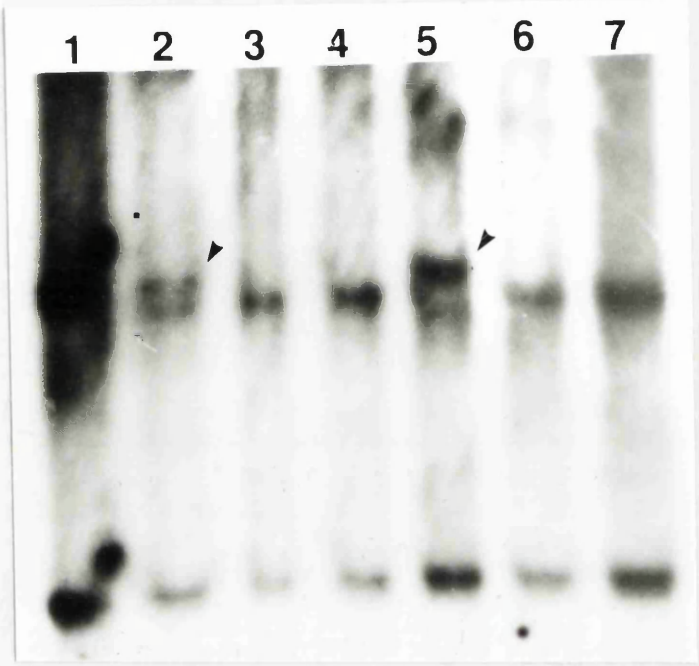


Figure 8: SSCP analysis of exons 15-16 of the RB gene from patients with bilateral retinoblastoma. A 285-bp fragment was amplified with the primers 5'-TAA GGT TTC AAT TAA ACA AC-3' and 5'-CTT TAA ATT GAA CAA AAG TG-3'. PCR products were run on a 0.5XMDE gel (AT Biochem.) containing 5% glycerol at 8 W at room temperature for 17 hours. Lane 2: DNA from patient AR showed an abnormal SSCP banding pattern, Lane 5: DNA from patient GM showed an abnormal SSCP banding pattern when compared with DNA from other patients, Lanes 1, 3, 4, 6, 7: DNA from other patients showed no conformation polymorphism.

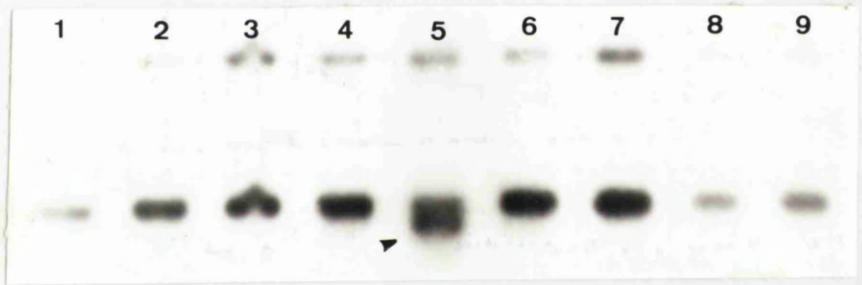


Figure 9: SSCP analysis of exon 20 and the flanking intronic region of the RB gene from patients with bilateral retinoblastoma. A 295-bp fragment was amplified with the primers 5'-AGA GGT TTC TGT TAA AAT GC-3' and 5'-AGAAGG TGA AGT GCT TGA TT-3'. PCR products were run on a 0.5XMDE gel containing 5% glycerol at 8 W room temperature for 16 hours. DNA from the patient EAS in lane 5 shows an additional band when compared with DNA from other patients.

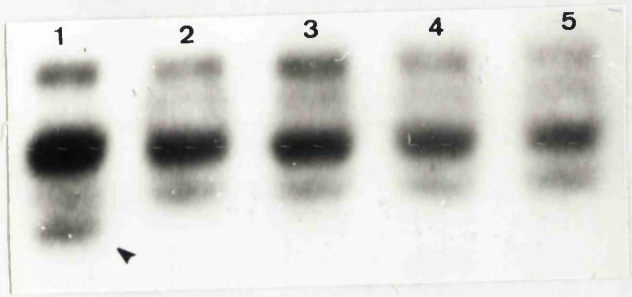


Figure 10: SSCP analysis of exon 21 of the RB gene from patients with bilateral retinoblastoma. The DNAs were amplified with the primers 5'-ATT CTG ACT ACT TTT ACA TC-3' and 5'-ATG AGA TCA AAT GAA TTA CC-3'. PCR products, 216-bp in length, were analysed on a 0.5X MDE gel containing 5% glycerol and run for 14 hours. DNA from patient MH in lane 1 shows an additional band when compared with DNA from other patients.

4.4 SSCP analysis of patients with bladder carcinoma.

To determine the efficiency of SSCP technique in bladder carcinoma, DNA extracted from tumour tissue of 40 patients with bladder carcinomas were screened for point mutations. 30 cycles of PCR were performed on the target DNA sequences using 10 ng of genomic DNA in 15 µl reaction solution. 5 pmol ^{32}P dCTP (60 µCi) was included in the reaction mixture to label the PCR products. After thermal cycling, the amplified product was diluted 1:7 in gel loading buffer. 2 µl of the samples were loaded on to 6.5% non-denaturing MDE gels (20cmx40cmx0.4cm) containing 5% glycerol. Electrophoresis was carried out at 5 W for 14-24 hours. Exon 12-22 and flanking intron sequences of the RB gene were analysed in all cases. 39 out of 40 samples analysed for 11 exons and flanking intron sequence showed no shift in the electrophoretic mobility, indicating no structural alterations including point mutations. The only exception was sample no.32 which showed a shift in the electrophoretic mobility (Figure 11).

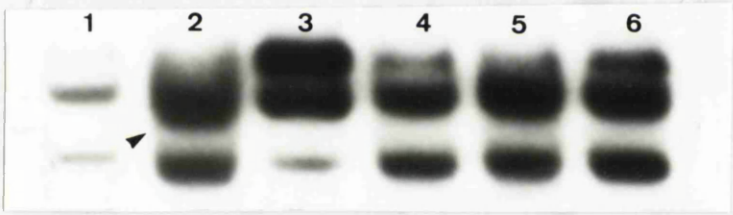


Figure 11: SSCP analysis of exon 12 of the RB gene from patients with Bladder carcinoma. A 295-bp fragment was amplified with primers 5'-GAT ACA TTT AAC TTG GGA GA-3 and 5'-CAT GTT AGA TAG GAG ATT AG-3'. 3 µl of each PCR products was run on a 0.5XMDE gel containing 5% glycerol for 16 hr. DNA from a patient no. 32 (lane 2) shows an abnormal SSCP pattern when compared with DNA from other patients.

4.5 SSCP analysis of patients with breast carcinoma.

The 11 regions amplified by the primers listed on Table 3 were analysed for sequence variations by means of single strand conformation polymorphism analysis (SSCP). A systematic screening was performed using genomic DNA as the template for the PCR reaction from thirty-nine breast tumours by SSCP. A 30 cycle PCR was performed on the target DNA sequences using 10 ng of genomic DNA in 15 µl reaction solution. 5 pmol ^{32}P dCTP (60 µCi) was included in the reaction mixture to label the PCR products. After thermal cycling, the amplified product was diluted 1:7 in gel loading buffer. The sample was heated at 95°C for 2 minutes to denature the DNA. The denatured DNA was then placed directly into ice bath for several minutes. Then, 2 µl of the sample was loaded on to 6.5% non-denaturing MDE gel (20cmx40cmx0.4cm) containing 5% glycerol. Electrophoresis was carried out at 5 W for 14-24 hours. The RB gene from 39 breast carcinoma was analysed exon-by-exon by the PCR-SSCP technique. No unusual SSCP pattern was found in any of the tumours indicating no structural alterations or point mutations detected.

4.6 Heteroduplex analysis of the family of patient MH.

In the current study a modified heteroduplex detection protocol using MDE gel electrophoresis was used to evaluate its efficiency in the detection of a point mutation in the RB gene. The heteroduplex technique was applied to PCR-amplified exon 21-containing DNA from the patient showing a positive SSCP result which was found to be a G insertion by sequence analysis. To confirm whether the observed SSCP pattern in the region of exon 21 from patient MH with Rb was a new mutation or inherited from one of the parents, heteroduplex analysis was also performed on parents DNA.

This technique takes advantage of the formation of heteroduplexes in the PCR between different alleles from heterozygous individuals. These heteroduplexes can be detected on non-denaturing gels because they migrate slower than their corresponding homoduplexes due to a more "open" double-stranded configuration surrounding the mismatched bases. Heteroduplexes containing a single base deletion or insertion are easier to detect than single base substitutions. The reason for this a most likely because a larger opening is created by the absence or addition of a nucleotide on one strand than by two mismatched bases opposing on another (White et al., 1992).

After the final thermal cycle was completed, the reaction was inactivated by adding EDTA to 5 mM. Amplified DNA fragments were heated to 95^{o}C for 3 minutes and then allowed to cool down to room temperature over a period of 30 minutes to form heteroduplexes (Keen et al., 1991). Since Rb is an autosomal dominant disease, heteroduplex formation will occur by PCR amplification in the presence of mutations. 1 kb ladder as a size marker was also included on the gel as well as control homoduplex DNA. Approximately 100-200 ng of DNA was loaded in each lane. Before loading the samples, 1 µl of gel loading buffer (described in the methodology)

for each sample was added and mixed thoroughly. The electrophoresis was performed at a maximum constant voltage of 800 Volts for 15 hr. The gel was then stained with ethidium bromide to visualise the bands on the gel without using any radioactivity. Typically the heteroduplex band migrated slower than the homoduplex. Analysis of the family with this technique showed that only the patient MH (the son) has the mutation but not the parents as shown in Figure 12, indicating either the parents are not carriers or one of the parents has gonadal mosaicism.. In order to evaluate its efficiency, this region from all family members was screened by direct sequencing in the presence of the patient as a control. No other member of the family showed the mutation. Screening the family for this known mutation using this technique was found to be very fast, easy and reliable.

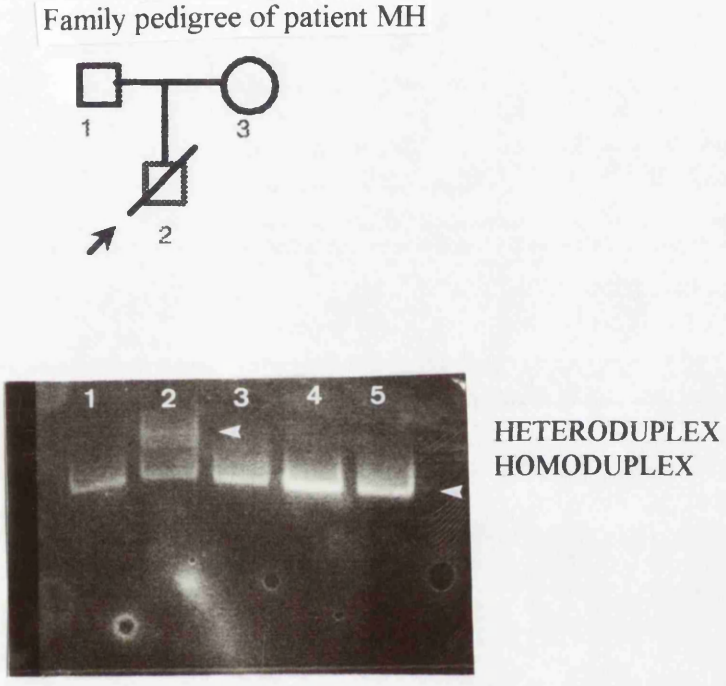


Figure 12: Result of the heteroduplex technique applied to PCR-amplified exon 21-containing DNA from MH showing a positive SSCP result and his parents. The slower moving heteroduplex DNA in lane 2 stains with ethidium bromide to about 1/3 of the intensity of the faster moving homoduplex DNA. The MDE gel was run at about 800 Volts for 16 hours. Lane 1: father; lane 2: patient MH; lane 3: mother; lanes 4 and 5: homoduplex normal controls.

4.7 RNA extraction and quantitation.

RNA was extracted from lymphoblastoid cell lines and lymphocytes in all patients and controls. The extraction was performed by the guanidium thiocyanate method (Chomczynski and Nicoletta, 1987). The basic principle of RNA extraction is very similar to that for DNA but the methods used are different in many specific aspects. In general, the cells are lysed, the cell lysate de-proteinized and the RNA separated from DNA. However, RNA is much more liable than DNA and more susceptible to nuclease degradation. Furthermore, RNases are much more resistant to protein denaturants than DNases. These factors make the isolation of undegraded RNA relatively difficult. Therefore, in order to obtain good quality RNA, it is necessary to lyse the cells and inactivate RNases simultaneously. Guanidium thiocyanate is among the most effective protein denaturants, thus disintegrating cellular structures causing nucleoproteins to dissociate from nucleic acids as the protein secondary structure lost. As a strong inhibitor of ribonucleases, guanidium thiocyanate as well as β-mercaptoethanol were included in the primary extraction buffer to protect the RNA from degradation by RNase (Chirgwin et al., 1979). Since there is a significant amount of RNase on the skin, gloves were worn during isolation of RNA. Whenever possible all water used was treated with DEPC and RNase inhibitors was used in the extraction solutions, the subsequent steps in the experiments, and for short-time storage of RNA in aqueous solution.

This extraction method was found to be satisfactory and convenient. Usually the extraction was performed in a day. The concentration of RNA ranged from 300-1000 μg/ml with a mean of 700 μg/ml. RNA quantitation was carried out by spectrophotometry. The optical density (OD) of the RNA was determined both at 260 and 280 nm. The purity as determined by the mean value of OD_{260}/OD_{280} was 2 indicating it was free of protein contamination. The preparations were run on RNA

checking gels with formaldehyde to determine the integrity of the RNA. The RNA was denatured by heating in the presence of formamide and formaldehyde in order to disrupt aggregates and hydrogen bonded structures. After running the samples, the gels were soaked in water for 1 hour to wash out the formaldehyde for better resolution of the rRNA bands. Undegraded RNA typically shows a 2:1 ratio of 28S:18S. Results of fractionation of the RNA preparations are shown in Figure 13. The RNA samples were found to be ideal. The extraction provided high yield and the extracted RNA was both pure and undegraded. Due to its simplicity and elimination of ultracentrifugation, the guanidium thiocyanate method allows simultaneous processing of a large number of samples. The method proved to be useful for RNA isolation from as few as 10^6 cells or 3 mg of tissue. The degradation and loss of RNA was minimised by the limited handling involved in the technique.

The RNA was divided into two aliquots. To one tube, 0.1 volume of 2 M potassium acetate and 2.5 volumes of ethanol were added, the mixture was then stored at -70°C until required. To recover the RNA the mixture was centrifuged at 13000 g for 10 min and washed in 75% ethanol to remove the salt and the pellet was then dissolved in TE buffer. The other aliquot was kept at -20°C to be used for RT-PCR. Repeated freeze and thaw cycles were avoided. The amplified RB cDNA was also stored at -20°C until required in the second round of amplification.

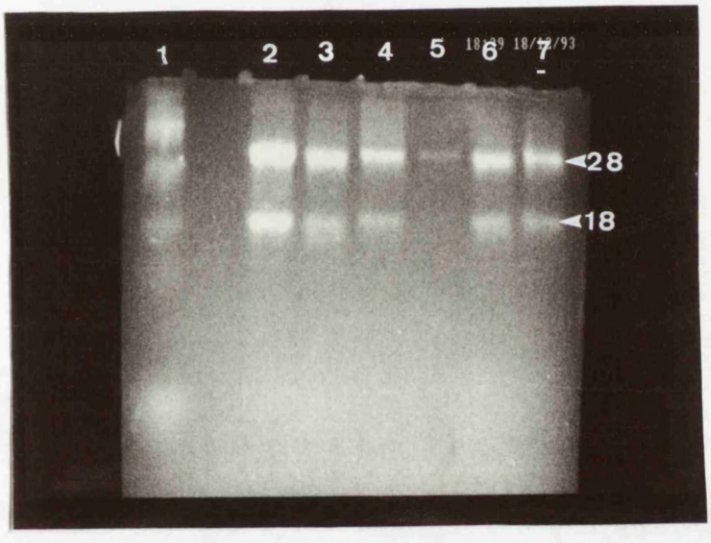


Figure 13: RNA electrophoresis of Rb patients and normal control. Lane 1: 1 μg of *Esherichia coli* ribosomal RNA used as a marker. The other lanes are RNA samples from patients and control representing 28S rRNA, 18S rRNA.

4.8 RT-PCR of the retinoblastoma gene mRNA.

Total cellular RNA from lymphoblastoid cells of patients with bilateral Rb was used to synthesise cDNA using oligonucleotide primers with nucleotide sequences complementary to various regions of RB mRNA. The single-stranded cDNA fragments carrying portions of the RB mRNA thus obtained were then amplified by the PCR to double-stranded DNA products using sets of two oligonucleotide primers, one of which was the same as that used for reverse transcription. The regions thus amplified, designated as fragments A, B, C, D, and E, with nucleotide lengths of 302 bp to 1433 b, are shown in Figure 5. These 5 fragments overlapping each other and almost cover the whole coding sequence of the RB cDNA. Approximately 3938 bp were amplified, then subjected to AMD analysis. Oligo^{TM} (MEDPROBE) computer program was used to design primers set to amplify cDNA fragments. Even with this guideline, the construction of successful PCR primers can be empirical, and more than one primer set needed to be tested before a good combination was found. As a result, several sets of primers were designed for some segments before a single band was seen on an agarose gels (Figure 14). Details of annealing temperature of the primers are given in Table 6.

Amplification of segment C of the cDNA of patients with bilateral Rb has revealed that one of the patients (namely EAS) has an truncated transcript, as shown in Figure 15, indicating either a deletion or a splice mutation.

Figure 14: Amplification of segments of RB cDNA.

Lane 1: 1 kb ladder. Lane 2: RT-PCR amplification with primers A5 and A3. Lane 3 RT-PCR amplification with primers B5 and B3. Lane 3: RT-PCR amplification with primers C5 and C3. Lane 4: RT-PCR amplification with primers D5 and D3. Lane 5: RT-PCR amplification with primers E5 and E3.

Segment of cDNA of the RB Gene	Size of amplified product (bp)	Temperature of PCR rx (^{o}C)
A	302	53
B	400	50
C	707	52
D	1433	53
E	1370	53

Table 6 Size of amplified segments of cDNA of the RB gene and their annealing temperature.

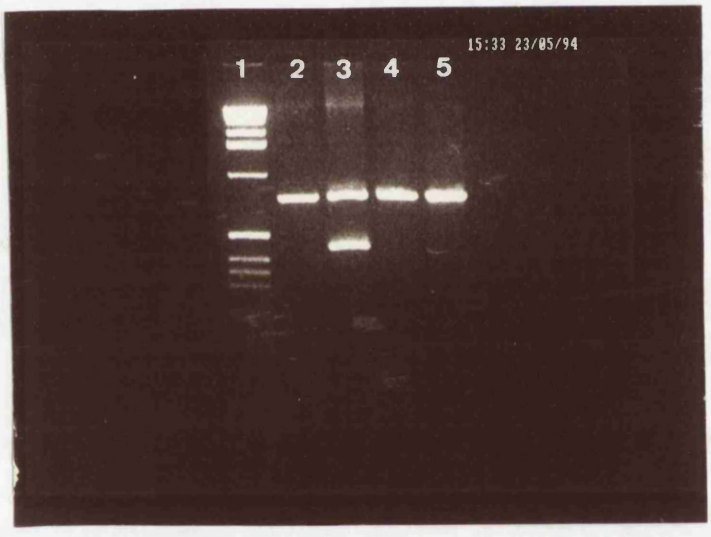


Figure 15: Amplification of C segments from patients with bilateral retinoblastoma Lane: 1 kb ladder. Lane :2 patient with normal size of 707 bp C segment. Lane 3: patient EAS with 707 bp and 511 bp product Lane 4: patient with normal size of 707 bp C segment. Lane 5: patient with normal size of 707 bp C segment.

4.9 Mutation detection using amplification and mismatch detection (AMD) analysis.

AMD detects all classes of point mutations (Cotton et al., 1988). AMD analysis was used to screen all cDNA fragments (A, B, C, D, E) from patients with Rb, as well as 12-16 exons and the flanking intron regions. PCR-generated probes can be prepared by incorporating labelled dNTP either during PCR, or after amplification in a separate labelling reaction (as described in the methodology section). When comparing these two methods, the latter approach can be preferred for several reasons. Synthesis of probes with high-specific activities it is necessary to keep the concentration of unlabelled counterparts of the ^{32}P dNTP as low as possible. However, PCR amplification becomes inefficient when any of the dNTPs are in low concentration. Generation of high specific activity probes labelled during PCR amplification required the use of a full 250 μCi of labelled dNTP in a single PCR reaction to achieve an adequate concentration of all four dNTPs. This is generally undesirable due to cost. In contrast, one standard PCR amplification can provide nearly 0.5 to 1 μg of cDNA fragment, more than enough material for numerous post-PCR probe preparations using 50-100 ng of cDNA with only 50 μCi of labelled dNTP per probe. A critical step in preparing high specific activity probes by PCR amplification was the removal of unincorporated dNTPs from the PCR reaction. For this purpose, the geneclean method was found to be effective and rapid.

In the current study, the use of PCR amplification in the presence of suitable end-labelled primers yielded substrates for chemical cleavage reactions was found to be more efficient than using internally-labelled probes. In order to prepare 5' end-labelled probe, PCR reaction was carried out in the presence of two end-labelled primers as described before. The advantage of this method is that one of the strands of the wild-type cDNA can be labelled by the incorporation of radiolabelled oligonucleotide

primer during the PCR reaction: either the sense or the antisense strand can be labelled. This strategy can be used to locate the position of the cleavage. After amplification of the probe by 5' end-labelled primers, the product was purified by Geneclean. Reference DNA or cDNA probe was mixed with excess test DNA or cDNA in ratio of 1/10; the mixture was then melted and then cooled to allow reannealing and thus heteroduplex formation with mismatched or unmatched base pairs at the position of the mutation. The probe is modified at mismatched C and T bases by reaction with hydroxylamine and osmium tetroxide, respectively, and subsequently cleaved by piperidine treatment. Fragments are sized on gels to locate the point of cleavage and hence, the mutation. In the cases of point mutations, mismatched G and A will not be directly detected, but they are transposed to mismatched C and T bases, respectively, by the use of probes of the opposite sense for detection. However, matched bases adjacent or close to mismatched or unmismatched bases become reactive by transmission of the distortion (Cotton et al., 1988; 1989), and can signal the presence of the mutation and hence allow indirect detection. This allows detection of insertions (Cotton et al., 1989). Unmatched C and T bases are also reactive, allowing detection of deletions. It should be emphasised that this is a screening method developed to avoid the need for sequencing kilobases of DNA to detect a single mutation. Once the site of the mutation is detected, only a small portion of the mutated gene needs to be sequenced. For subsequent detection in individuals, families or populations, one of a series of simple mutation detection methods, such as oligonucleotide hybridisation can be used.

Because all classes of C and T mismatches (C.C, C.T, C.A, T.T, T.G, and T.C) are cleaved (Cotton et al., 1988), complete screening of double-stranded target for point mutations can be achieved using probes of both strands. Deletions will be detected by a cleavage of mutation of unmatched C and T bases or indirectly because of reactive bases nearby. Insertions will be detected indirectly by the increased reactivity of

nearby matched C and T bases in the probe next to or near the loop of the unlabelled DNA in the heteroduplex (Cotton et al., 1991).

The technique has two modes of use with either uniformly labelled probe or end-labelled probe. Either mode can be used when the variation expected is minimal, such as one mutation in the region covered by a probe. However, if multiple differences are expected, e.g., 1 base in 10 is likely to vary, an end-labelled probe will generated a single and unique band for each reactive C or T in the probe, thus generating a pattern of difference between the pieces of nucleic acid. When using end-labelled probe, the low reactivity of all matched C and T bases with hydroxylamine and osmium tetroxide, respectively, offers a background C and T track to help locate the mismatch.

When screening kilobase lengths, probes need to be overlapped by 20-30 bases to avoid missing mismatches at the overlap. This is because of considerable breathing of the duplex at each end (Cotton et al., 1989). Pilot work needs to be done on the time needed for analysis of particular quantities of unlabelled DNA/DNA probe. If most of the probe remains uncleaved, there has been too little reaction, and if it is all cleaved, there may be too much reaction. This is because matched bases are modified and cleaved at a rate of about 1/100 of that of mismatched bases, allowing nonspecific probe destruction if the incubation time is too long (Cotton et al., 1991). Formation of a heteroduplex with a known mismatch should be included as a control that will be cleaved if in fact that heteroduplex has been formed. Potency of osmium tetroxide between two laboratories has varied. A range of concentrations should be tried when setting up the method. Solutions should be freshly made each three months before the solution takes on a green hue, although it is still active at this time. Eppendorf tubes darken as a result of the reaction.

Analysis of fragment C from a patient (PC) with Rb showed a hydroxylamine reaction. It is logical that cleavage kinetics should be different when mismatches are present in one or both alleles. Thus, in the case of homozygotes, the probe band should disappear to nothing at a uniform rate, but in the case of heterozygotes, the probe band should rapidly diminish to half intensity and then diminish more slowly and in parallel with the band signalling the mismatch (Cotton, 1991). This situation was observed in the heterozygous mutation. However, a test has recently been developed for testing allele status (Dianzani et al., 1991). This involves making the probe from the test DNA and hybridising it with unlabelled test DNA. If the wild-type allele is present in the test sample, it is heterozygote and cleavage will occur, but not if it is homozygous for the mutation in question. The band at 481 bp (Figure 17) represents direct detection of a mismatched G found in the heteroduplex as a result of a T→G mutation. It is notable that this mutation is heterozygous, since Rb is an autosomal dominant condition. As mentioned above, the kinetics of the band change is typical of a heterozygote.

The fragments of the RB cDNA from patients with bilateral Rb and some of the exons amplified from genomic DNA were subjected to AMD analysis. In order to determine point mutations by the mismatch technique, PCR amplified wild type DNA or cDNA was either end-labelled or internally-labelled and annealed to cold target DNA or cDNA. The resulting heteroduplex was treated with either hydroxylamine or osmium tetroxide and treated with piperidine. The samples were then subjected to electrophoresis.

4.9.1 AMD analysis of cDNA fragments

Since the coding region is too large (4.7 kb) to routinely sequence in its entirety, in order to determine the mutations, AMD technique was applied to scan PCR-

amplified cDNA from RB mRNA for mismatches relative to the wild-type molecule. Before its application on clinical cases. AMD analysis of PCR amplified products was first performed on a panel of 14 patients with acute intermittent porphyria (AIP) to determine the efficiency of this method for mutation screening and to evaluate its potential for accurate carrier screening. Those patients had been previously studied and their molecular pathologies were described by Mgone et al. (1992). AIP is an autosomal disorder caused by partial deficiency of the enzyme porphobilinogen deaminase (PBG-D) (Mgone. 1991). Analysis of these known mutations was necessary to establish, develop and optimise the various procedures used in the detection of mutations. Initially the study concentrated on AMD analysis of cDNA templates. The patients included in this study were known to carry the following mutations: 1. Two patients with G(606)→T (V202V); 2. G(135)→A; 3. ΔCT(730-731); 4. C(100)→A (Q34K) and G(606)→A; 5. C(100) →A; 6. C(610)→T(Stand) (unpublished data); 7. G(848)→A (Kennedy) (unpublished data); 8. T(761)→C (Bossdow) (unpublished data); 9. G(754)→A (Roberts) (unpublished data); 10. G(789)→A (Boubou) (unpublished data); 11. G(1002)→A (Olifant) (unpublished data).

Approximately 1.1 kb of cDNA from the PBG-D gene was synthesised and amplified by RT-PCR as described (Mgone, 1991) and then Geneclean used to purify the PCR products. These purified PCR products served as the unlabelled test cDNA to be used in the preparation of heteroduplexes. Control cDNA was labelled at the 5' end using 25 pM of each primer in a PCR reaction solution and used as a probe. It was purified using the Geneclean. 10 ng of labelled probe and 100 ng of each test cDNA was mixed in a 1 X annealing buffer, boiled for 5 minutes and incubated at 65^{o}C overnight. After precipitation of the heteroduplexes, they were treated with osmium tetroxide and hydroxylamine as described in the methodology section. 50 µl of 10% piperidine were added to each

tube which was immediately closed and kept on ice to prevent evaporation of the piperidine. The tubes were heated at 90°C for 30 min and then precipitated. Finally the pellet was dissolved in 10 µl of loading buffer and loaded onto an 8% denaturing polyacrylamide gel. Analysis of the patients with porphobilinogen deaminase (PBG-D) deficiency by the AMD technique revealed that three of them had heteroduplexes cleaved by hydroxylamine. One cleavage was at the expected position of 754 of the cDNA product and two of them at position 100 (Figure 16).

After optimisation of the technique, AMD analysis was applied for detection of mutations in patients with bilateral retinoblastoma. Analysis of all 5 fragments of cDNA of the RB gene from patients with bilateral Rb revealed no cleavage, indicating no structural alterations or point mutations. The only exception was fragment C. Analysis of fragment C from patient PC showed that the patient had a band represents a cleavage at position of 481 bp of the C fragment (707 bp) after hydroxylamine modification (Figure 17).

In order to compare the efficiency of end-labelling versus internal-labelling of the probes, AMD analysis of wild-type/mutant duplex RT-PCR amplified DNA was performed using either 5'end-labelled or internally labelled probes. Internally labelled probe was derived from an RT-PCR reaction in the presence of 20 pmol ^{32}P dCTP (60 µCi). Patient PC shows a cleavage product at expected position in lane 2 of Figure 18. Comparison of the results obtained are shown in Figures 17 and 18.

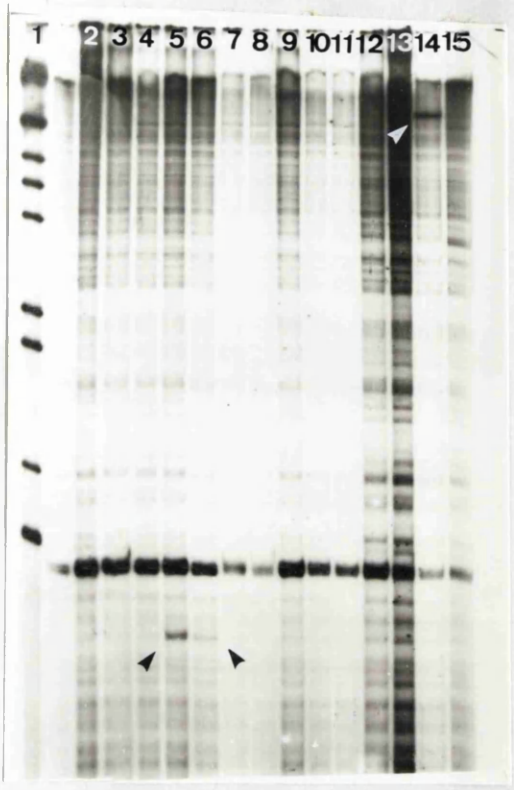


Figure 16: Detection of mutations by AMD analysis of patients with porphobilinogen deaminase (PBG-D) deficiency. Lane 1: Size marker (1 kb ladder). Lane 2: patient with G(606)→T mutation. Lane 3: patient with G(135)→A. Lane 4: patient with ΔCT(730-731) mutation . Lane 5: patient with C(100)→A and G(606)→A mutations shows a cleavage at position of 100 bp. Lane 6: patient with C(100)→A mutation shows a cleavage at position of 100 bp. Lane 7: patient with C(610)→T mutation. Lane 8: patient with unknown mutation. Lane 9: patient with unknown mutation. Lane 10: patient with G(606)→T mutation. Lane 11: patient with G(848)→A mutation. Lane 12: patient with T(761)→C. Lane 13: patient with G(1002)→A mutation shows a cleavage at position of 754 bp. Lane 14: patient with G(754)→A. Lane 15: patient with G(789)→A mutation.

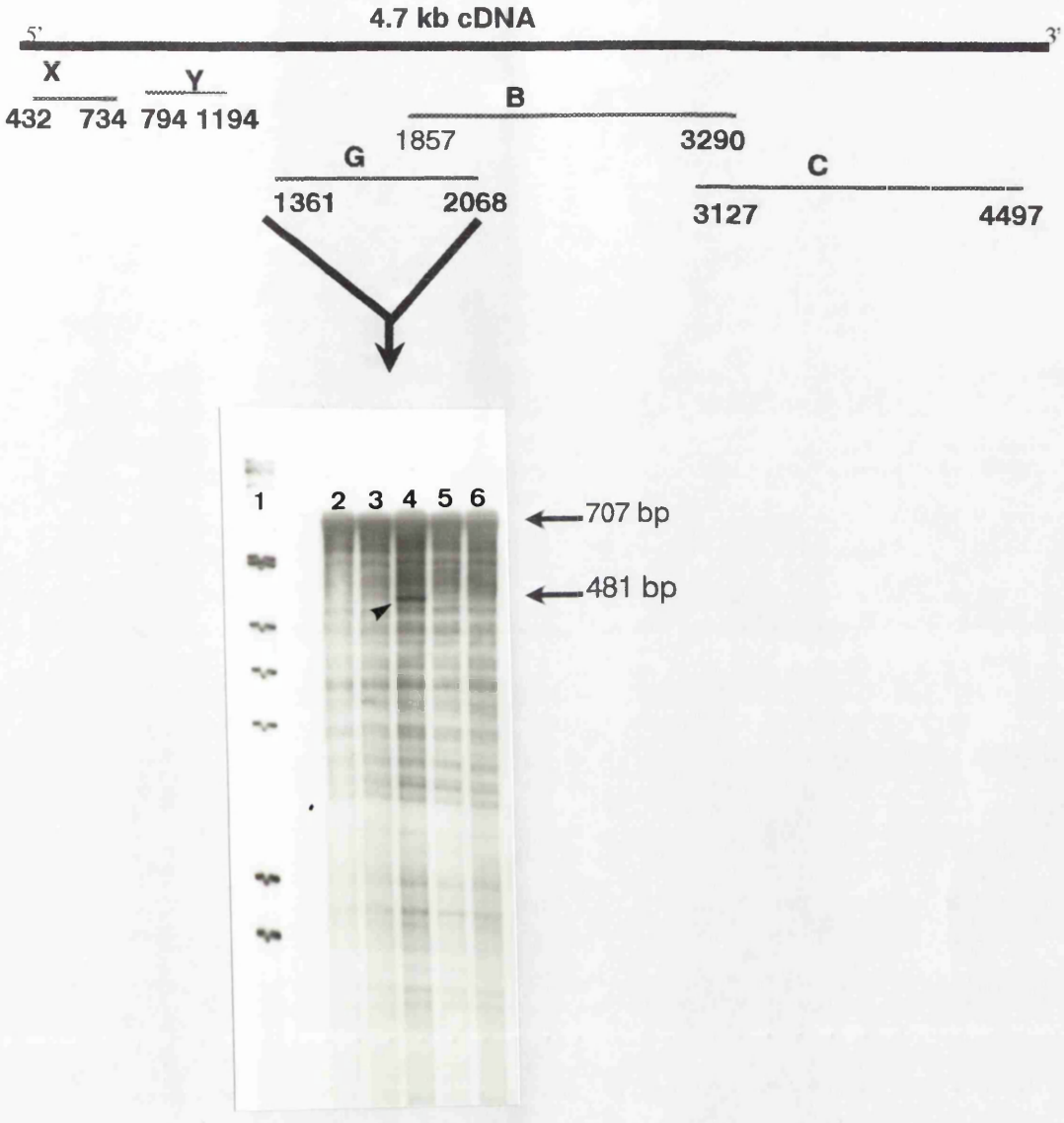


Figure 17: AMD analysis of segment C from patients with retinoblastoma using hydroxylamine reaction with 5'end-labelling.

A schematic representation of cleavage site in the RB cDNA is shown in the upper part of the figure. Lane 1: Size marker (1 kb ladder) Lane 4: patient PC shows a band represents a cleavage at 481 bp, Lanes 2, 3, 5 and 6 show no cleavage.

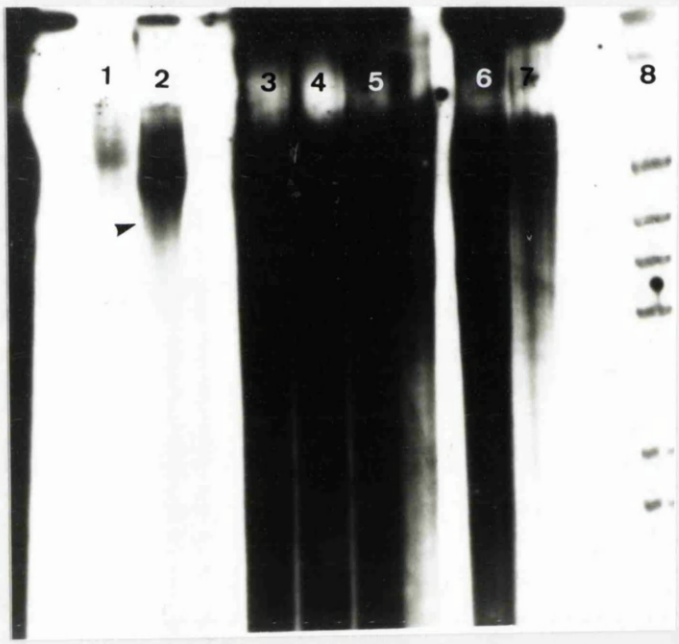


Figure 18. Hydroxylamine reaction with internal labelling of theC fragment of RB gene. Lane 2: cleavage product present at the expected position of 481 bp, Lanes 1, 3, 5, 6, 7, show no cleavage. Lane 8: Size marker (1 kb ladder) (not technique of choice).

4.9.2 AMD analysis of DNA fragments.

Since not all mutations occur within the coding sequence, AMD analysis was used in order to screen for splice mutations and other mutations in the RB gene in patients with bilateral Rb by analysing the DNA exon by exon. Exons 12 to 16 including the flanking intronic region (Table 3) of the RB gene were screened for sequence changes with AMD analysis. Systematic screening was performed using DNA as a template in 20 patients. PCR amplified wild type DNA was end-labelled and annealed to cold target DNA using the method described in methodology. The resulting heteroduplex was treated with either hydroxylamine or osmium tetroxide and treated with piperidine. The samples were then subjected to electrophoresis. Analysis of DNA fragments from 20 patients revealed that patient E.A.S showed a cleavage in the hydroxylamine reaction.(Figure 19)

Figure 19: AMD analysis of exon 17 and the flanking intronic region from patients with retinoblastoma by hydroxylamine reaction with 5'end-labelled probe. A 300-bp fragment was amplified with the primers shown in Table 3 and subjected to AMD analysis. Lane 1: Size marker (1 kb ladder) Lane 15: (patient EAS) cleavage product present at 245 bp, other lanes show no cleavage.

	Mutation	Sequence	Location of mutation	Restriction enzyme
1	A→C transversion	wt GAAGgattt mt GAAGgcttt	-2 of acceptor site of intron 16	CvijI
2	T→G transversion	wt TTTCATATG mt TTTCAGATG	nucleotide position 1587 within exon 16	*Nde*I
3	1bp insertion	wt TGTAG *TATT mt TGTAGGTATT	nucleotide position 2251 within exon 21	*Fok*I
4	A→C transversion	wt aatgACGA mt aatgCCGA	nucleotide position 1636 in exon 16	*Cvij*I
5	A→G transition	wt ttttaacgag mt ttttagcgag	intron 19	*Tsp* 509 I
6	T→C transition	wt AATGTTGGA mt AATGCTGGA	nucleotide position 1617 in exon 16	None
7	G→C tranversion	wt gaatgCAATT mt gaatcCAATT	+1 of the donor site of intron 12	*Mae*II, *Bpu*101, *Dde*I

Table 7: 1-6; Mutations of the RB gene, detected in retinoblastoma patients.

7; Mutation of the RB gene, detected in bladder carcinoma.

(Capitals: exonic, lower case: intronic)

4.10 Sequencing of DNA fragments in which a shift was present in electrophoretic mobility.

Sequencing of DNA fragments in which a shift was present in electrophoretic mobility was performed in order to detect sequence alterations. Direct sequencing of double-stranded PCR products is usually more difficult than single-stranded templates, because artefacts are seen more often in the sequencing of double- rather than single-templates. Templates have a strong tendency to reanneal which is responsible for the high background seen in the sequencing of double-stranded templates (Mgone, 1991). Single-stranded PCR products were generated using an asymmetric PCR amplifying exons of the RB gene. The homogeneity of the asymmetric PCR product was increased by reamplification of 1 μg of the product of a standard PCR. In these experiments, a standard PCR was run on an agarose gel and the gel was stained with ethidium bromide and the appropriate band was then excised. The amplified DNA was eluted by soaking overnight in TE buffer. The amplified product was used directly in asymmetric PCR without further purification. The most common source of contamination is the products of previous PCRs, and the best solution to the problem is extreme caution when handling the PCR reagents. To check for contaminants, a negative control reaction without any DNA template was run in parallel with all PCR reactions.

In asymmetric PCR, the amplification primers were included in a ratio of 50:1 to 100:1, one primer at the usual concentration and one diluted in order to generate an excess of ssDNA complementary to the limiting primer. The best ratio was generally determined by trial and error. Since the efficiency of asymmetric PCR is low when compared with standard PCR, the number of PCR cycles was increased to 40 instead of 30. To overcome the low efficiency of asymmetric PCR, more *Taq* DNA polymerase was added in the late cycles of the PCR.

In order to sequence both strands, two PCR reactions were carried out with reciprocal ratios of the limiting primers. Prior to sequencing, the amplified DNA was purified by removing excess dNTPs, primers and salts because of their interference in sequencing reactions. This purification was done by selective alcohol precipitation in 2 M ammonium acetate (pH 5.2) and 2 volume of propan-2-ol as described in methodology. The presence of excess primers interferes with annealing and extension steps in sequencing reactions. Excess of primers leads to unlabelled primers which compete with radiolabelled primers for binding sites on the templates. Excess of dNTPs can serve as substrates that can interfere with the reaction. Ammonium acetate which used in precipitation works by inhibiting the co-precipitation of dNTPs. It is also necessary to remove salts which interfere with the reactions by inhibiting the sequencing enzyme. Regions of DNA with strong secondary structure may give rise to two problems: first, low efficiency of the PCR, due to the high frequency of templates that are not being fully extended by the *Taq* polymerase, second, compression of the DNA sequences in the sequencing reaction.

One of the major problem in sequencing regions close to the primers is the presence of faint bands. To avoid this problem, two approaches were employed. In the first approach, less nucleotide was used in the labelling step. The labelling mix was diluted in a ratio of 1/10 or 1/20 of the stock reagent and both reaction times kept at 3-5 min as described before. When reading sequences within 20 nucleotides of the 3' end primer, the amount of template DNA and primer concentration was increased. In the second approach, the MN buffer (0.15 M Sodium Isocitrate, 0.1 M $MnCl_2$) provided with the Sequenase Version 2.0 DNA sequencing kit was used. This reagent takes advantage of the activity of the Sequenase enzyme in the presence of Mn^{2+} ions (Tabor and Richardson, 1989). The addition of Mn^{2+} to normal sequencing reactions reduces the average length of DNA synthesised in the termination step, intensifying bands corresponding to sequences close to the primer.

Asymmetric PCR and direct sequencing of fragments showing an altered mobility on PCR-SSCP analysis were performed in all 4 cases: patient AR with Rb, patient GM with Rb, patient MH and patient no.32 with bladder carcinoma. The sequence obtained was compared with the sequence of normal controls and with the published sequence ((McGee et al., 1989). Single-stranded PCR products were generated using an asymmetric polymerase chain reaction amplifying the exons of the RB gene. Results of both asymmetric and standard PCR are shown in Figure 20. PCR products were purified by selective precipitation in 2 M ammonium acetate and propan-2-ol. Sequencing was performed with Sequenase Version 2.0 DNA polymerase using 50% of each purified PCR product and 1 pmol of the limiting primer as a sequencing primer in a 10 µl reaction mixture containing 40 mM Tris.HCI (pH 7.5), 20 mM $MgCI_2$ and 50 mM NaCI and annealed by heating to 65°C for 2 min and allowed to cool down to below 30°C over a period of 20 min. Thereafter the rest of the reactions were carried out as described in the methodology section. The sequencing reactions were stagger loaded and electrophoresed for 3 h. Both the sense and anti-sense strands were generated and sequenced. From these experiments, it emerged that the optimal amount of PCR products to be used in sequencing reactions was 50% of each PCR reaction sequenced with 1 p mol of the sequencing primer.

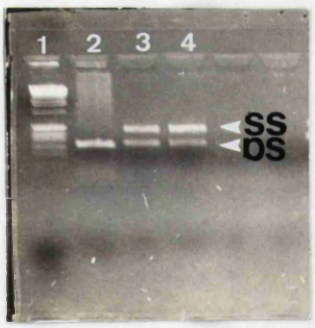


Figure 20: Agarose gel analysis of DNA generated by asymmetric PCR.

Lane 1: 1 kb ladder, Lane 2: double stranded DNA, Lane 3, 4: single and double stranded DNA.

Sequencing of exon 20 and flanking intron regions from patient EAS with bilateral Rb revealed mutation an A→G transition at position 77 towards exon 20 of the 19th intervening sequence (IVS19) (Figure 21). The change abolishes a recognition site for the restriction *Tsp* 509 I enzyme. Although this exon and flanking intronic region was screened by SSCP analysis using samples from 20 patients with Rb, 40 patients with bladder carcinoma and 39 patients with breast carcinoma, analysing of the restriction site recognised by *Tsp* 509 I was also performed for the same samples. In the normal allele *Tsp* 509 I digests the region into 6 segments of 9 bp, 27 bp, 30 bp, 36 bp, 83 bp and 108 bp. In the mutation, the abolition of the recognition site leads to the production of 27 bp, 36 bp, 39 bp, 83 bp, 108 bp. In heterozygosity, carrier will carry mutant products as well as 9 bp and 30 bp products originate from normal allele (Figure 22). Screening was performed by amplifying the exon and flanking intronic region and fractionated the products on agarose gels. Following ethidium bromide staining, the PCR products were visualised under UV. light. No other samples were found to have this mutation. This screening can be performed to help in counselling of affected family members. Using two different approaches for the same change proves that SSCP analysis is an effective method to screen for known mutation. On the other hand, single base substitutions within introns could result in preferential alternative splicing of the mRNA molecules at the site of the mutation. A common form of β thalassaemia that occurs in the Mediterranean population results from a single nucleotide substitution, G→A, at position 110 of the first intervening sequence (IVS1) of the β globin gene. This change produces an AG sequence that happens to be preceded by a stretch of pyrimidines and thus forms a functional 3' acceptor consensus sequence (Weatherall, 1991). The mRNA produced as the result of the abnormal splicing contains intron sequences and is therefore useless as a template for globin chain synthesis. Therefore it was necessary to check the mRNA product including this region from the patient with bilateral Rb. The single-stranded cDNA fragment carrying a portion of the RB mRNA (fragment D shown in Figure 5) was

amplified by PCR to double-stranded DNA products using sets of two oligonucleotide primers, one of which was the same as that used for reverse transcription. This amplification revealed no shorter RNA transcript. Therefore this mutation does not effect splicing of the mRNA and can be considered a silent mutation. In some cases, these so called silent mutation may be responsible for pathology. This especially occurs in highly expressed genes where despite degeneracy of the code, a particular codon may be preferred for a certain amino acid than others. In a such cases, changing one codon to another, even if still codes for same the amino acid, may lead to inefficient incorporation of that particular amino acid residue. While analysing this region by sequencing, two base pair mistakes in the published sequence by McGee et al. (1989) were found. The sequence at positions 78 and 79 towards exon 20 of the IVS19 was AAC(78)GA(79)GATTAA, whereas the published sequence was AAT(78)GT(79)GATTAA (Appendix A). On the other hand, one of these sequence differences could be a polymorphism.

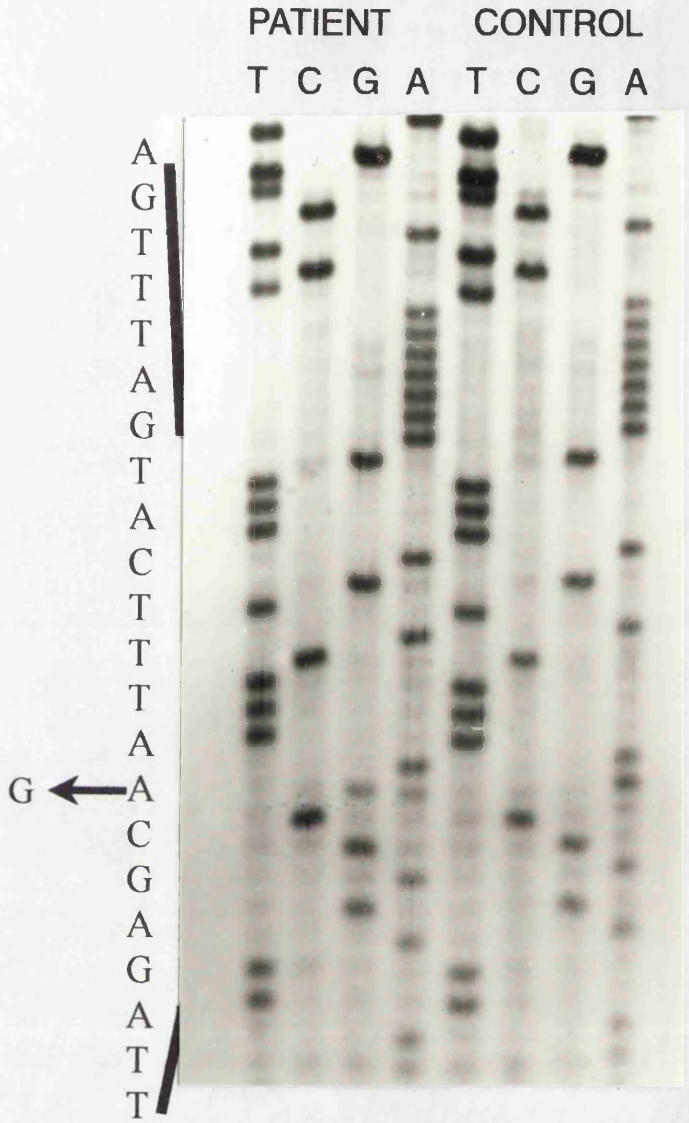


Figure 21: Partial sequence of intron 19 of the RB gene from patient EAS with bilateral retinoblastoma, showing a A→G transition. The wild-type sequence on the right reads, AGAGCAATTTCAT, whereas the mutant is, AGAGCGATTTCAT. The change creates a recognition site for the restriction *Tsp* 509 I enzyme.

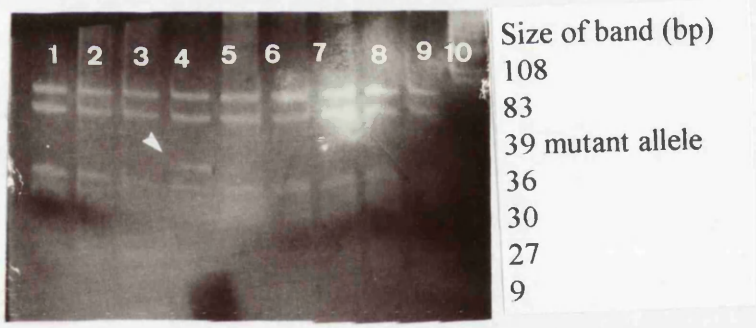


Figure 22: Screening for the recognition site of the restriction enzyme *Tsp* 509 I. Lane 4: A heterozygote patient shows 9 bp, 27 bp, 30 bp 36 bp, 39 bp, 83 bp, 108 bp, Lane 1, 2, 3, 5, 6, 7, 8, 9: show 6 segments of normal allele of 9 bp, 27 bp, 30 bp, 36 bp, 83 bp and 108 bp. Lane 10: 1 kb Ladder.

A $+G_{2251}$(Exon 21) mutation was detected from patient MH with bilateral Rb. Sequencing of the exon and flanking intronic region revealed an insertion of a G at nucleotide position 2251 within exon 21 resulting in a novel stop codon (TAA) at codon 719 (nucleotide position 2295) within exon 21 (Figure 23) thus deleting the domain interacting with the SV40 T antigen. The translated protein is most probably too short to be functional. In order to screen the mutation in the family, the heteroduplex technique was applied to PCR-amplified exon 21-containing DNA from the patient and the parents. Neither of the parents was a carrier indicating that the mutation occurred as a new germinal change (Figure 12). To determine the efficiency of the heteroduplex analysis, the parents were also screened by direct sequencing which showed no change in the region. No other samples from the patients show the same SSCP pattern. On the other hand, the mutation abolishes a restriction site for the restriction enzyme *Fok* I which could be used to screen the population for this mutation.

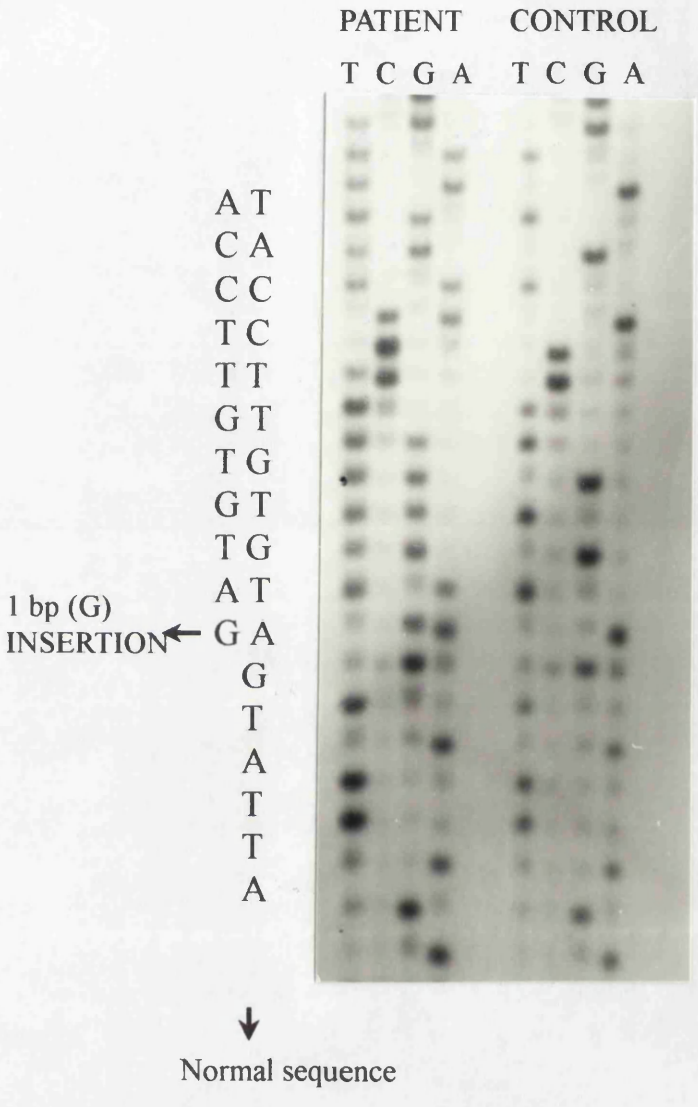


Figure 23: Sequence analysis of exon 21 of patient MH with bilateral retinoblastoma. The mutant sequence arises from an insertion of a G at nucleotide position 2251 resulting in a novel stop codon (TAA) at codon 719. The mutation abolishes a restriction site for the restriction enzyme *Fok*I.

A $R_{507}R(A_{1636}\rightarrow C)$ mutation found from patient AR with bilateral Rb was an A to C transversion at position 1636 in exon 16 causing AGA to CGA codon change (Figure 24), both coding for the same amino acid: Arginine, therefore this mutation does not cause an amino acid substitution. The mutation site is located in the last base of the exon 16, therefore it could affect splicing of the mRNA of RB gene. The location of the mutation lies within an RB domain (aminoacids 393-572), therefore the mutation could be the cause of the disease. SSCP analysis was performed for about 100 patients, none of them had the same change. The mutation abolishes a restriction site for the restriction enzyme *Cvij*I.

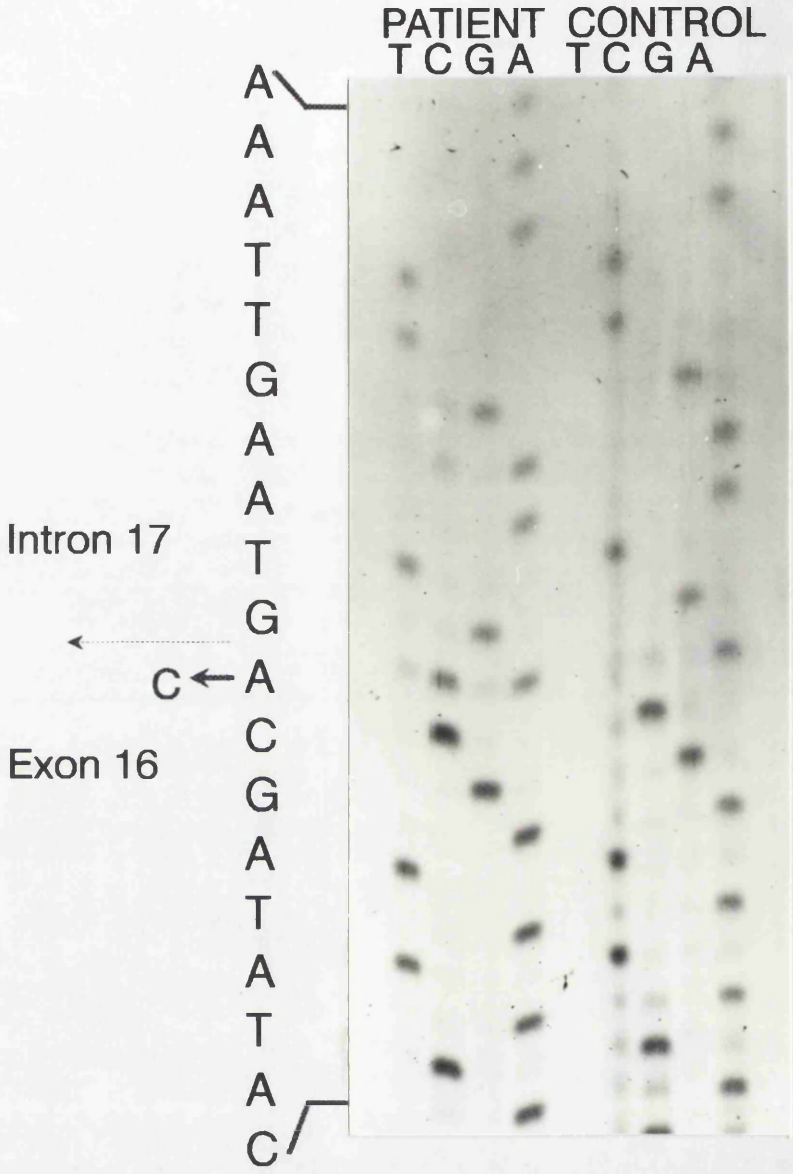


Figure 24: Sequence analysis of exon 16 of the RB gene from patient AR with bilateral retinoblastoma in which a shift in the electrophoretic mobility was detected. The mutation is an A to C transversion at position 1636 in exon 16. The mutation changes the codon AGA to CGA, both coding for the same amino acid: Arginine. The sequence on the right, AGCAGTAA is from a normal control and the one on the left from an Rb patient (AR). The mutant sequence reads, AGCCGTAA. The mutation creates a restriction site for the restriction enzyme *Cvij*I.

$V_{500}\rightarrow V(T_{1617}\rightarrow C)$ caused by a T to C transition in exon 16, changes codon GTT (GUU) to GTC (GUC) both coding for the same amino acid: valine (Figure 25). This mutation does not lead any change or recognition sites for restriction enzymes. Analysis of a cDNA from the patient GM revealed no alteration or rearrangement of the region. Analysis of all patients with SSCP showed none of the patients carry this mutation. Therefore. this mutation is very unlikely to be the cause of Rb. In addition, since the mutation does not alter a recognition site for restriction enzymes, this is not useful in the diagnosis or linkage studies for tracking of this condition in affected family.

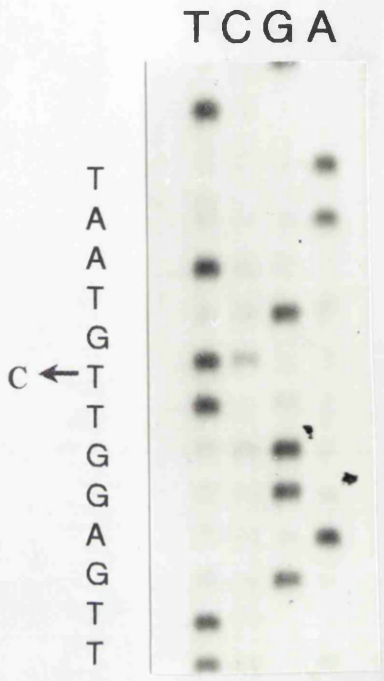


Figure 25: Sequence analysis of exon 16 of the RB gene from patient GM with bilateral retinoblastoma. The mutation is a T to C transition at position 1617 in exon 16. This altered the codon GTT (GUU) to GTC (GUC) both coding for the same amino acid: valine. The mutant sequence reads TTGAGGTCGTAAT. This mutation, which was observed in one individual, does not result in any change, either in the amino acid pattern or recognition sites for restriction enzymes.

The only mutation found from a patient with bladder carcinoma was a G to C transversion at position +1 of the donor site of intron 12 (Figure 26). This mutation represents a somatic mutation in heterozygous form and most probably causes abnormal splicing of the RNA. A second mutation was assumed that it lie in a part of the retinoblastoma gene that was not analysed, since in somatic cases two hits (Figure 1) in the RB gene are expected. On the other hand, the sample was obtained by transurethral resection, it could be contaminated with non tumour tissue resulting in a heterozygote mutation. Intact RNA cannot always be recovered from the type of clinical materials used in this study, therefore the diagnostic strategy for bladder carcinoma was chosen not to be based on the analysis of RNA transcript. In order to determine the role of RB gene in tumourogenesis, 40 DNAs extracted from bladder carcinoma were screened with SSCP analysis. Horowits et al., (1990) found that inactivation of the retinoblastoma protein, $p105^{RB}$, which is universal in retinoblastoma cells, is present in most small cell lung cancers and in one-third of bladder cancers but is infrequent in other tumours. RB (like p53) has been implicated in a tumour-suppressing role in a wide range of tumours including bladder carcinoma (Dunn et al., 1989; Horowitz et al., 1989). Horowitz et al. (1989) reported a splice acceptor mutation at the 5' end of exon 21 from bladder carcinoma cells J82. This mutation resulted in fusion of exon 20 into exon 22. In the current study only 12 exons of the RB gene were analysed. If the entire gene could be analysed, more mutation may be detected. In this individual mutation of RB found may represent the initiating event in tumour development.

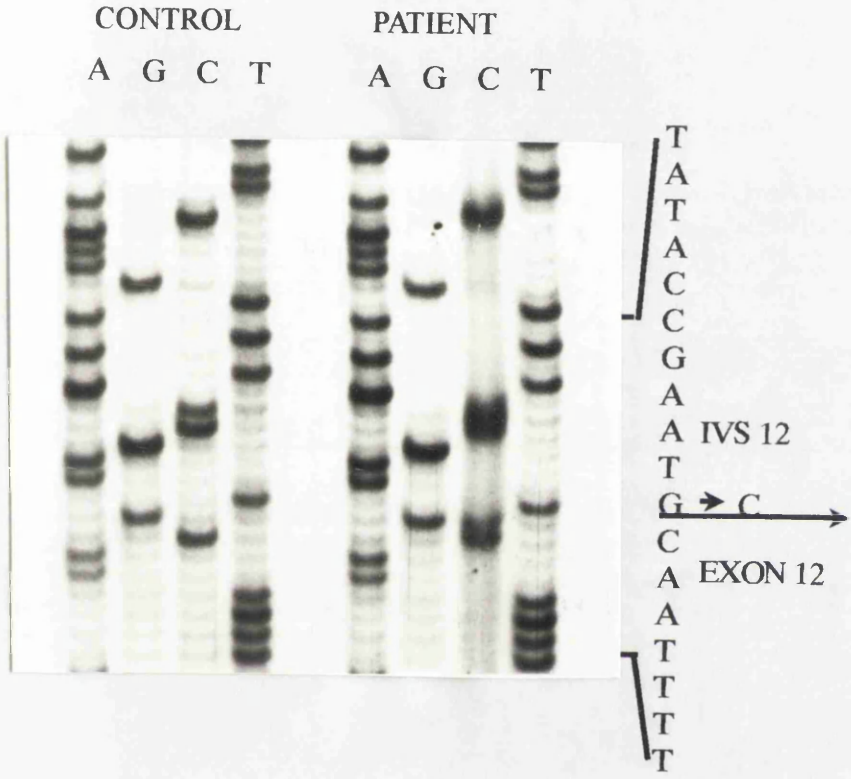


Figure 26: Sequence analysis of exon 12 of the RB gene from a patient with bladder carcinoma. The mutation arises from a G to C transversion at position +1 of the donor site of intron 12. The normal sequence is, TTTTAACGTAAGC whereas the mutant sequence reads, TTTTAACCTAAGC. The mutation abolishes a restriction sites for the *Mae*II, *Bpu*101, *Dde*I restriction enzymes.

4.11 Sequencing of DNA and cDNA fragments in which a cleavage was detected.

RT-PCR was performed with the primers a standard manner to produce double-stranded products. The desired band was then excised and the cDNA eluted and reamplified with the same primers used for RT-PCR. Such eluted cDNA could be stored at -20^{o}C for several months. The cDNA was then reamplified asymmetrically. The adequacy of the single-stranded products was monitored by running on 1% agarose gels as described earlier. It was possible to produce both cDNA strands with equal efficiency. The products were purified by selective alcohol precipitation. Sequencing reactions were performed with Sequenase T7 DNA polymerase with chain termination and extension reactions being carried out at 37^{o}C. Sequencing was carried out with one of the sequencing primers at 1 pmol. The amount of the template cDNA was varied. In some reaction whole purified product was used in a sequencing reaction whilst sometimes half of the product was enough to get result. This was varied for each of the cDNA segments. 40 amplification cycles were carried out. The single-stranded products were subsequently sequenced directly.

The first mutation involved -2 of the acceptor site of intron 16. The mutation was caused by an A→C transversion. One PCR amplification utilising primers C1 and C2 yielded a truncated transcript, indicating a deletion associated with the reading frame (Figure 15). This amplification reaction delineated a deletion in RNA of approximately 65 nucleotides between bases 1361 and 2068. This deletion was more precisely defined by AMD analysis of exons and flanking intron regions of the region. Analysis of exon 17 and the flanking intronic region revealed a cleavage by hydroxylamine modification. This exon, together with the flanking intronic sequence was subjected to detailed sequence analysis. The observed mutation converts a canonical splice acceptor sequence, 5'-TTTTTAG-3', present in the wild-type allele into the sequence 5'-TTTTTCG-3' (Figure 27). This indicated that the short RNA transcript was not due to a deletion. Presumably, the point mutation found in the RB gene abrogates normal splicing at this site. As a consequence, exon 17 is discarded during the biogenesis of the RB mRNA, resulting in the fusion of exon 16 directly to exon 18. Because the short transcript band on the agarose gel was approximately 196 bp shorter than the original band and exon 17 is 196 bp in size. This leads in turn to the production of a truncated RB protein. In contrast to wild-type p105-RB, this aberrant, destabilised protein is unable to bind E1A oncoprotein. In addition, the mutant RB protein may fail to complex with SV40 large T antigen. It is likely that this defective RB protein is also deficient in carrying out its normal function within the cell. No similar mutant allele was recovered when genomic DNAs from 19 unrelated individuals were screened by AMD analysis and sequenced in this region. The mutation abolishes a restriction site for the restriction enzyme *CviJI*.

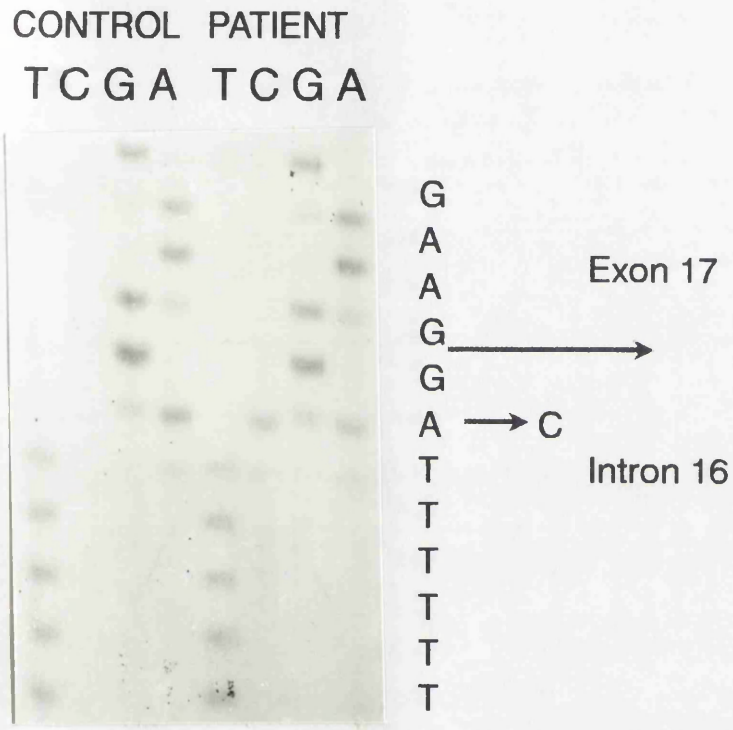


Figure 27 Sequence analysis of exon 17 of the RB gene from patient EAS with bilateral retinoblastoma. The normal sequence reads, GAAGGGATTTTT whilst the mutant reads GAGGCTTTTTT because of a substitution of C for A at position -2 of the acceptor site of intron 16. The change causes the skipping of exon 17. The change creates a restriction sites for the restriction enzyme *Cvij*I.

A second mutation, namely H502→Q (T_{1587}→G) was detected by AMD analysis of segment C of the RB mRNA. The mutation, caused by a T to G transversion at nucleotide 1587 (Figure 28), was seen in one patient with bilateral RB. This changes codon CAU (CAT) to CAG thus substituting histidine for glutamine at amino acid residue 483 (McGee et al., 1989). The missense mutation identified within exon 16 suggests, may or may not have a functional effect.. This residue lies within an RB domain (aminoacids 393-572) identified recently by in vitro deletion mutants to be required for oncoprotein binding (Hu et al., 1990). The His-483→Gln substitution appears to simultaneously abrogate RB phosphorylation and affinity for viral oncoproteins. Since the patient is the only affected member of the family, the substitution has occurred as a new germinal change, not present in the father which was the only parent available to analysis. Analysis of 19 unrelated patients with bilateral RB by AMD analysis showed no other patient carrying this mutation. In addition, this mutation abolishes a recognition site for the *Nde* I restriction enzyme allowing this to be used for screening purposes.

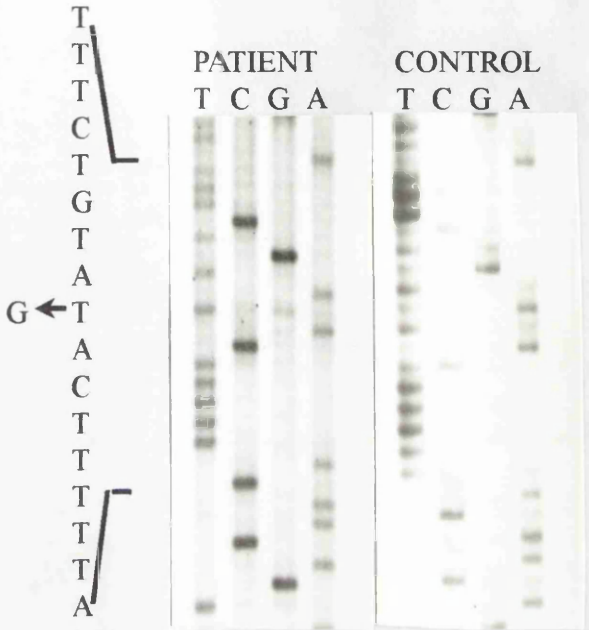


Figure 28 Sequencing of the cDNA fragment of the RB gene of patient PC who has shown a cleavage after hydroxylamine modification. The mutation arises from a T to G transversion at nucleotide position 1587 within exon 16. This changes codon CAU (CAT) to CAG thus substituting conserved amino acid, histidine for glutamine. The mutant sequence, TTTTTCAGATGTCTTT on the left arises from a T to G transversion. This mutation creates a restriction site for the *Nde* I. restriction enzyme.

4.12 Mutations of the RB gene, detected in patients with retinoblastoma and other tumours in the current study.

The techniques of single strand conformational polymorphism (SSCP) analysis, amplified mismatch detection (AMD) analysis and heteroduplex analysis together with direct sequencing were used to detect mutations in 20 patients with retinoblastoma. Analysis revealed six mutations in patients with bilateral RB and one mutation from a patient with bladder carcinoma. Of those patients with RB, one had two different mutations. The six mutations from patients with RB were : a G insertion at nucleotide position 2251, a missense mutation (T to G transversion) at nucleotide position 1587, a splice site mutation (A to C transversion) at position 1636 in exon 16, a splice acceptor site mutation at position -2 of intron 16, an A→G transversion in intron 19 and a T to C transition at position 1617. A G to C transversion at position +1 of the splice donor site of intron 12 was detected in a patient with bladder carcinoma (Table 8).

Patient	Mutation	Resulting Change	The technique used
1. EAS	A→C (-2 acceptor site of IVS16)	truncated RNA transcript, skipping exon 17	RT-PCR, AMD analysis
2. EAS	A_{D77}→G (IVS19)	does not result in any change	SSCP analysis
3. PC	H483→Q (T_{1587}→G)	mutation causing the substitution of histidine to glutamine	AMD analysis
4. MH	+G_{2251} (Exon 21)	frameshift in exon 21 leads to a stop codon at codon 719 (nucleotide 2295)	SSCP analysis
5. AR	R_{500}R (A_{1636}→C)	does not cause any amino acid substitution, affecting splice site	SSCP analysis
6. GM	V_{493}→V (T_{1617}→C)	causes no change in the amino acid pattern	SSCP analysis
7. Bladder no.32	G→C (+1 donor site of IVS12)	affected splice site	SSCP analysis

Table 8. Analysis of the RB structural changes likely to arise from detected mutations and the techniques used.

CHAPTER FIVE: DISCUSSION

5. DISCUSSION.

5.1. Comparison of screening strategies; SSCP and AMD analysis for detection of point mutations in the RB gene .

The present study focused on application of screening strategies in the RB gene in patients with retinoblastoma, bladder carcinoma and breast carcinoma. The techniques of single strand conformational polymorphism (SSCP) analysis, amplified mismatch detection (AMD) analysis were applied for screening mutation on PCR amplified samples of cDNA and DNA from blood samples in 20 patients with retinoblastoma and in tumour genomic DNA from 40 patients with bladder carcinoma and 39 patients with breast carcinoma. In those patients who showed a band shift on SSCP analysis or a cleavage with AMD analysis, direct sequencing of the PCR products was performed. This approach revealed six mutations in patients with bilateral Rb and one mutation in the tumour from a patient with bladder carcinoma.

There are very few comparable published studies. Various methods have been employed for screening large numbers of constitutional mutation carriers in retinoblastoma (Cowell et al., 1992, Horsthemke et al., 1992, Sakai et al., 1991a; Kloss et al., 1991), but no single approach has detected mutations in more than 10-15% of the germ line cases investigated. For instance Blanquet et al. (1993) used denaturant gradient gel electrophoresis (DGGE) to detect mutations in a pool of 120 unrelated retinoblastoma patients including 15 patients with familial form, 65 patients with sporadic bilateral, and 40 patients with unilateral. They characterized 10 sequence alterations. The majority of mutations were found either in tumour tissue or in established tumour cell lines. This could suggest a different mutation rate in germ cells versus tumour cells. No specific region or hot spot has been identified and the mutations seem to vary in both type and location. This lack of any common mutation for the carriers is major drawback for informed genetic counselling.

Janson and Nordenskjold, (1994) used pulsed-field gel electrophoresis and cDNA probes in screening 20 unrelated cases with bilateral Rb. They found one patient with aberrant fragments due to a mutation as well as a normal allele for a number of restriction endonucleases. Cytogenetic analysis revealed a balanced translocation t(4;13)(q21q14).

Published mutations that have been detected using SSCP technique in RB gene include base substitution, small insertions and deletions, and rearrangements (Hogg et al., 1992). This technique has also been applied to the detection of DNA polymorphisms at various other loci of the human genome (Orita et al., 1989). Shimizu et al. (1994) and Kreipe et al. (1993) have also screened the RB gene exon-by-exon using SSCP analysis. These two group found mutations in 14 of 24 and 12 of 12 Rb tumours respectively. PCR-based single-strand conformation polymorphism is a simple and fast method of screening for the presence of sequence changes. However, SSCP does not detect all sequence changes; Sarkar et al. (1992) reported that an average of 83% of single-base changes were detected in a segment of 183 bp, and only 58% were detected for a segment of 307 bp. They found only 35% of the mutations when segments of the factor IX gene ranging from 180-497 bp were screened in a prospective fashion in nine segments. They also described dideoxy fingerprinting (ddF), a method that detected 100% of single-base changes in the human factor IX gene when tested in retrospective and prospective manners. Dideoxy fingerprinting provides information about the location of the sequence change, and the efficiency of detection is independent of the length of the amplified product. In this technique, a ladder of bands is generated by performing one of the four standard dideoxy sequencing reactions and resolving the products by electrophoresis on a nondenaturing polyacrylamide gel. But the efficiency of this technique for RB gene is unknown. Murakami et al. (1991) have used PCR-SSCP analysis in combination of reverse transcriptase reaction for analysis of cDNA fragments of the RB gene

obtained from mRNAs. They found a transcript with a mutated exon 2 sequence, but not a transcript carrying the normal sequence of exon 2 in small cell lung carcinoma (SCLC) cells and confirmed its loss by SSCP analysis of genomic DNA.

The inheritance of a disease causing gene can be determined either indirectly, by studying the segregation of DNA markers closely linked to the mutant allele, or by direct identification of a disease causing mutation carried by individual subject. The linked marker approach is valuable when there is a family history of a disease or when the disease is caused by a small number of mutant alleles present at relatively high frequency in the general population. Generally, a study of linked markers is not helpful when a new mutation has occurred in a previously unaffected family, and hence has only limited application in detecting disorders associated with high rates of new mutation. The results of AMD and SSCP analysis of the presence of a disease-causing mutation are more applicable in the study of disease such as retinoblastoma, which are characterised by high rates of new mutations. Many autosomal dominant and X-linked recessive disorders are most often caused by new mutations and the lesions responsible for these new familial cases may be very heterogeneous. Although RFLP analysis remains the method of choice in studying families predisposed to retinoblastoma that have more than one affected member, the diagnostic strategy used in this project is not dependent on a positive family history and thus can be routinely applied to a disease with no family history. Southern blotting can serve the same purpose, but its application is limited to the 10-20% of all cases of retinoblastoma caused by relatively large DNA rearrangements or deletions in the retinoblastoma gene. Allele-specific oligonucleotide (ASO) hybridisation and restriction fragment length polymorphism (RFLP) can be used to identify known genetic variations over relatively short stretches (one to 20 nucleotides) in the amplification products.

Direct sequencing of either single- or double stranded PCR amplified DNA (or cDNA) fragments is highly accurate method for detection of essentially all genetic variations that may occur within the amplified fragment. With recent improvements, sequences can be read more consistently than before. However, all forms of direct sequencing are labour-intensive per base pair analysed when compared to the other techniques. This approach is especially expensive when it is necessary to analyse large numbers of samples for rare genetic variations. However, direct sequencing remains the method of choice for characterising the variant sequences that are detected by more rapid techniques, such as SSCP analysis (Hogg et al., 1992), AMD analysis (Cotton et al., 1988).,

In the current study, SSCP analysis was applied to screen functionally important RB gene regions. The oncoprotein binding sites of $p110^{RB}$ are known to lie between aminoacids 393-572 and 646-772 which fall in exons 12-22 inclusive. Therefore exons 12 to 22 and flanking intron sequences of the RB gene were screened for sequence change with PCR-SSCP analysis. This technique was carried out to screen for point mutations. All mutations detected by SSCP were confirmed by sequencing. The five mutations detected by SSCP analysis were $A_{D77}\rightarrow G$ (IVS19) from patient EAS, $+G_{2251}$(Exon 21) from patient MH, $R_{507}R(A_{1636}\rightarrow C)$ from patient AR, $V_{500}\rightarrow V(T_{1617}\rightarrow C)$ from patient GM, G→C(+1 donor site of IVS12)(patient with bladder carcinoma). No false-positive results from SSCP analysis were observed although false negatives were not excluded because, as with most methods, the sensitivity is not 100% (Hayashi et al., 1993). The mobility shifts cannot determine either the precise position or the exact nature of the base changes. Nevertheless, SSCP analysis is useful for screening for point mutations as it is simple, fast, and efficient. When mutated cases are identified, other methods such as direct sequencing must be used to determine the base substitution. It is still not clear whether this approach will successfully identify all mutations in the RB gene. However, in addition to the initial

studies on the *ras* oncogenes (Orita et al., 1989), it has been used successfully to detect mutations in the cystic fibrosis gene (Dean et al., 1990) and the p53 gene (Mazars et al., 1991). Multiplex SSCP could provide a quicker way to screen a gene. However, digestion of the samples following co-amplification is not feasible because enzyme sites are often present in an inappropriate position and an extremely complex gel pattern would be generated. Hence, full-length PCR products must be used and sensitivity may be decreased for fragments >200 bp in length (Hogg et al., 1992). Although this approach can be applied only to disease loci for which the intron-exon structure and surrounding DNA sequences are known, the number of monogenic disorders that fail into this category is increasing rapidly (Kidd et al., 1989). A particular advantage of this method is that it does not require the presence of intact RNA transcript or functional protein, and therefore it can be used to examine tumours or other tissues in which no transcripts or gene products are expressed or from which RNA cannot be recovered. This is important because a relatively large fraction of retinoblastoma tumours produce no RNA transcript from the retinoblastoma gene (Friend et al., 1986; Fung et al., 1987; Goddart et al., 1988). In addition, direct sequencing of genomic DNA allows the detection of mutations that may occur at splice sites or other sequences that are excluded from the RNA transcript (Horowitz et al., 1989; Wong et al., 1987; Tromp et al., 1988).

Like all screening techniques mentioned in the introduction section, the detection of mutants by AMD analysis does not discriminate between mutations resulting in amino acid change and silent mutations or natural polymorphism. To answer this questions, direct sequencing of PCR products or of the excised and reamplified mutant band need to be done. As shown by its use for detection of point mutations, both AMD and SSCP analysis are sensitive enough to detect small changes of nucleotide sequence. AMD technique could be used in various kinds of mRNA analyses.

To characterise all types of mutations in the coding sequence of the RB gene, the entire cDNA has to be analysed. In this study AMD analysis was applied to screen for mutations. This required amplification of cDNA and mRNA of the RB gene with at least 5 primer sets to cover almost the entire gene. Although AMD analysis can detect all types of mutations, in the current study only one mutation could be detected by analysing cDNA with this technique. The other mutation (A→C, -2 acceptor site from patient EAS) found with AMD analysis was detected by amplification of an exon and its flanking intron region. Since Southern blot analysis identified structural abnormalities of RB in 10%-40% of the hereditary and sporadic Rb tumours (Cowell and Hogg, 1992) and approximately 10% of mutations affect splice junction sites (Bookstein et al., 1990), the chance of detecting mutations by AMD analysis is less than 100%, unless the segments of cDNA do not contain the beginning of the abnormalities. Two mutations detected by AMD analysis were an A→C (-2 acceptor site of IVS16)(patient EAS), H502→Q (T_{1587}→G)(patient PC). Weir-Thompson et al. (1991) also used AMD analysis and identified a point mutation in the RB gene in a small-cell lung carcinoma. There have been no false positives identified so far using this technique. The main disadvantage of the method is the highly toxic reagents which are dangerous to handle and difficult to dispose of. This technique also proved its efficiency when three mutations were found in 14 AIP patients with known mutations. This strategy, greatly saved time and labour and in addition, was found to be reliable. Furthermore this procedure of AMD analysis of PCR amplified of cDNA can be used as a prototype to investigate the molecular pathology of any other single gene disorder as shown in this study by being applied to demonstrate the mutations in acute intermittent porphyria. PCR-SSCP analysis was found to be a rapid and effective method for detecting point mutations because it was carried out on DNA. Simplicity is the major advantage of the PCR-SSCP analysis. However, in one case a mutation (T→G transversion at nucleotide position 1587 within exon 16) was not found using SSCP analysis which was identified later by AMD analysis. The

advantage of SSCP analysis in screening mutation is that analyse exon by exon and short segments can be analysed. AMD analysis probably more sensitive but slower and uses toxic reagents such as osmium tetroxide, hydroxylamine and piperidine. Strategy for mutation analysis for another disorders would be rapidly screened by SSCP analysis and use AMD analysis in those patients who have no mutation found with SSCP analysis.

5.2. Heterogeneity of molecular pathology in retinoblastoma gene.

At the time (1991) this project was initiated only a few point mutations had been discovered in the RB gene. The RB gene was cloned and mapped in 1987. Although the gene is well characterised, over a period of 7 years nearly 80 mutations including small insertion and deletions have been published (Appendix B) involving all or part of the retinoblastoma gene but, most of this type of work has been done on tumour material. Still very few germline mutations have been characterised.

Of the seven mutations found in this study one was due to a base insertion leading to a frameshift, six were due to single base substitutions. All mutations are novel, emphasising the heterogeneity of this condition. Among the mutations found, three mutations affect splice sites, one of which was detected from a patient with bladder carcinoma. One mutation causes an amino acid substitution. Of those patients with Rb, one had two different mutations which were a C substitution of the A at position -2 of the acceptor site of intron 16 and an A→G transition in intron 19. a G insertion at position 2251 mutation resulting in a novel stop codon (TAA) at codon 719 (nucleotide position 2295) within exon 21 and a T to G transversion at nucleotide position 1587 within exon 16 which results in substitution of conserved amino acid histidine to glutamine. All these are likely to be the cause of Rb in these individuals. Two mutation are considered to be silent mutations.

Deletion of exons 13 to 17 is a frequent observation in various types of tumours, including retinoblastoma, breast cancer, and osteosarcoma (Hong et al., 1989). Based on this observation, Hong (1989) suggested the presence of a potential "hot spot" for recombination in this region. Data (Figure 29) constructed from published mutations in exons 1-27 and flanking intronic region of the RB gene fail to show bias towards mutation in the two regions of suspected oncogene binding site. Uniform heterogeneity of the mutation distribution is clearly demonstrated (Figure 29).

Kaye et al. (1990) found a single point mutation within exon 21 that resulted in a nonconservative amino acid substitution (cysteine to phenylalanine) at codon 706. Stable expression of this mutant RB cDNA in a human cell line lacking endogenous RB demonstrated that this amino acid change was sufficient to inhibit phosphorylation. In addition, this mutation also resulted in loss of RB binding to the SV40 large tumour and adenovirus E1A transforming proteins. Yandell and Dryja (1989) have screened 3,712 bp of genomic DNA from each of nine individuals and found four DNA sequence polymorphism using PCR amplification and direct genomic sequencing. They found all four polymorphisms in introns.

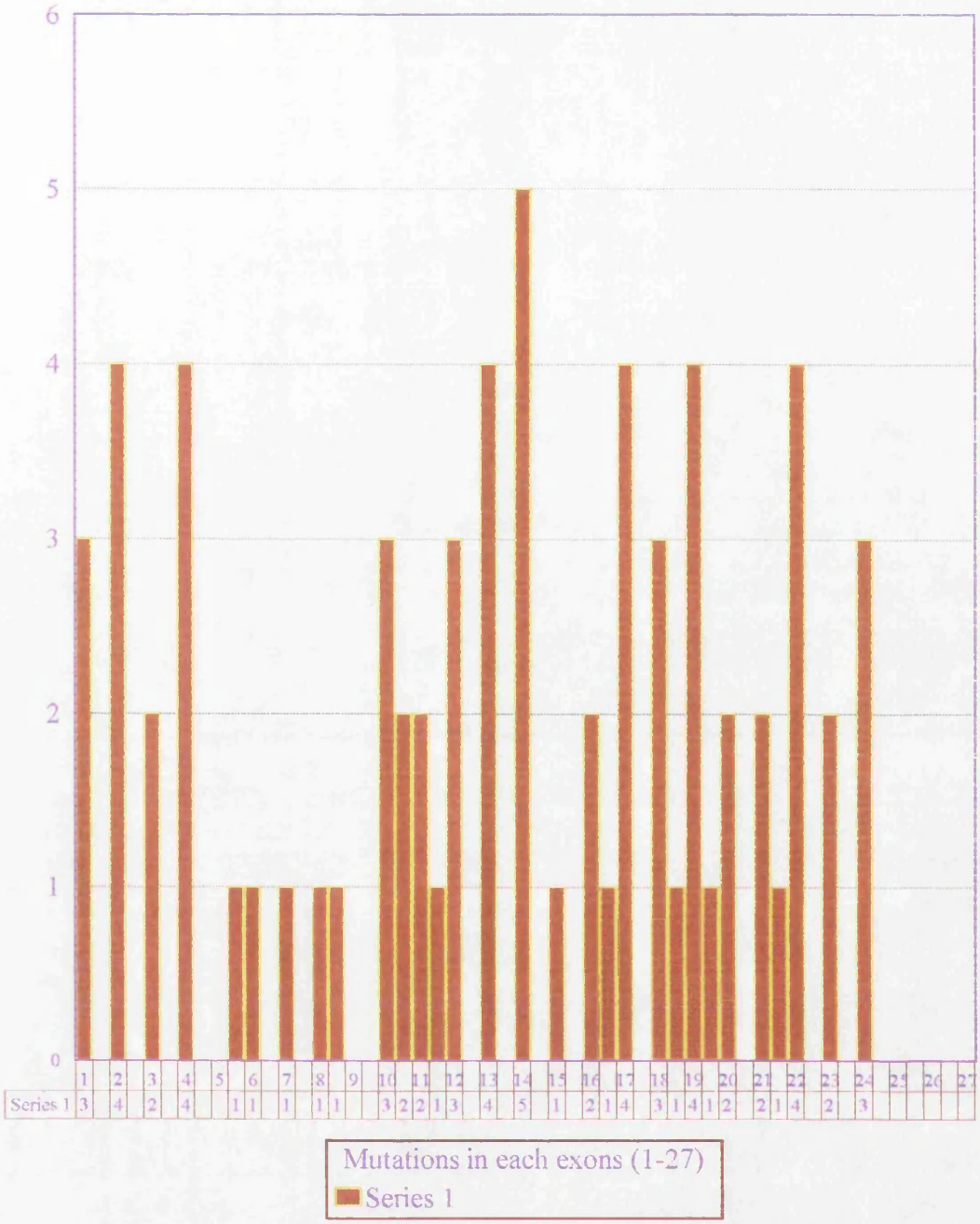


Figure 29. Distribution of germ-line and somatic mutations of the RB gene.

This demonstrates the considerable heterogeneity of the mutation in this gene. Bars represent the number (Series I) of mutations, which have been reported in the literature, in each exon (upperline numbers 1-27) and intron.

Bookstein et al. (1992), analysed mRNA from a prostate carcinoma cell line, identified an in-frame deletion of exon 21, but the splice junction sequences were normal. Mori et al. (1990) showed that the mRNA from an SCLC cell line was missing exon 22. A two base pair mutation GC→TT, within the exon, apparently converted a glutamine to a stop codon, but, in fact, resulted in the entire exon being spliced out. Presumably these mutations generate cryptic splice sites that result in the endogenous splice sites being ignored (Cowell and Hogg, 1992).

Sakai et al. (1991) screened DNA samples from 111 patients with retinoblastoma for mutations of the 5' untranslated region or the promoter region of the RB gene using SSCP technique. DNA was derived from lekuocytes from patients known to have a germ-line mutation (bilateral disease or a positive family history) or from primary tumour fragments. Two cases showed a sequence variation within the region extending 327 bases, upstream of the initiation codon. In both instances, the variation was present in leukocyte DNA from all affected members or obligate carriers of families with hereditary retinoblastoma that they examined. In one family, the mutation was a G to T transversion 189 bp upstream of the initiating methionine codon, and in other family, it was due to a G to A transition 9 bp further upstream. The penetrance of these mutations appeared to be low: both cariers in one family had only unilateral Rb, and there was at least three obligate carriers who had no Rb in other family. A concurrent analysis of the majority of the coding region of the RB gene also using SSCP technique has revealed no other abnormalities in the DNA sequence in these two cases.

Deletions as small as 1 bp can be detected by enzymatic DNA amplification of short gene segments and high-resolution acrylamide gel electrophoresis (Lohmann et al., 1992). Small deletions appear to comprise another 35% of germline mutations. Point mutations, which exist in approximately 50% of patients with hereditary

retinoblastoma, can be detected by single-strand confirmation analysis (Sakai et al., 1991), and sequencing of amplified gene segments (Yandell and Dryja, 1989). In contrast to the results of Yandell et al. (1989) and Onadim et al. (1992) reporting C to T transition as germline mutations and other studies in different disease in which the RB gene is involved, no missense mutations were found in the population which Blanquet et al. (1993) screened with DGGE. Blanquet et al. (1993) characterised 10 sequence alterations generating stop codons, leading to amino acid substitution, or affecting splice sites in the RB gene, as well as four polymorphisms. Among them, five were alterations of the reading frame generating premature stop codons and hence aborted proteins. Because four of the premature stop codons appear in exon 3,6 and 7, they speculated that the translated proteins were most probably too short to be functional. In the fifth case the stop codon was generated in exon 19 thus deleting the domain interacting with the SV40 T antigen. The sixth putative causal mutation is an A substitution of the conserved G at position +1 of the donor site of intron 6, probably impairing normal splicing of the RNA. The three other nucleotide changes occurring in splice sites do not modify the consensus specificity. Hogg et al. (1992) used the single strand conformation polymorphism (SSCP) technique to screen for mutations, exon by exon, in the RB gene and characterised two mutations. These are 1 bp insertion of a T in the coding strand of exon 20 and a G to A transition in the coding strand of exon 14. Dunn et al. (1988, 1989) used tumour cell lines to isolate RNA and, with the use of RNase protection, identified mutations in 50%-60% of cases. They found that small deletions were the most common abnormality but suggested this may reflect limitations in the RNase protection procedure. Small deletions were also found most frequently in a proportion of SCLC cell lines studied by Mori et al. (1990). Murakami et al. (1991) identified a mutation C→A transversion in exon 2 causing a stop codon. They speculated that the mutation caused a short polypeptide of 81 residues instead of the complete RB protein.

Kato et al. (1994) found two intragenic deletions (exon 18-19 and exon 24) and two point mutations (one missense mutation in exon 21 and one mutation at splice-donor site for exon 13) in the RB gene in somatic and tumour cells of patients with hereditary Rb. They found that three mutations were located in a domain essential for binding to oncoproteins encoded by DNA tumour viruses (Hu et al., 1990; Huang et al., 1990). One mutation (deletion of exon 24) was outside this domain but it is in the region essential for binding to transcriptional factor E2F, and for suppression of malignant phenotypes (Qian et al., 1992). A minisatellite-like sequence and short repeated sequences were located at the breakpoint of the deletion of exon 24, and they speculated that two deletions on both sides of the minisatellite-like sequence may be generated by a DNA slippage and misalignment mechanism. Shimizu et al. (1994) screened 12 patients with bilateral Rb and 12 patients with unilateral Rb by PCR-SSCP analysis in the entire coding region and promoter region. They identified mutations in 14 of 24 tumours, of which 6 were single base substitutions, 4 were small deletions, 3 were small insertions, and 1 was a complex alteration due to deletion-insertion. A majority (57%) of mutations were found in E1A binding domains.

Kreipe et al. (1993) have also screened the RB gene exon-by-exon using SSCP analysis. They found mutations in all 12 Rb tumours and one-third of the tumours had independent mutations in both alleles neither of which were found in the germ line, confirming their true sporadic nature. In the remaining two-thirds of the tumours only one mutation was found, consistent with the loss of heterozygosity theory of tumourigenesis. Point mutations, the majority of which were C→T transitions, were the most common abnormality and usually resulted in the conversion of an arginine codon to a stop codon. Small deletions were the second most common abnormality and most often created a downstream stop codon as the result of a reading frameshift. They found that deletions and point mutations also affected splice junctions and

direct repeats were present at the breakpoint junction in the majority of deletions, supporting a slipped-mismatch mechanism

Too few naturally occurring mutations in the Rb patients have been identified, so far, to allow correlations to be drawn between genotype and phenotype. One issue that is still virtually unaddressed is how pRB controls the differentiation of immature retinal cells into mature photoreceptors. Hong et al. 1989 found that the sequence extending from 196 to 249 bases upstream of the initiation codon is essential for transcription. Additional evidence that this region is important comes from the report of a prostate carcinoma with a deletion of 103 bases that overlaps this region (Bookstein et al., 1990)

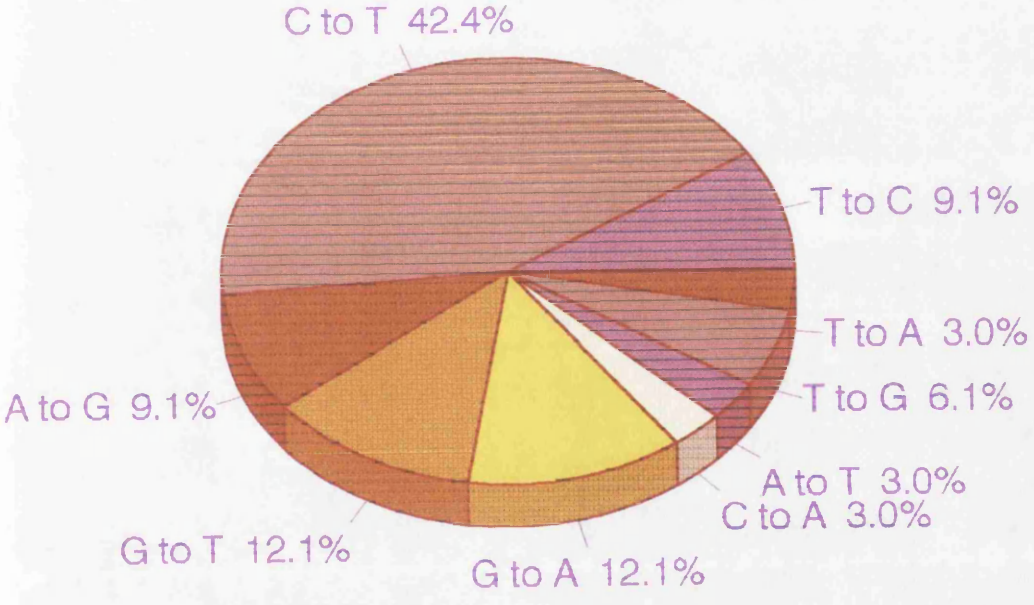


Figure 30. Point mutations in the RB gene.

Analysing of point mutations reported in Fig 29, according to base changes, showing dominance of C→T transition.

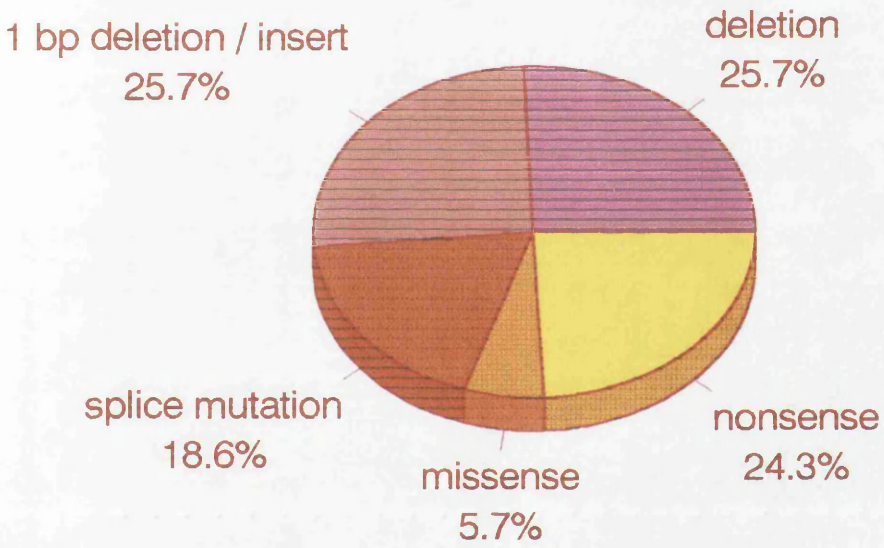


Figure 31: Categorization of the mutations of the RB gene

Documentation of over 72 published mutations revealed that point mutations (70% of all mutations in the gene), the majority of which are C→T transition, are the most common types of mutation in the RB gene (Figure 30). Hogg et al. (1993) analysed the RB gene from 12 Rb tumours using SSCP analysis. They found that one-third of the tumours had independent mutations in both alleles neither of which were found in the germ line, confirming their true sporadic nature. In the remaining two-third, of the tumours only one mutation was found, consistent with the loss-of-heterozygosity.

Point mutations (Appendix B), the majority of which were C→T transitions, were the most common abnormality and usually resulted in the conversion of an arginine codon to a stop codon. Nearly fourty three percent of all mutations were C→T transitions (Figure 30), which is consistent with observations in other genes and presumably due to the high mutability of 5-methylated cytosines in CpG dinucleotides (Yandell et al., 1989). This suggest that T→G mismatch repair is error-prone during both spermatogenesis and mitosis (Yandell et al., 1989). In RB tumours, it appears that premature stop codons are required to inactivate the RB protein. Otherwise, a larger proportion of amino acid substitutions, caused by C→T mutations in CpG dinucleotides, would be observed (Hogg et al., 1993). The C→T mutation in a CGA arginine codon is the only way a single base pair change in a CpG dinucleotide can convert an amino acid codon directly to a stop codon and is the most common finding. The CGA mutation in exon 11 has been reported in a different RB tumour (Yandell et al., 1989). However, mutations of either of the two CGA residues in exon 27 or the two CGA residues in exon 8 has net been reported. This may simply reflect the small number of tumours which have been studied so far.

A shorter transcript is usually consistent with decreased gene activity. On the other hand, less than six percent of the mutations described were due to missense point mutation (Figure 31). How such mutations could effect gene activity is still unclear.

They may creat a new splice site or they may be critical to the conformational structure of RB protein. Blanquet et al. (1993) found a single missense mutation occuring in exon 4 in a patient with bilateral Rb. In this patient, a A to T transition found at codon 137 and resulted in a glutamic acid to aspartic acid change. Although the charge and the type of amino acid remains unchanged, the folding and therefore the structure and the stability of the protein could be altered. In vitro studies are necessary to confirm the oncogenetic nature of this amino acid substitution. Yandell et al. (1989) reported another missense mutation which was a serine residue in exon 18 replaced by a leucine residue. This mutation is very unlikely to be a phenotypically silent polymorphism. The substitution occured as a new germinal change, not present in either parent, that was retained after reduction to homozygosity during formation of the tumour. Furthermore, the retinoblastoma protein is known to be phosphorylated in vivo at one of several serine residues, and this phosphorylation is probably a critical event in the normal cellular role of the retinoblastoma protein. Kato et al. (1994) has also reported another missense mutation. In the current study, a missense mutation namely H502→Q (T_{1587}→G)(patient PC) was found.This changes codon CAU (CAT) to CAG thus substituting conserved amino acid, histidine for glutamine. In this mutation, the charge and type of amino acid was changed. Since the patient was the only affected member of the family, the substitution has occurred as a new germinal. This mutation was likely to be the cause of Rb in these individuals. Data (Figure 31) suggesting that more than 94% of the RB gene mutations will create one way or another, a shorter transcript.

The possibility that other genes are involved in the genesis of this tumour can not be ruled out. For example, there seems to be variable resistance to tumour development even in patients inheriting retinoblastoma susceptibility. Further, heterozygous RB null mice do not develop retinoblastoma, but develop a characteristic brain tumour instead (Goodrich et al., 1993). The precise mechanism of action of RB is unknown,

but a broad outline is beginning to emerge RB seems to influence negatively tumour cell growth by participating in regulation of the cell division cycle. RB has also been implicated in differentiation; its effect on the cell division cycle and its effects on differentiation may be different manifestations of the same function. In the current study, analysis of mutations in RB gene could also help to understanding of the function of the gene.

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APPENDIX A

SEQUENCE OF THE RETINOBLASTOMA cDNA

5'.....TTC CGG TTT TTC TCA GGG GAC GTT GAA ATT ATT TTT GTA ACG GGA GTC GGG AGA GGA CGG GGC GTG CCC CGC GTG CGC GCG CGT CGT CCT CCC CGG CGC TCC TCC ACA GCT CGC TGG CTC CCG CCG CGG AAA GGC GTC ATG CCG CCC AAA ACC CCC CGA AAA ACG GCC GCC ACC GCC GCC GCT GCC GCC GCG GAA CCC CCG GCA CCG CCG CCG CCG CCC CCT CCT GAG GAG GAC CCA GAG CAG GAC AGC GGC CCG GAG GAC CTG CCT CTC GTC **AGG(275/2)CTT** GAG TTT GAA GAA ACA GAA GAA CCT GAT TTT ACT GCA TTA TGT CAG AAA TTA AAG ATA CCA GAT CAT GTC AGA GAG AGA GCT TGG TTA ACT TGG GAG AAA GTT TCA TCT GTG GAT GGA GTA **TTG(402/3) GGA** GGT TAT ATT CAA AAG AAA AAG GAA CT(primer A5)G TGG GGA ATC TGT ATC TTT ATT GCA CGA GTT GAC CTA GAT GAG ATG TCG TTC ACT TTA CTG AGC TAC AGA AAA ACA TAC GAA ATC **AG(518/4)T GTC** CAT AAA TTC TTT AAC TTA CTA AAA GAA ATT GAT ACC AGT ACC AAA GTT GAT AAT GCT ATG TCA AGA CTG TTG AAG AAG TAT GAT GTA TTG TTT GCA CTC TTC AGC AAA TTG GAA **AG(638/5)G ACA** TGT GAA CTT ATA TAT TTG ACA CAA CCC AGC AGT **TC(677/6)G ATA** TCT ACT GAA ATA AAT TCT GCA TTG GTG CTA AAA GTT TCT TGG ATC ACA TTT T(primer A3)TA TTA GCT AAA G(745/7)GG GAA GTA TTA CAA ATG GAA GAT GAT CTG GTG ATT TCA TTT CAG TTA A(primer B5)TG CTA TGT GTC CTT GAC TAT TTT ATT AAA CTC TCA CCT CCC ATG TTG CTC AAA GAA CCA TAT **A(856/8)AA ACA** GCT GTT ATA CCC ATT AAT GGT TCA CCT CGA ACA CCC AGG CGA GGT CAG AAC AGG AGT GCA

CGG ATA GCA AAA CAA CTA GAA AAT GAT ACA AGA ATT ATT GAA GTT CTC TGT AAA GAA CAT GAA TGT AAT ATA GAT **GAG(999/9) GTG** AAA AAT GTT TAT TTC AAA AAT TTT ATA CCT TTT ATG AAT TCT CTT GGA CTT GTA ACA TCT AAT GGA CTT CCA **GAG(1077/10) GTT** GAA AAT CTT TCT AAA CGA TAC GAA GAA ATT TAT CTT AAA AAT AAA GAT CTA GAT CGA AGA TTA TTT TTG GAT CAT GAT AAA ACT CTT CAG ACT GAT TCT ATA GAC **AG(1187/11)T TTT** GAA ACA C(primer B3)AG AGA ACA CCA CGA AAA AGT AAC CTT GAT GAA GAG GTG AAT ATA ATT CCT CCA CAC ACT CCA GTT **AG(1265/12)G ACT** GTT ATG AAC ACT ATC CAA CAA TTA ATG ATG ATT TTA AAT TCT GCA AGT GAT CAA CCT TCA GAA AAT CTG ATT TCC TAT TTT **AA(1353/13)C AAC** TGC A(primer C5)CA GTG AAT CCA AAA GAA AGT ATA CTG AAA AGA GTG AAG GAT ATA GGA TAC ATC TTT AAA GAG AAA TTT GCT AAA GCT GTG GGA CAG GGT TGT GTC GAA ATT GGA TCA **CAG(1470/14) CGA** TAC AAA CTT GGA GTT CGC TTG TAT TAC CGA GTA ATG GAA TCC ATG CTT AAA **TCA(1527/15) GAA** GAA GAA CGA TTA TCC ATT CAA AAT TTT **AG(1559/16)C AAA** CTT CTG AAT GAC AAC ATT TTT CAT ATG TCT TTA TTG GCG TGC GCT CTT GAG GTT GTA ATG GCC ACA TAT AGC **A(1636/17)GA AGT** ACA TCT CAG AAT CTT GAT TCT GGA ACA GAT TTG TCT TTC CCA TGG ATT CTG AAT GTG CTT AAT TTA AAA GCC TTT GAT TTT TAC AAA GTG ATC GAA AGT TTT ATC AAA GCA GAA GGC AAC TTG ACA AGA GAA ATG ATA AAA CAT TTA GAA CGA TGT GAA CAT CGA ATC ATG GAA TCC CTT GCA TGG CTC **TCA(1833/18) GAT** TCA CCT TTA TTT GAT CTT AT (primer D5)T AAA CAA TCA AAG GAC CGA GAA GGA CCA ACT GAT CAC CTT GAA TCT GCT TGT CCT CTT AAT CTT CCT CTC CAG AAT

AAT CAC ACT GCA GCA GAT **AT(1952/19)G TAT** CTT TCT CCT GTA AGA TCT CCA AAG AAA AAA GGT TCA ACT ACG CGT GTA AAT TCT ACT GCA AAT GCA GAG ACA CAA GCA ACC TCA GCC TTC CAG ACC CAG AAG CCA TTG AAA (primer C3)**TCT ACC TCT CTT TCA CTG TTT** TAT AAA AAA **G(2098/20)TG TAT** CGG CTA GCC TAT CTC CGG CTA AAT ACA CTT TGT GAA CGC CTT CTG TCT GAG CAC CCA GAA TTA GAA CAT ATC ATC TGG ACC CTT TTC CAG CAC ACC CTG CAG AAT GAG TAT GAA CTC ATG AGA GAC AGG CAT TTG GAC **CAA(2244/21) ATT** ATG ATG TGT TCC ATG TAT GGC ATA TGC AAA GTG AAG AAT ATA GAC CTT AAA TTC AAA ATC ATT GTA ACA GCA TAC AAG GAT CTT CCT CAT GCT GTT CAG **GAG(2349/22) ACA** TTC AAA CGT GTT TTG ATC AAA GAA GAG GAG TAT GAT TCT ATT ATA GTA TTC TAT AAC TCG GTC TTC ATG CAG AGA CTG AAA ACA AAT ATT TTG CAG TAT GCT TCC ACC **AGG(2463/23) CCC** CCT ACC TTG TCA CCA ATA CCT CAC ATT CCT CGA AGC CCT TAC AAG TTT CCT AGT TCA CCC TTA CGG ATT CCT GGA GGG AAC ATC TAT ATT TCA CCC CTG AAG AGT CCA TAT AAA ATT TCA GAA GGT CTG CCA ACA CCA ACA AAA ATG ACT CCA AGA TCA **AG(2627/24)A ATC** TTA GTA TCA ATT GGT GAA TCA TTC **GGG(2658/25) ACT** TCT GAG AAG TTC CAG AAA ATA AAT CAG ATG GTA TGT AAC AGC GAC CGT GTG CTC AAA AGA AGT GCT GAA GGA AGC AAC CCT CCT AAA CCA CTG AAA AAA CTA CGC TTT GAT ATT GAA GGA TCA GAT GAA GCA GAT GGA **AG(2801/26)T AAA** CAT CTC CCA GGA GAG TCC AAA TTT CAG CAG AAA CTG GCA GAA ATG **A(2851/27)CT** TCT ACT CGA ACA CGA ATG CAA AAG CAG AAA ATG AAT GAT AGC ATG GAT ACC TCA AAC AAG GAA GAG AAA TGA GGA TCT CAG GAC CTT GGT GGA CAC TGT GTA CAC CTC TGG ATT CAT TGT

CTC TCA CAG ATG TGA CTG TAT AAC TTT CCC AGG TTC TGT TTA TGG CCA CAT TTA ATA TCT TCA GCT CTT TTT GTG GAT ATA AAA TGT GCA GAT GCA ATT GTT TGG GTG AGT CCT AAG CCA CTT GAA ATG TTA GTC ATT GTT ATT TAT ACA AGA (primer E5)TTG AAA ATC TTG TGT AAA TCC TGC CAT TTA AAA AGT TGT AGC AGA TTG TTT CCT CTT CCA AAG TAA AAT TGC TGT GCT TTA TGG ATA GTA AGA ATG GCC CTA GAG TGG GAG TCC TGA TAA CCC AGG CCT GTC TGA CTA CTT TGC CTT CTT TTG TAG CAT ATA G (primer D3)GT GAT GTT TGC TCT TGT TTT TAT TAA TTT ATA TGT ATA TTT TTT TAA TTT AAC ATG AAC ACC CTT AGA AAA TGT GTC CTA TCT ATC TTC CAA ATG CAA TTT GAT TGA CTG CCC ATT CAC CAA AAT TAT CCT GAA CTC TTC TGC AAA AAT GGA TAT TAT TAG AAA TTA GAA AAA AAT TAC TAA TTT TAC ACA TTA GAT TTT ATT TTA CTA TTG GAA TCT GAT ATA CTG TGT GCT TGT TTT ATA AAA TTT TGC TTT TAA TTA AAT AAA AGC TGG AAG CAA AGT ATA ACC ATA TGA TAC TAT CAT ACT ACT GAA ACA GAT TTC ATA CCT CAG AAT GTA AAA GAA CTT ACT GAT TAT TTT CTT CAT CCA ACT TAT GTT TTT AAA TGA GGA TTA TTG ATA GTA CTC TTG GTT TTT ATA CCA TTC AGA TCA CTG AAT TTA TAA AGT ACC CAT CTA GTA CTT GAA AAA GTA AAG TGT TCT GCC AGA TCT TAG GTA TAG AGG ACC CTA ACA CAG TAT ATC CCA AGT GCA CTT TCT AAT GTT TCT GGG TCC TGA AGA ATT AAG ATA CAA ATT AAT TTT ACT CCA TAA ACA GAC TGT TAA TTA TAG GAG CCT TAA TTT TTT TTT CAT AGA GAT TTG TCT AAT TGC ATC TCA AAA TTA TTC TGC CCT CCT TAA TTT GGG AAG GTT TGT GTT TTC TCT GGA ATG GTA CAT GTC TTC CAT GTA TCT TTT GAA CTG GCA ATT GTC TAT TTA TCT TTT ATT TTT TTA AGT CAG TAT GGT CTA ACA CTG GCA TGT TCA AAG CCA CAT TAT TTC TAG TCC AAA ATT ACA AGT AAT CAA GGG TCA TTA TGG GTT AGG CAT TAA TGT TTC TAT CTG ATT

TTG TGC AAA AGC TTC AAA TTA AAA CAG CTG CAT TAG AAA AAG AGG CGC TTC TCC CCT CCC CTA CAC CTA AAG GTG TAT TTA AAC TAT CTT GTG TGA TTA ACT TAT TTA GAG ATG CTG TAA CTT AAA ATA GGG GAT ATT TAA GGT AGC TTC AGC TAG CTT TTA GGA AAA TCA CTT TGT CTA ACT CAG AAT TAT TTT TAA AAA GAA ATC TGG TCT TGT TAG AAA ACA AAA TTT TAT TTT GTG CTC ATT TAA GTT TCA AAC TTA CTA TTT TGA CAG TTA TTT TGA TAA CAA TGA CAC TAG AAA ACT TGA CTC CAT TTC ATC ATT GTT TCT GCA TGA ATA TCA TAC AAA TC (primer E3)A GTT AGT TTT TAG GTC AAG GGC TTA CTA TTT CTG GGT CTT TTG CTA CTA AGT TCA CAT TAG AAT TAG TGC CAG AAT TTT AGG AAC TTC AGA GAT CGT GTA TTG AGA TTT CTT AAA TAA TGC TTC AGA TAT TAT TGC TTT ATT GCT TTT TTG TAT TGG TTA AAA CTG TAC ATT TAA AAT TGC TAT GTT ACT ATT TTC TAC AAT TAA TAG TTT GTC TAT TTT AAA ATA AAT TAG TTG TTA AGA GTC.....3'

APPENDIX B

Reported mutations of the RB gene in Rb patients and other tumours

	MUTATION	LOCATION OF MUTATION Exon	bp	codon	RESULTING CHANGE	Material or type of mutation	TECHNIQUE	AUTHOR
1	a G deletion	24	2657	840	frameshift in exon 24 leads to a stop codon in exon 25	Rb tumour germline?	direct sequencing	Yandell et al., 1989
2	T→C transition	intron 19			loss of splice-donor site for exon 19	Rb tumour unilateral	direct sequencing	Yandell et al., 1989
3	C→T transition	14	1462	445	arginine→a new stop codon	Rb tumour germline	direct sequencing	Yandell et al., 1989
4	C→T transition	18	1838	567	serine→leucine	Rb tumour germline	direct sequencing	Yandell et al., 1989
5	C→T transition	23	2498	787	arginine→a new stop codon	Rb tumour germline	direct sequencing	Yandell et al., 1989
6	1-bp deletion	22	2381	748	frameshift in exon 22 leads to a stop codon in exon 22	Rb tumour somatic	direct sequencing	Yandell et al., 1989
7	G→T	intron 10			loss of splice-donor site for exon 10	Rb tumour somatic	direct sequencing	Yandell et al., 1989
8	C→T transition	11	1119	358	arginine→a new stop codon	?	direct sequencing	Yandell et al., 1989
9	A→G	21			loss splice-acceptor site for exon 21	somatic (bladder)	direct sequencing	Yandell et al., 1989
10	G→T	22	2379	748	glutamic acid→a new stop codon	somatic (SCLC	direct sequencing	Yandell et al., 1989
11	G→A	intron 11			splice donor site of exon12	somatic	RNase protection	Dunn et al., 1989
12	a 5 bp deletion	8			a frameshift causing termination codon	germline	RNase protection	Dunn et al., 1989
13	55 bp duplication	10			a frameshift causing termination codon	germline	RNase protection	Dunn et al., 1989
14	a 10 bp deletion	18			a frameshift causing termination codon	germline	RNase protection	Dunn et al., 1989
15	a 9 bp deletion	19			a frameshift causing termination codon	germline	RNase protection	Dunn et al., 1989
16	GC→TT	22	2307		absence of exon 22	somatic (SCLC	RT-PCR	Mori et al., 1990
17	Δ4	20	1979		frameshift in exon 20→stop codon in exon 20	somatic (SCLC	RT-PCR	Mori et al., 1990
18	Δ1	23	2326		frameshift in exon 23→stop codon in exon 23	somatic (SCLC	RT-PCR	Mori et al., 1990
19	G→T transversion	21			(cysteine to phenylalanine) at codon 706.	somatic (SCLC	RNase protection	Kaye et al., 1990
20	G→T transversion			promo ter	causes no change	germline	SSCP	Sakai et al., 1991
21	G→A transition			promo ter	causes no change	germline	SSCP	Sakai et al., 1991
22	G→A	intron 21			missing exon 21	somatic (SCLC	AMD analysis	Thompson et al., 1991
23	C→T	2			premature stop codon at codon 82	somatic (SCLC	SSCP	Murakami et al., 1991
24	T insertion	20	52 (exon)		premature stop codon at codon 672	germline	SSCP	Hogg et al., 1992
25	C→T transition	14	31 (exon)		Arginine(CGA)→stop codon(TGA)	germline	SSCP	Hogg et al., 1992

	MUTATION	LOCATION OF MUTATION Exon	bp	codon	RESULTING CHANGE	Material or type of mutation	TECHNIQUE	AUTHOR
26	2Δ	2	57		frameshift in exon 2 leads a stop codon at codon 109	Rb tumour, ?	DGGE	
27	2bp deletion	3	24 (exon)		frameshift in exon 3 leads a stop codon at codon 109	Rb tumour, ?	DGGE	Blanqued et al., 1993
28	1 bp deletion	intron 2			affected splicing site	Rb tumour, ?	DGGE	Blanqued et al., 1993
29	A→T transition	4	31 (exon)		glutamic acid→aspartic acid	Rb tumour, ?	DGGE	Blanqued et al., 1993
30	1 bp insetion	6	(15)		frameshift in exon 6 leads to a stop codon at codon 192	Rb tumour, ?	DGGE	Blanqued et al., 1993
31	G→A transition	intron 5			affected splicing site	Rb tumour, ?	DGGE	Blanqued et al., 1993
32	1bp deletion	7	70 (exon)		frameshift inexon 7 leads to a stop codon at codon 228	Rb tumour, ?	DGGE	Blanqued et al., 1993
33	T→C transition	intron 8			affected splicing site	Rb tumour, ?	DGGE	Blanqued et al., 1993
34	1bp deletion	18	12 (exon)		frameshift in exon 18 leads to a stop codon at codon 642	Rb tumour, ?	DGGE	Blanqued et al., 1993
35	T→C	intron 18			affected splicing site	Rb tumour, ?	DGGE	Blanqued et al., 1993
36	Δ22	13	1353		a frameshift causing termination codon	somatic	SSCP	Hogg et al., 1993
37	C→T	1	179		splicing ?	somatic	SSCP	Hogg et al., 1993
38	Δ2	19	2076		a frameshift causing termination codon	somatic	SSCP	Hogg et al., 1993
39	T→G	14	1508		causing splice acceptor site or stop codon	?	SSCP	Hogg et al., 1993
40	C→T	11	1210		arginine→stop codon	somatic	SSCP	Hogg et al., 1993
41	C→T	14	1501		arginine→stop codon	somatic	SSCP	Hogg et al., 1993
42	Δ1	4	638		splice donor deletion of exon 4	?	SSCP	Hogg et al., 1993
43	TT→C	4	622		a frameshift causing termination codon	somatic	SSCP	Hogg et al., 1993
44	Δ4	24	2658		splice donor→frameshift→ stop codon	somatic	SSCP	Hogg et al., 1993
45	C→T	17	1804		arginine→stop codon	somatic	SSCP	Hogg et al., 1993
46	C→T	10	1096		arginine→stop codon	?	SSCP	Hogg et al., 1993
47	C→T	12	1173		glutamine→stop codon	somatic	SSCP	Hogg et al., 1993
48	Δ4	4	538		a frameshift causing termination codon	somatic	SSCP	Hogg et al., 1993
49	G→T	10	1105		glutamic acid→stop codon	somatic	SSCP	Hogg et al., 1993
50	C→T	17	1791		arginine→stop codon	somatic	SSCP	Hogg et al., 1993
51	◊1	16	1576		a frameshift causing termination codon	somatic	SSCP	Hogg et al., 1993
52	Δ2	3		422	a frameshift causing termination codon	leukocyte DNA, germ line	SSCP	Onadim et al., 1993
53	Δ1	17			a frameshift causing termination codon	leukocyte DNA, germ line	SSCP	Onadim et al., 1993
54	◊1	13			a frameshift causing termination codon	leukocyte DNA, germ line	SSCP	Onadim et al., 1993

	MUTATION	LOCATION OF MUTATION Exon	bp	codon	RESULTING CHANGE	Material or type of mutation	TECHNIQUE	AUTHOR
55	Δ7&◊20	17		502	a frameshift causing termination codon	Rb tumour bilateral	SSCP	Schmizu et al., 1994
56	A→G	11			acceptor site	Rb tumour bilateral	SSCP	Schmizu et al., 1994
57	T→G	17			donor site	Rb tumour bilateral	SSCP	Schmizu et al., 1994
58	1Δ	13		444	stop in xon 14	Rb tumour bilateral	SSCP	Schmizu et al., 1994
59	C→T	12		384	Glutamine→stop	Rb tumour bilateral	SSCP	Schmizu et al., 1994
60	1Δ	24		833	stop in exon 25	Rb tumour bilateral	SSCP	Schmizu et al., 1994
61	C→T	14		455	Arginine→stop	Rb tumour unilateral	SSCP	Schmizu et al., 1994
62	1◊	2		71	stop in exon 3	Rb tumour unilateral	SSCP	Schmizu et al., 1994
63	1Δ	13		412	stop in exon 13	Rb tumour unilateral	SSCP	Schmizu et al., 1994
64	C→A	16		489	cysteine→stop	Rb tumour unilateral	SSCP	Schmizu et al., 1994
65	T→A	12		389	leusine→stop	Rb tumour unilateral	SSCP	Schmizu et al., 1994
66	1◊	22		771	stop in exon 23	Rb tumour unilateral	SSCP	Schmizu et al., 1994
67	4◊	15		472	stop in exon 16	Rb tumour unilateral	SSCP	Schmizu et al., 1994
68	2◊	19		646	stop in exon 19	Rb tumour unilateral	SSCP	Schmizu et al., 1994
69	A→C	intron 16			splice acceptor site mutation, trucated RNA transcript, skipping exon 17	leukocyte DNA, germ line	RT-PCR, AMD	Dündar, 1994
70	A→G	inton 19			does not result any change	leukocyte DNA, germ line	SSCP	Dündar, 1994
71	T→G	16	1587	483	Histidine→glutamine	leukocyte DNA, germ line	AMD	Dündar, 1994
72	◊G	21	2251		a frameshift in exon 21 leads to a stop codon 719	leukocyte DNA, germ line	SSCP	Dündar, 1994
73	A→C	16	1636	500	affecting splice site	leukocyte DNA, germ line	SSCP	Dündar, 1994
74	T→C	16		493	causes no change in the amino acid pattern	leukocyte DNA, germ line	SSCP	Dündar, 1994
75	G→C	intron 12			splice donor site mutation	tumour, bladder	SSCP	Dündar, 1994

◊: insertion, Δ: deletion

PUBLICATIONS.

1. Papers.

1.1. Scottish frequency of the common G985 mutation in the Medium-chain Acyl-CoA Dehydrogenase (MCAD) Gene and the role of MCAD deficiency in Sudden Infant Death Syndrome (SIDS). *J Inher Metab Dis* 1993; 16: 991-993.

2. Abstracts.

2.1. Screening for the G985 medium chain acyl-CoA dehydrogenase deficiency mutation in Scottish women. *British Medical Genetics Conference*, Nottingham September 1992.

2.2. Detection of germline mutations in the RB1 gene using amplification mismatch detection (AMD) analysis and polymerase chain reaction sequencing. *British Medical Genetics Conference*, York 1994 (submitted)

2.3 Molecular pathology of the RB1 gene in retinoblastoma, breast and bladder tumours. *British Medical Genetics Conference*, York 1994 (submitted)