INVESTIGATION OF THE ROLE OF DNA METHYLATION IN THE AETIOLOGY OF RETINOBLASTOMA.

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg in fulfillment of the requirements for the Degree of Master of Science.

ABSTRACT

Retinoblastoma (RB) is a malignant intraocular tumour that affects newborns and young children. The disease results from inactivation of both alleles of the Rb-1 tumour-suppressor gene within chromosome band 13q14 by deletion, mutation or gene silencing. About 40% of RB cases are of the hereditary type and about 60% are sporadic. The incidence in the Caucasoid population is 1 : 12 000.

Various mutations have been found throughout the 180 kb gene, ranging from point mutations to large deletions, but no "hot spot" or specific region for mutations has been identified. However, many mutations appear to occur as a result of C → T transitions at CpG dinucleotides.

The promoter region and exon 1 of the Rb-1 gene encompass a CpG rich region. Although CpG islands are not methylated in normal tissues, they have been demonstrated to become methylated in various tumour types.

In this study, DNA from paraffin-embedded tumour specimens of southern African Negroid RB individuals was analyzed using a

methylation-specific PCR-based method, targeting specific methylation sites in order to ascertain differences of methylation patterns in RB tumours as opposed to normal retinal tissue. Methylation of certain CpG sites in the 5′ region of Rb-1 may result in transcriptional silencing and thereby contribute to loss of function of the gene.

All of the tumours analysed were methylated at a minimum of one site, while DNA from the normal fetal retina was unmethylated at all of the methylation sensitive CpG sites analyzed.

The study provided an effective means for screening methylation changes in the Rb-1 gene that had occurred during tumorigenesis, as well as the methylation pattern of normal retinal tissue.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

MELANIE BERNICE JUDITH ISRAELSTAM

25TH day of MARCH 1998.

To my mother with my love and thanks.

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LIST OF ABBREVIATIONS AND SYMBOLS

A	adenine
bp	base pair
C	cytosine
°C	degrees Celsius
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
g	gram
G	guanine
kb	kilobase
kD	kiloDalton
M	molar
mCpG	methyl-CpG
MeCP	methyl-CpG binding protein
mRNA	messenger RNA
mg	milligram
ml	millilitre

mM	millimolar
μg	microgram
μl	microlitre
PCR	polymerase chain reaction
%	percentage
pmol	picomole
q	long arm of a chromosome
RB	Retinoblastoma
Rb-1	Retinoblastoma susceptibility gene
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
T	thymine
U	uracil
V	volts

1. INTRODUCTION

1.1 RETINOBLASTOMA - THE CLINICAL CONDITION

1.1.1 DEFINITION OF RETINOBLASTOMA

Retinoblastoma (RB) is an intraocular malignant tumour that arises from retinal progenitor cells. It affects children mostly under the age of five years - the majority being affected before they are two years old. Individuals can present with RB at birth, indicating that tumours have been growing since early fetal life (1,2,3,4).

1.1.2 CLINICAL IDENTIFICATION OF RB

RB has a reported incidence of 1 : 12 000 in the Caucasoid population (1). The disease may affect one or both eyes and so may be unilateral or bilateral. There may be one tumour (unifocal) or several tumours (multifocal). RB can be detected at a relatively early stage of tumour development as leukokoria

(a white reflection through the pupil), (see Figure 1.1) and can be treated by a variety of therapeutic measures (4,5). If the tumour attains a relatively large size, the affected eye must be enucleated (1,5). Tumours left to develop in the eye will eventually invade the optic nerve, sclera and orbit, or metastasise resulting in a poor prognosis (1,4), (see Figure 1.2).

FIGURE 1.1: The Retinoblastoma tumour is visible as leukokoria - a white reflection through the pupil (49).

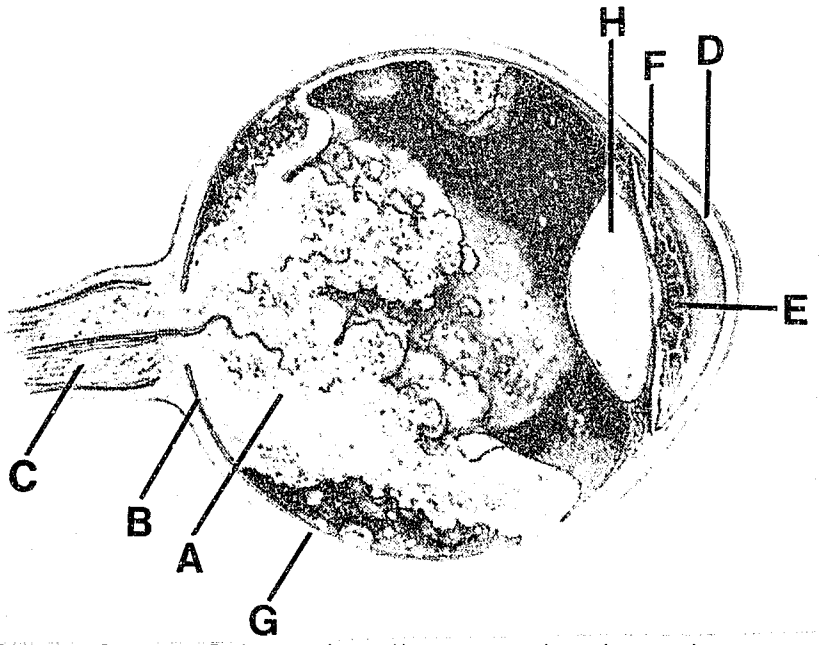


FIGURE 1.2: Schematic diagram showing the Retinoblastoma tumour with multiple seedings and optic nerve invasion (49).

KEY:

A = retinoblastoma tumour
B = retina
C = optic nerve
D = cornea
E = pupil
F = iris
G = sclera
H = lens

1.1.3 CLASSIFICATION OF RETINOBLASTOMA

The disease occurs in both sporadic and heritable forms. In the Caucasoid population 60% of RB cases are of the sporadic type and represent the majority of unilateral, unifocal cases, although bilateral cases have been observed (1,3,6). The remaining 40% of cases in the Caucasoid population show a hereditary predisposition for RB. These familial cases more frequently have bilateral, multifocal tumours with an average of 3 - 5 tumour foci (1,4,7). The latest data indicate that RB shows no variation between races, countries, or levels of industrialization (55). Very little data are currently available specifically on the incidence of RB in the African Negroid population.

1.2 THE RETINOBLASTOMA SUSCEPTIBILITY GENE (Rb-1)

1.2.1 DEFINITION OF THE Rb-1 GENE

The retinoblastoma susceptibility gene (Rb-1) is a tumour - suppressor gene. Its product is an ubiquitous phosphoprotein found primarily in the nuclei of most mammalian cells, and codes for a cell - cycle regulatory protein (1,3,7,10,11,40).

The Rb-1 gene is termed a tumour - suppressor gene because the wild type allele prevents tumour formation (1).

1.2.2 ISOLATION OF THE Rb-1 GENE

Understanding of the genetics of retinoblastoma occurred with the identification and characterization of the Rb-1 gene. Cytogenetically observable deletions on the long arm of chromosome 13 were noted in 5 - 8% of patients with RB (1). The isolation of the Rb-1 gene was accomplished using various molecular genetic techniques (1,8,38). The genetic loci of the Rb-1 and esterase D genes were assigned to band q14 of chromosome 13 after cytogenetically detectable deletions were seen in RB and esterase D deficient individuals (1,4,7,38). The close linkage of the two loci was confirmed by studies of RB pedigrees. The Rb-1 gene was isolated using "chromosome walking" from other chromosome 13 markers. Candidate genes for Rb-1 were identified by their location on the chromosome and their recessive behaviour, that is: an intact Rb-1 gene should be expressed in normal retinal tissue, but not in tumours. The initial starting point was the esterase D gene, its cDNA was used as a probe to isolate genomic DNA clones.

Distal DNA segments of these genomic clones were used to isolate additional genomic clones. Unique sequences were isolated within 20 kb DNA regions and used as probes to isolate (DNA clones from fetal retina and placental libraries. Candidate Rb-1 genes were then used as probes in RNA blotting to detect relevant mRNA transcripts. Polyadenylated RNA was prepared from human fetal retinas and the Rb-1 gene hybridised to a 4,7 kb mRNA transcript found in both libraries (8,38,39). Rb-1 gene deletions were also found to be altered in retinoblastoma patients using Southern blot analysis with the Rb-1 cDNA as a probe. Absence of bands were noted when homozygous deletions were present. Under-represented fragments were observed when heterozygous deletions (deletion of the region on one chromosome only) were present. Partial deletions resulted in fragments of altered size being seen (8). Isolation of the gene has permitted molecular analysis of genetic alterations associated with RB (7,8). The Rb-1 gene spans approximately 180 kb of genomic DNA and consists of 27 exons, ranging in size from 31 bp to 1873 bp and encoding 928 amino acids. The 26 introns range from 80 bp to 70 500 bp in size (1,4,5,9,39).

1.2.3 FUNCTION OF THE Rb-1 GENE

A growing cell undergoes a cell-cycle which comprises two periods: a very short stage of cell division, the M phase (mitosis) and a long intervening interphase which comprises three phases. These are: S phase (DNA synthesis), G_1 phase (the gap between M phase and S phase) and G_2 phase (the gap between S phase and M phase). Cells normally enter S phase only if they are committed to mitosis. Non-dividing cells remain in a modified G_1 stage called G_0. The events responsible for the initiation of S phase occur during G_1. To regulate the cell-cycle, the synthesis of certain proteins, for example cyclins, is required. These proteins are regulated by cell-cycle dependent phosphorylation (37).

The Rb-1 gene produces a 100 kD nuclear phosphoprotein, $p110^{Rb1}$, which is regulated by cell cycle dependent phosphorylation. The hypophosphorylated form exists during the G_0 and G_1 phases of the cell cycle and is active in growth suppression. During late G_1 phase when $p110^{Rb1}$ is hyperphosphorylated by cyclin - dependent kinases,

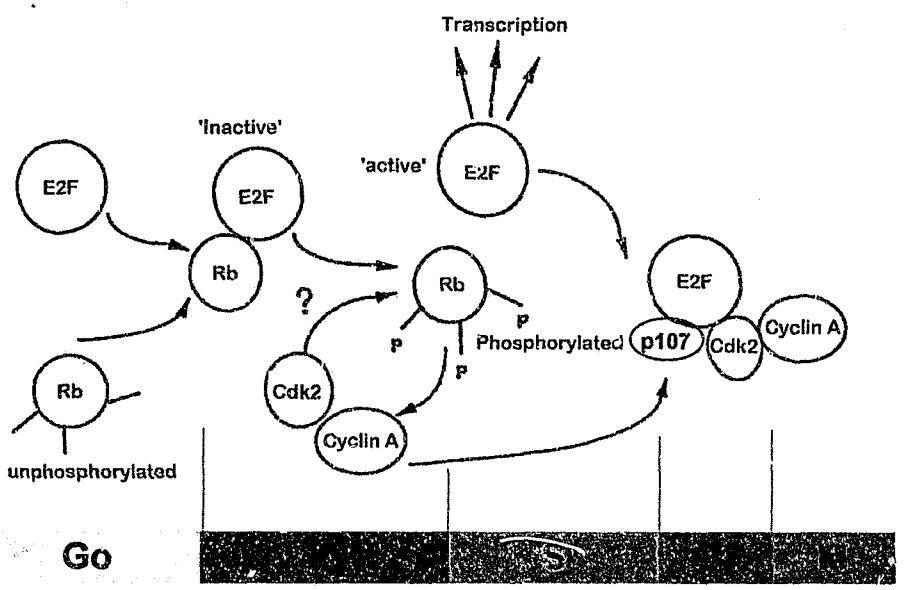


Figure 1.3: Events in the Cell-cycle involving the Rb Protein. (Modified from Ref 47).

E2F is a transcription factor implicated in the control and regulation of expression of critical G_1 genes. E2F prevents premature entry into the G_1 phase of the cell cycle. The availability of E2F is under the tight control of Rb during the cell-cycle. In the G_1 phase, the Rb-E2F complex is inactive. In the G_0 and the early G_1 phases, Rb is hypophosphorylated. As the cell moves into mid/late G_1 phase, Rb undergoes hyperphosphorylation by cyclins and cyclin-dependent kinases (cdks), cyclin A and cdk2 (these are important regulators of progression of the cell-cycle and promote cellular proliferation). Rb remains phosphorylated during the S phase and until the end of mitosis. The Rb-E2F complex dissociates at the G_1 -S boundary. E2F is released to actively transcribe cellular promoters. In the G_2 and M phases, E2F again forms complexes with kinases and p107 (a protein homologous to pRb) and becomes inactive in order to control and maintain normal cell growth.

it releases transcription factors, allowing them to activate the gene transcription necessary for progression through the cell cycle (1,12,13,14,15). This hyperphosphorylated state is maintained through S, G_2 and M phases. The protein is then dephosphorylated by phosphatase activity in the late M phase (14,15). The lack or alteration of this protein in RB tumours is indicative of the regulatory role of $p110^{Rb1}$ in cellular proliferation. Absence of this protein is related to tumour formation and, therefore, its normal function appears to be to limit or constrain cell proliferation or to regulate cell differentiation (12,14).

It has been found that $p110^{Rb1}$ binds specifically to the E2F transcription factor. E2F is a transcription factor implicated in the control and regulation of expression of critical G_1 genes, such as thymidine kinase and dihydrofolate reductase, required for cellular proliferation (48). Its regulation is important for maintenance of G_0 and for the initiation of cell growth (43).

To regulate transcription, E2F must form a stable complex with specific cellular proteins to bind specific DNA sequences in the promoter regions of the genes it controls (47). E2F was originally identified as a cellular factor recruited for the transcription of viral genes by the adenovirus E1A oncoprotein (40,41,42,43). E2F recognition sites have also been found in the promoters of several cellular genes whose functions are essential for DNA replication and whose products respond to proliferation signals and are required for movement of cells from G_0 to S phase (40,41,42,43). E1A acts to dissociate Rb - E2F complexes, which shows that one function of $p110^{Rb1}$ is to sequester free E2F thereby inhibiting the transcription of genes necessary for the DNA synthesis (S) phase of the cell cycle (12,14,15,40,42,44). E2F is a positive element in the absence of an active form of $p110^{Rb1}$. Switching E2F sites from activators to repressors of transcription is a function of $p110^{Rb1}$, indicating that the Rb-E2F is in an active complex that inhibits the activity of other promoter elements by down-regulating their expression and thus silencing their transcription (40,44,45). For schematic representation of the involvement of the Rb protein in the cell cycle, see Figure 1.3.

1.2.4 MUTATIONS IN THE Rb-1 GENE

Point mutations, submicroscopic deletions, duplications and translocations have been detected in some RB patients (1,4). These mutations have been found throughout the gene and no intragenic "hot spot" or specific region for mutations has been identified (1,2,7). Many patients with large, cytogenetically observable deletions are mentally and/or physically retarded suggesting that either the Rb-1 gene or genes in close proximity to it on chromosome 13 is/are responsible for causing mental and physical retardation, but these still remain to be identified (1,55).

1.3 TUMORIGENESIS

1.3.1 MECHANISM OF TUMORIGENESIS

Two genetic events are required for RB tumour development in both hereditary and sporadic forms, leading to loss of function of both Rb-1 alleles (4,16,17). The initial event is a mutation of one Rb-1 allele. Mechanisms leading to loss of function of the second allele, as seen in Figure 1.4 include:

(i) Mitotic Non-Disjunction : which results in hemizygosity for all loci on chromosome 13.

(ii) Mitotic Non-Disjunction Followed By Reduplication: which results in loss of constitutional heterozygosity at both loci. On RFLP analysis, the remaining allele at each locus in the mutant cell can be seen to have twice the band intensity of that seen for either allele in DNA from a normal cell. This is because the mutant locus has a double dose of the remaining allele.

(iii) Mitotic Recombination: between homologues with a breakpoint between the Rb-1 locus and the centromere with both Rb-1 bearing chromosomes moving into one daughter cell.

This would result in heterozygosity at loci in the proximal region and homozygosity throughout the rest of the chromosome, including the Rb-1 locus (8,16,17,18).

(iv) Small Deletions: which would result in a pathogenic loss of genetic material at the Rb-1 locus.

(v) Point Mutations: which would result in a pathogenic loss of genetic material at the Rb-1 locus.

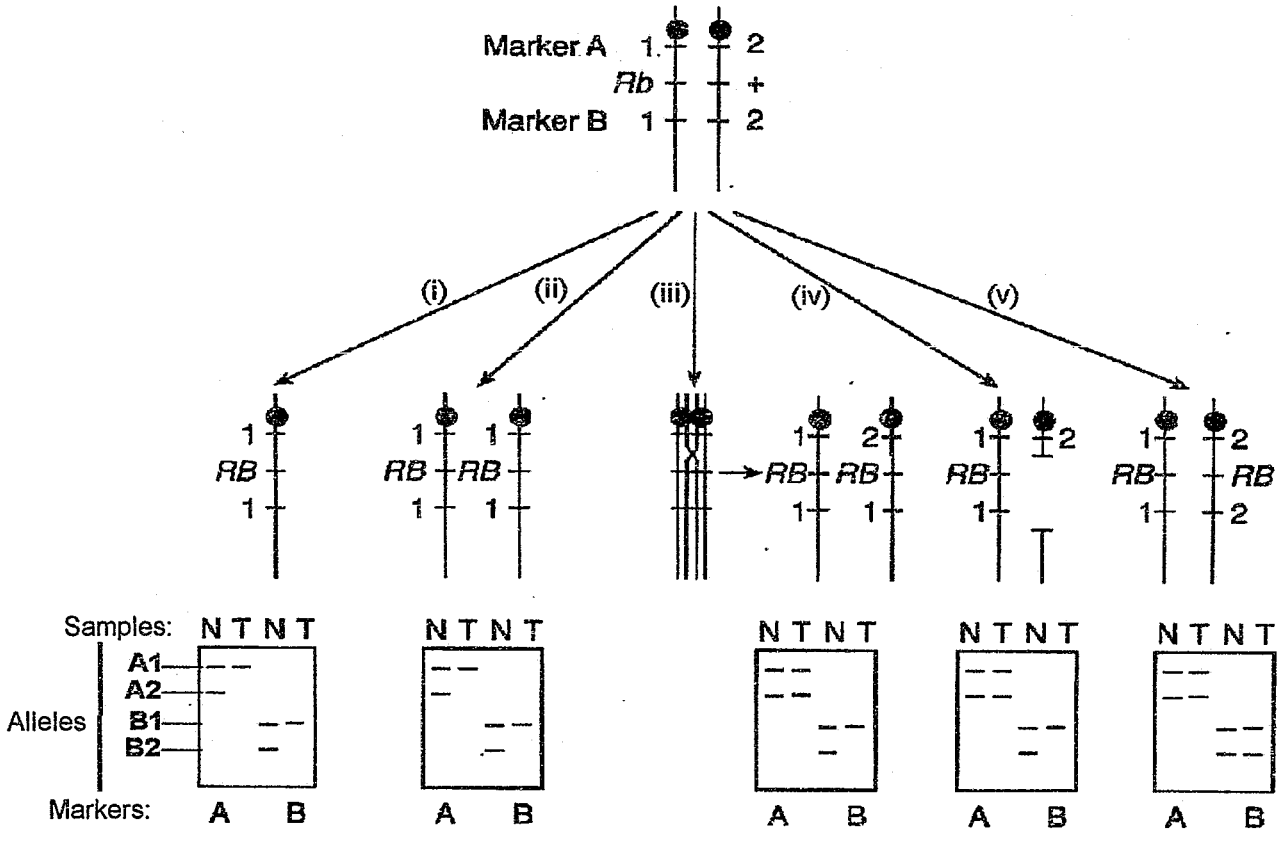


Figure 1.4: Mechanisms of loss of wild type allele in RB (Modified from ref.37)

FIGURE 1.4: Mechanisms of loss of wild type allele in RB (Modified from Ref 37).

The figures below show results of RFLP analysis of typing normal (N) and tumour (T) DNA for two markers A and B located as shown. Note the patterns of loss of heterozygosity.

(i) Loss of whole chromosome by mitotic nondisjunction.
(ii) Loss followed by reduplication.
(iii) Mitotic recombination proximal to the RB locus, followed by segregation of both Rb-1 bearing chromosomes into one daughter cell.
(iv) Deletion (if large enough) of the wild-type allele
(v) pathogenic point mutation of the wild-type allele.

1.3.2 TUMORIGENESIS IN RB

Hereditary predisposition to RB is caused by a germline mutation at the Rb-1 locus, which is transmitted as an autosomal dominant trait with 90% penetrance. The second event is the somatic inactivation of the remaining functional allele, often causing loss of heterozygosity (1,4,6,12,16, 17,36). Since the first mutation is already present in the germline, the second mutation is likely to occur in more than one cell, so most hereditary cases develop bilateral disease with multifocal tumours (1).

In cases of non - hereditary RB, the same retinoblast must acquire two somatic mutations, on on each chromosome 13, as a result of sequential sporadic events (1,4,12,16,18). These patients usually develop only one tumour as the occurrence of two mutations in more than one cell does not occur frequently (1).

1.4 DNA METHYLATION

1.4.1 DNA METHYLATION AND GENE REGULATION

Chemical modification of DNA has been demonstrated to be an important mechanism of gene regulation (19). The most common eukaryotic DNA modification is the addition of a methyl group to the 5 position on the cytosine ring at CpG dinucleotides (19,20,21,22). DNA methylation is an important part of the heritable epigenetic system influencing expression or silencing of genes necessary for normal differentiation and proliferation. The relationship between DNA methylation, chromatin structure and gene activity is best shown by suppression of transcription of one of the X chromosomes in females. The inactive parts of the X chromosome are hypermethylated and heterochromatic, whereas the transcriptionally active X chromosome is hypomethylated and euchromatic (21,27). Transcriptional activity may also be silenced by methylation of upstream regulatory regions. Reactivation of genes is generally associated with demethylation (17,21,23,24).

1.4.2 DNA METHYLATION AND TRANSCRIPTIONAL REPRESSION

Transcription of genes by RNA polymerase II can be inhibited by methylation of CpG dinucleotides (30). In normal tissues, when a CpG island becomes methylated, the associated gene is inactivated, through a combination of direct inhibition of a transcription factor binding and because methylation directs chromatin into an inactive structure (27,68). The inactivation of CpG island associated genes by DNA hypermethylation is mediated by methyl - CpG binding proteins MeCP-1 and MeCP-2, both of which bind specifically to DNA containing methyl-CpG pairs. MeCP-1 binds *in vitro* to DNA containing at least 12 symmetrically methylated CpGs (26,68). The binding of this protein to DNA containing multiple symmetrically methylated CpG's is thought to cause transcriptional repression by blocking the binding of a particular transcription factor to its target sequence, thus altering or preventing gene expression (20,21,24,26,30). MeCP-2 binds to a single methylated CpG pair (26,68). It is associated with alteration of chromatin structure and contributes to long-term gene repression and nuclease-resistance of methyl-CpGs (26,68).

A crucial determinant of transcriptional repression is the density of methyl - CpG's in the promoter region. Islands have a high density of CpGs and are therefore high affinity substrates for MeCP binding when methylated; hence repression of an associated gene is severe (20,24,26,30).

1.4.3 CpG ISLANDS

CpG islands are short regions of approximately 1kb of DNA which account for approximately 2% of the genome and have distinctive properties when compared to the rest of the genome. CpG islands consist of 60 - 70% GC - rich DNA which is unmethylated. Islands do not show any suppression of the CpG dinucleotide because of lack of methylation (27). By contrast, bulk genomic DNA is heavily methylated, with only approximately 40% consisting of CpG dinucleotides. The CpG dinucleotide is under-represented about four-fold from what is expected in genomic DNA (22,24,25,27).

A consequence of cytosine methylation by methyltransferase is the deamination of 5 - methyl - cytosines (5-mC) to form thymine (T), responsible for the high mutability of the CpG

dinucleotide in DNA (46). These C → T transitions cause point mutations which over evolutionary time transform mCpG's to TpG's. G:T mismatch resulting from the deamination of 5-mC is believed to be more difficult to repair than the G:U mismatch resulting from C deamination. If U (uracil) is recognised as being incorrect, it is removed by uracil DNA glycosylase, followed by excision repair. If U is allowed to remain undetected in the DNA, it has the hydrogen bonding properties of T, and will pair with A in the next round of replication and become incorporated into the DNA. When 5-mC is deaminated, T (thymine) residue results. If DNA repair is delayed until the next cell cycle, the cell will not recognise the T residue as an incorrect base and it will not be excised. This base is normally found in DNA therefore not easily recognised as being mutant by DNA repair mechanisms and is not excised. Hence these regions become depleted of CpG's (22,24,25,27,46).

Two possible pathways of C → T transition mutations at CpG sites is shown in Figure 1.5:

(i) hydrolytic deamination of 5-mC to form thymine.

(ii) enzymatic deamination of C to form uracil. Uracil has the hydrogen bonding properties of thymine, and if allowed to remain in the DNA, will pair with adenine in the next round of replication. So, after another round of replication, this would result in a G-C → A-T transition.

Both hydrolytic and enzymatic deamination of cytosine create temporary premutagenic mismatches in the DNA, which if not recognised by DNA repair systems prior to DNA replication, become fixed mutations. Despite the existence of efficient repair systems, C → T transition mutations are the most common cause of somatic and germline mutations and of mutation in cancer, for example in p16, p53 and APC (adenomatous polyposis coli) (22,46).

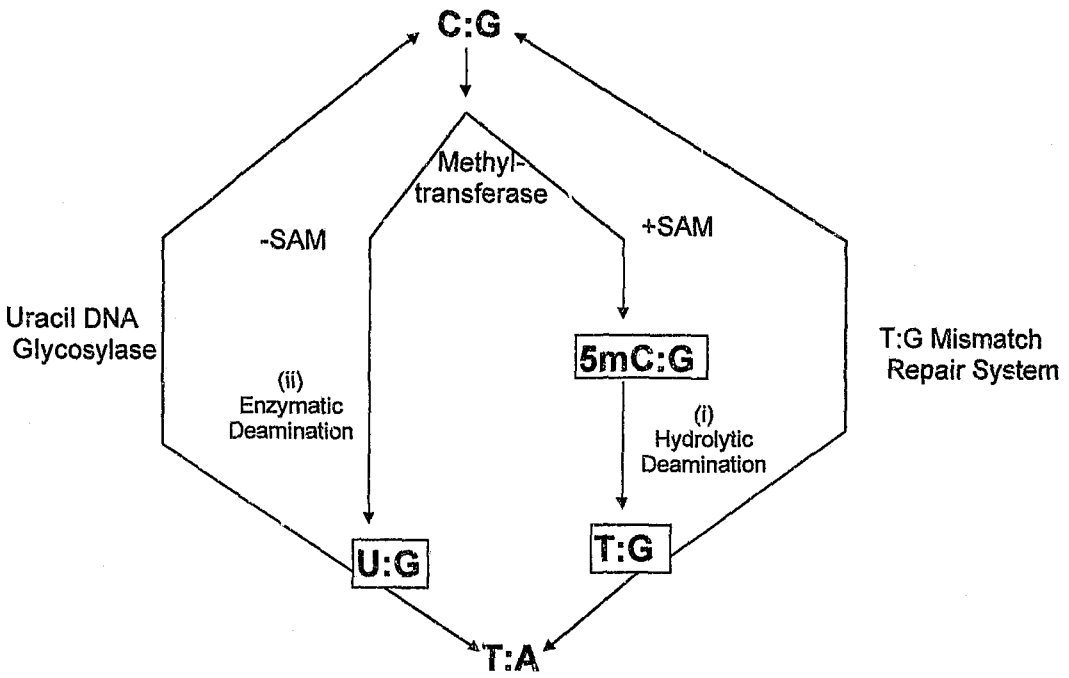


Figure 1.5: Two possible pathways for C → T transition mutations.
(Ref 46: Yang,A.S. *et al.* Nucleic Acids Research, 23:1380-1387,1995)

The deamination of products of C and its methylated derivative 5-mC are differentially recognised by DNA repair enzymes.
(i) Hydrolytic deamination of 5-mC results in the conversion of CpGs to TpG and CpA dinucleotides.
(ii) Enzymatic deamination of C results in the conversion of CpGs to TpU and CpA dinucleotides. Their repair is blocked by the binding of methyltransferases which have a high affinity for G:C DNA mismatches. But, if the enzyme uracil DNA glycosylase is present, uracils are removed and replaced with cytosines. Hence, C:G is present in this pathway.

KEY:

SAM = S-adenosylmethionine, which is a methyl donor.

+SAM = The donor allows for methyl transfer to form mCpG .

-SAM = In the absence of the methyl donor, methyl transfer does not occur, resulting in a uracil residue when cytosine deamination occurs.

CpG islands are often associated with the 5′ regions of many frequently transcribed "housekeeping" genes and less often with genes with a tissue-restricted pattern of expression (19,21,22,23,24,27). About 56% of human genes are associated with the estimated 45 000 CpG islands in the human genome (18,24,27). CpG islands are associated with a specific sub - set of chromosomal R (reverse) bands - these correspond to replicating chromosome domains, rich in G-C base pairs and less condensed than G (giemsa) bands. The subset of R bands are called T bands. They are extremely heat resistant, have long regions of homogeneous base composition, and the highest GC content. Of the mapped CpG islands, approximately 65% are found in T bands (27). Their location most often coincides with transcriptional initiation, and includes the first exon of the gene. Hence, the methylation of regulatory sequences in CpG islands, in or near the promoter region of a gene is a form of epigenetic control of gene expression (11,21,23,24,27,28,29). The structure of the Rb-1 CpG island can be seen in Figure 1.6.

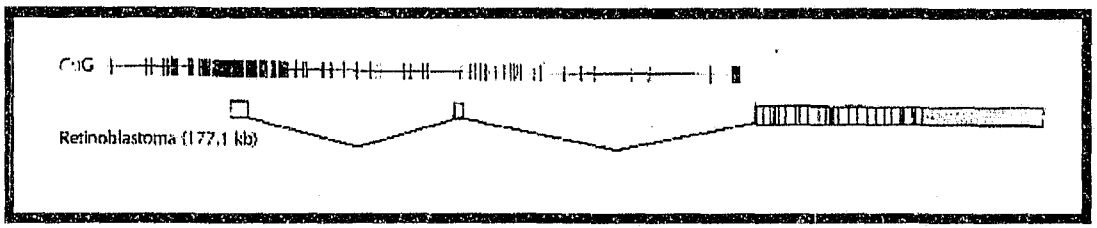


FIGURE 1.6: Structure of the RB CpG Island (27).

The island consists of the high density of CpGs in the 5' region of the gene.
Vertical lines show the positions of CpGs in the first 10kb of the gene, The locations of exons are shown by boxes. Any exons not present in the first 10kb of DNA are shown fused together to the right. The total length of the gene (in kb) is given brackets.

1.5 METHYLATION IN CARCINOGENESIS

1.5.1 METHYLATION AND TUMOUR - SUPPRESSOR GENES

The inactivation of tumour - suppressor genes such as p53, p16 (MTS1), Wilm's tumour (WT-1), neurofibromatosis type 1 (NF1) and type 2 (NF2), von Hippel-Lindau (VHL) and adenomatous polyposis coli (APC), contributes to the formation and/or progression of many human cancers (31,32,48). These genes encode growth-inhibitory proteins that normally act to limit cell proliferation (48).

It has been observed that hypermethylation of promoter regions of tumour -suppressor genes occurs. There is consequent gene inactivation. This results in silencing of genes that control the cell cycle and maintain DNA integrity (21,28, 28,31,32,33). It has been postulated that methylation of tumour-suppressor genes is a mechanism for oncogenesis, since methylation can stably repress their transcription (21,22,28,31). Clonal selection of changes that confer a growth advantage to a single cell line over surrounding cells is the driving force of tumour

progression (34). Methylation inactivates tumour-suppressor genes conferring a growth advantage for tumour cells. Cellular memory then plays a role in the maintenance of methylation, and so the pattern of methylation is stably passed from a somatic cell to its descendants, giving rise to a clone of cells (23,28,34).

1.5.2 Rb-1 GENE METHYLATION AND CARCINOGENESIS

The 5′ end of the Rb-1 gene has a high G + C content, a lack of CpG suppression and an apparent absence of CpG methylation, consistent with a CpG island (9,28,29). Inactivation of both copies of the Rb-1 gene by deletions, insertions, point mutations or translocations in the body of the gene has a role in the genesis of RB (1,28). It is also possible that 5-methyl cytosine deamination may contribute to germline structural alterations. The modified base is significantly more susceptible to mutagenesis. Mutation resulting from deamination of 5-mC increases the molecular complexity within the major groove of DNA, resulting in conformational changes

that have the potential to influence the affinity of DNA binding proteins. This may predispose to tumorigenesis, since some inherited RB mutations occur at the CpG dinuleotide (21).

Hypermethylation of the Rb-1 CpG island was observed in prevoius studies, in 5 patients (16% of cases), without detecting structural abnormalities (1,29,33,34). The extent of the methylated region varied slightly, but always included exon 1. Hypermethylation is an epigenetic event by which instructions are passed faithfully to daughter cells to enable clonal evolution following mitosis (1,21). No differences in the nucleotide sequence of the hypermethylated regions and the same unmethylated regions of tumour tissue have been found. The hypermethylated regions also appear to be structurally normal (29). These data suggest that hypermethylation is the mechanism by which the Rb-1 gene is inactivated and by which carcinogenesis progresses.

Inactivation of tumour-suppressor genes has been demonstrated as a result of epigenetic change - the hypermethylation in the CpG islands of certain genes, for example, p16 gene (24,28,33,34). Data suggest that once methylation is established in the 5′ regulatory region of a tumour - suppressor gene, clonal selection will follow. This leads to tumour progression (21,34). Changes in methylation of the 5′ region of the Rb-1 gene play an important role in the development and/or progression of RB tumours (1,11,28,29, 32,34).

1.6 AIMS OF THE PROJECT

In this study, I proposed to test the hypothesis that there may be differences in the methylation patterns of the Rb-1 gene in RB tumours as opposed to normal eye tissue. If the CpG sites within the 5' region of the Rb-1 gene were found to be methylated in tumours, but not normal cells, it would have suggested that these methylation sites may have an important role in tumour formation and in the silencing of the Rb-1 gene.

The specific aims of this project were:

(1) To extract good quality DNA from formalin - fixed, paraffin - embedded RB tumour specimens.

(2) To design primers flanking regions possibly affected by methylation in the 5' region of the Rb-1 gene and to optimise conditions for polymerase chain reaction (PCR) amplification of these regions.

(3) To analyse methylation patterns of the 5' region of the retinoblastoma gene in sporadic and hereditary forms of RB, in the southern African Negroid population using a methylation - specific restriction endonuclease assay and PCR.

2. SUBJECTS AND METHODS

2.1 SUBJECTS

2.1.1 SELECTION OF SUBJECTS

The subjects for the study were selected from patients that were seen and treated at the Haematology and Oncology Unit of the Department of Paediatrics and Child Health at the Chris Hani - Baragwanath Hospital (Soweto, Johannesburg) from 1993 - 1995.

Consent for use of patient data in hospital files in this study was obtained from the Committee for Research on Human Subjects (see Appendix B).

Subject files were extensively reviewed and relevant data extracted. Family history, RB tumour type, date of enucleation, whether the right or the left eye/ or both were involved, hospital number and histopathology specimen number, and type of treatment, was noted for each patient.

The sample size of the study was 19 tumours: 6 subjects with bilateral tumours, samples were obtained from both eyes in 3 cases (ie. 9 bilateral tumours) and 10 unilateral tumours. The limited number of bilateral RB tumour cases available determined the sample size as a similar number of bilateral and unilateral tumours were selected for the study.

2.1.2 SELECTION OF CONTROL

A normal retina sample was required as a control for comparison to tumour retina samples. A fetal retina was obtained at autopsy from a normal third trimester fetus following on a termination of pregnancy. Consent for use of this specimen was obtained from the Committee for Research on Human Subjects at the University of the Witwatersrand (see Appendix B).

2.1.3 TUMOUR HISTOLOGY

Consent for use of all tumour biopsy specimens utilized in this study was obtained from the Committee for Research on Human Subjects (see Appendix B).

2.1.3.1 IDENTIFICATION OF TUMOUR MATERIAL

All tumour material utilized was from paraffin-embedded wax blocks obtained from the Department of Anatomical Pathology. Slides were prepared for each tumour block. A very thin section was cut from each specimen, placed on a slide and stained with eosin. The histology of each specimen was reviewed microscopically by the Department of Anatomical Pathology and tumour material was identified in each. The blocks were then sectioned carefully to ensure that only pure tumour tissue (and no surrounding normal tissue) was obtained in each case.

2.1.3.2 CLASSIFICATION OF TUMOUR HISTOLOGY

Histology reports of enucleated tumours were obtained for all subjects. Each specimen was classified according to the stage of tumour progression with the aid of the Cytopathology Unit of SAIMR. The extent of tumour infiltration beyond the eye and whether metastasis had occurred was also noted.

2.2 METHODS

2.2.1 DNA EXTRACTION FROM PARAFFIN - EMBEDDED TUMOUR MATERIAL

DNA was extracted from archival paraffin-embedded tumour tissue using minor modifications of the method of Smith *et al* (50). Approximately 25 - 50μm histological sections were incubated at 55°C for 24 hours in 250 - 500μl of extraction buffer consisting of 10mM Tris pH 8.0, 50mM KCl, 1,5mM $MgCl_2$, 100μg/ml BSA, 0,45% Tween 20, 0,45% Nonidet NP40 and 100μg/ml Proteinase K. After cooling on ice, DNA was purified by removal of proteins using two phenol and one chloroform/isoamyl alcohol extractions. DNA was precipitated overnight at -20°C by adding 1/10 volume 3M NaAcetate and 2 volumes of cold absolute ethanol to the aqueous layer. After centrifugation, ethanol was removed, and the DNA pellet was then air-dried and resuspended in 20μl of TE. To reduce the salt concentration, the samples were dialysed against TE using Millipore VS filters (pore size 0,025μm), prior to restriction enzyme digestion.

2.2.2 VISUALIZATION OF DNA

2μl of DNA was electrophoresed on a 1% ethidium bromide stained agarose gel, with a molecular weight marker (1 kb ladder) alongside to determine fragment size. The quality of DNA obtained was either high molecular weight DNA, . single, slow travelling band; or sheared, semi-degraded DNA (approximately 300 bp)/ or a degraded DNA smear.

2.2.3 DNA EXTRACTION FROM THE CONTROL FETAL RETINA

The fetal retina sample was stored in liquid nitrogen at -70°C. Approximately 25mg of tissue was cut into small pieces. DNA was extracted from the tissue using the QIAmp Tissue Extraction kit. The extraction method followed the protocol provided with the kit (see Appendix E).

2.2.4 METHYLATION - SENSITIVE RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction enzymes (REs) used in this study were chosen because they will only cut if cytosines within CpG's in their

recognition sites are unmethylated. These rare-cutting REs, *BssH* II, *FnuD* II, *Sac* II and *Sma* I each had one or two CpG's in their recognition sites and were used to generate fragments (see Table 1).

Both tumour-derived DNA and control DNA were digested separately with the following restriction enzymes: *BssH* II, *FnuD* II, *Sac* II and *Sma* I (Boehringer Mannheim). The positions at which these enzyme cutting sites are present in the Rb-1 promoter region can be seen in figure 2.1. Restriction digestion was carried out accordir ı to the manufacturer's specifications for complete digestion. 1μl of DNA (although of variable concentration) was digested using 10 units of enzyme (1μl) and the specified buffer for the particular enzyme, in a final volume of 10μl made up with sterile double distilled water. The mixture was incubated for 2 hours at 37°C and the enzyme was inactivated by placing the mixture on ice for 10 minutes.

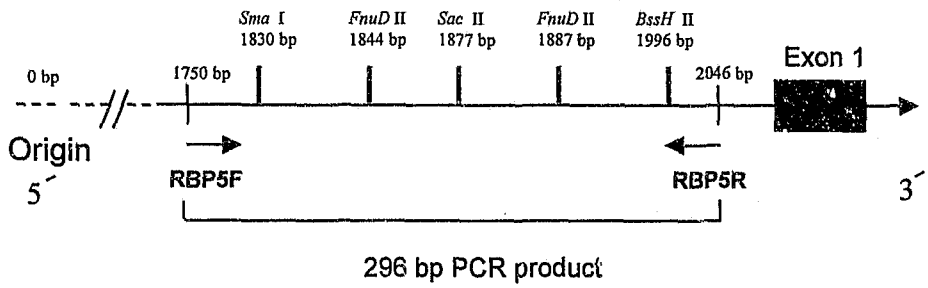


FIGURE 2.1 : Map of Rb-1 promoter region.

The region analysed in this study spans from 1750-2046bp. The positions of the methylation sensitive restriction enzyme sites are shown.

Table 1: Methylation-sensitive restriction endonuclease recognition sequence and cutting sites

Restriction Enzyme	Recognition sequence and cutting site
BssH II	+ + 5' G⇩CGCGC 3'
FnuD II	+ + 5' CG⇩CG 3'
Sac II	+ + 5' CCGC⇩GG 3'
Sma I	+ + 5' CCC⇩GGG 3'

KEY:

+
C = cytosine sensitive to methylation

⇩ = cutting site of restriction enzyme

2.2.5 POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR), first described by Saiki *et al* (51) enables rapid enzymatic amplification of DNA *in vitro*. DNA sequences can be amplified by simultaneous primed extension of complimentary strands of DNA. Only small quantities of DNA are required, and the method is usually highly specific. The main requirement is that the template DNA should be intact over the length that is being amplified, and the sequence of the region be known (52,53).

To enable strand formation, various components are required to make up the reaction mix: target DNA, primers, thermostable DNA polymerase, reaction buffer, precursor deoxynucleotide triphosphates (dNTPs) and divalent metal ions (Mg^{++}). The final volume of between 25 · 50μl is made up with double distilled water. Other substances such as DMSO may also be added to improve the quality of the PCR product by reducing secondary structure formation (52,53).

Two oligonucleotide primers (ranging between 18 - 25 bases) are synthesized, complementary to regions on either side of the target sequence which is to be amplified. The primers hybridize to these areas of DNA. The sequence between the two primers is then replicated many times by a thermostable DNA polymerase. A repetitive series of cycles involving template DNA denaturation (90 - 95°C), primer annealing (40 - 70°C) and the extension of the annealed primers (70 - 74°C) results in the exponential accumulation of target DNA. The primer extension products synthesized in a given cycle serve as a template in the next cycle, so the number of target DNA copies doubles every cycle (52).

2.2.6 PCR ANALYSIS OF TUMOURS AND CONTROL

2.2.6.1 PRIMER SELECTION

The sequences of the promoter region and exon 1 of the Rb-1 gene were obtained from a Genbank search (see Appendix C for sequence). Methylation-sensitive RE sites were selected for their position in the Rb-1 promoter region. Around these sites,

two sets of oligonucleotide primer pairs were designed with the aid of the "Oligo" computer program. This program shows: the number of bases between the forward and the reverse primers; G+C content; primer dimer formation; secondary structure formation; complementarity of the primers to each other at the 3′ ends and approximate annealing temperature for the primer pair chosen. Once the best possible pairs were obtained according to the above criteria, the primers with the least primer dimer formation; secondary structure formation; complementarity of the primers to each other at the 3′ ends and with approximately the same annealing temperature for each primer of the pair were chosen. These were synthesized by Genosys (U.K.).

The first primer pair was the closest to the 5′ end of the promoter region of the Rb-1 gene, at position 1518 and 1762 bases respectively from the origin of the Rb-1 gene sequence (at 0bp) contained in Figure 2.2 (and see Appendix C). Both primers were 20 bases long (20 mer) and called RBP1F (forward) and RBP1R (reverse). Their product was 244 bp in length.

The primer sequences are as follows:

RBP1F: 5′ CCAAGGAGGGAGAGTGGCGC 3′

RBP1R: 5′ TTAAACTGGGAAACCTGGCG 3′

The second primer pair was also in the promoter region but closer to exon 1, at position 1750 and 2046 bases respectively from the origin of the sequence contained in Figure 2.2 (and seen Appendix C). They were both 20 bp in length and called RBP5F (forward) and RBP5R (reverse). Their product was 296 bp in length.

Their sequences are as follows:

RBP5F: 5′ TTCCCAGTTTAATTCCTCAT 3′

RBP5R: 5′ GCGGGAGCCAGCGAGCTGTG 3′

The primers were designed so that they hybridized to opposite strands flanking the region of chosen target DNA sequence.

Primers were received in lyophilized form and rehydrated using sterile double distilled water, according to the suppliers' instructions (see Appendix D).

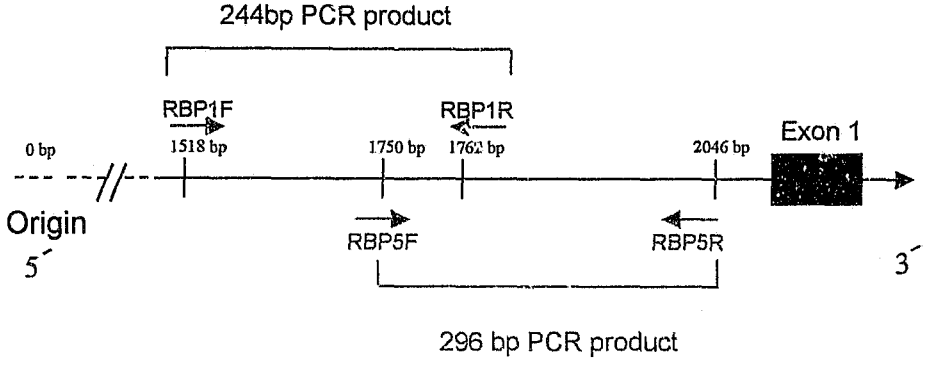


FIGURE 2.2 : Positions of Primer Pairs in the Rb-1 promoter region

SEQUENCE OF PRIMERS:

RBP1F	5' CCAAGGAGGGAGAGTGGCGC 3'
RBP1R	5' TTAAACTGGGAAACCTGGCG 3'
RBP5F	5' TTCCCAGTTTAATTCCTCAT 3'
RBP5R	5' GCGGGAGCCAGCGAGCTGTG 3'

2.2.6.2 PCR AMPLIFICATION

All PCR reactions were carried out in a Hybaid Omnigene thermocycler. The PCR reaction volume was 25μl. Each reaction included: 1 - 2μl of target DNA, 10x PCR buffer, dNTPs (each at a final concentration of 1,25mM), a pair of primers (50 pmol of each primer) for amplifying the target sequence and *Taq* DNA polymerase (1,5 units /25μl reaction). After some optimization, 5% DMSO was also added to the reaction mix. The reaction was overlaid with a drop of mineral oil to avoid evaporation during cycling.

Concentration of template DNA, annealing temperature and annealing and extension times were all varied during attempts to optimize PCR amplification in each of the two different primer pair systems.

Because of the high GC content of the region, a single denaturing cycle at 94°C for 3 minutes was performed initially

to ensure DNA strand separation. Amplification was performed for 35 cycles. Each thermal cycle consisted of a denaturing step at 94°C for 30 seconds, annealing for 1 minute and extension at 72°C for 1 minute. A final extension cycle at 72°C for 10 minutes ended each PCR run.

The annealing temperature for primer pair RBP1F and RBP1R was 61°C, and for primer pair RBP5F and RBP5R was 64°C.

2.2.6.3 NESTED PCR

The use of nested PCR increases the sensitivity and specificity ofthe PCR when poor quality DNA is used.

(a) **First round PCR**

In this amplification, **Expand High Fidelity PCR System** (Boehringer Mannheim) was used. The system is optimized to amplify DNA fragments up to 5 kb in length. It is composed of a unique enzyme mix containing thermostable *Taq* DNA and *Pwo* DNA polymerases. This mix is designed to give PCR products

with high yield, high fidelity and high specificity from genomic DNA (up to three times increased fidelity of DNA synthesis and twice the yield compared to *Taq* DNA polymerase).

For each PCR amplification, 1 - 2μl of RE digested DNA was used in the first round of amplification. The primers used for the first round PCR were RBP1F and RBP5R, which generated a PCR product of 2028 bp.

Two reaction mixes are required. The first mix of 12,5μl contains: 0,5μl of 10mM each of dATP, dCTP, dGTP and dTTP; 1μl (50 pmol) each of the upstream and downstream primers; 1 - 2μl of target DNA and double distilled water to make up the volume.

The second mix of 12,5μl contains: 2,5μl of 10x Expand buffer (with 15mM $MgCl_2$); 1,185μl of Expand enzyme (2,6 units) and the balance as double distilled water.

The two mixes were combined on ice in a 0,5ml PCR tube and covered with a layer of mineral oil to prevent evaporation. The thermal cycling was done in a Hybaid Omnigene thermocycler. The cycling profile was as per the instructions for the **Expand** System, and consisted of: a single denaturation at 95°C for 3 minutes, followed by 10 cycles of denaturation at 94°C for 15 seconds, annealing at 59°C for 30 seconds (this varied depending on the primer pair chosen) and elongation at 72°C for 45 seconds. From cycle 11 to cycle 20, 2 seconds/cycle were added to the elongation step. The final extension was at 72°C for 7 minutes.

Due to the low number of cycles it was not possible to visualize the PCR product on an agarose gel.

(b) Second round PCR

For each PCR reaction, 1 - 2μl of PCR product from the first round PCR was used as template for amplification. The primers used for the second round of PCR were

RBP5F and RBP5R which generated a PCR product of 296 bp. A separate PCR reaction was done for each tumour DNA specimen digested with each of the methylation-sensitive restriction enzymes chosen in this region of the Rb-1 promoter (see figure 2.1 for positions of RE sites).

The PCR reaction mix was the same as that used in section 2.2.6.2

The number of cycles required to give the PCR product required in this system was deduced by doing PCR amplifications of 14, 16, 18, 20, 22 and 24 cycles, running the PCR product on agarose gel and noting the initial appearance of a faint band at low cycle number and spurious band formation when the cycle number was too high. It was determined that 20 cycles was optimum for this system.

The thermal cycling consisted of: a single denaturation step at 94°C for 3 minutes, followed by 20 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 45 seconds and elongation at 72°C for 45 seconds. The run was ended with a final extension at 72°C for 10 minutes. PCR product was visualized on agarose gel.

2.2.7 AGAROSE GEL ELECTROPHORESIS

The matrix created by agarose acts as a filter, allowing for separation of DNA fragments according to size. Large fragments migrate slower through the gel than small fragments as they encounter more resistance when travelling through the matrix. The concentration of an agarose gel is varied depending on the size of fragments to be separated.

To visualize the RB tumour DNA and the control DNA, a 2,5% agarose gel was prepared by dissolving Seakem HGT agarose in TBE buffer by boiling. After the solution had cooled to 55°C,

ethidium bromide was added to a final concentration of 0,5μg/ml. Ethidium bromide contains a planar group that intercalates between the bases of DNA. Ultraviolet irradiation induces emission of fluorescence by ethidium bromide molecules and allows visualization of DNA (54).

The gel was poured into a perspex gel mould with a gel comb in place to form loading wells, and allowed to set at room temperature.

The gel was submerged in the buffer, and aliquots of 10μl of PCR product (containing loading dye) were loaded into individual wells prior to electrophoresis. Alternate lanes contained RE digested and undigested PCR samples of the same tumour specimen respectively. A 1 kb ladder DNA marker was loaded into the first well of each gel so that the size of the fragment/s could be determined.

Electrophoresis was carried out at a constant voltage of 100V for 1 hour at room temperature. DNA was visualized on a UV transilluminator and photographed [illegible] type 667 film.

3. RESULTS

3.1 SUBJECTS

The sample size of the study was 16 individuals, and 19 tumours (from 3 subjects, two tumour specimens were studied). These consisted of nine bilateral and 10 unilateral cases and a normal control fetal retina. The details of the sex, age, eye/s enucleated and date of enucleation can be seen in Table 2 - for bilateral cases and Table 3 - for unilateral cases.

3.2 TUMOUR HISTOLOGY

Examination of both the histology reports and slides made from paraffin-embedded tumour specimenswas done. Only tumours later utilized in the methylation sensitive assay were classified histologically according to the stage of tumour progression and the extent of tumour infiltration beyond the eye. These results are shown in Table 4. Photographs of histological examples of a well differentiated tumour and a poorly differentiated tumour can be seen in Figure 3.1 and Figure 3.2 respectively.

Table 2: Data on patients with bilateral RB - Age, sex, eye/s enucleated and year of enucleation

PATIENT NUMBER	AGE	SEX	EYE	TYPE	ENUCLEATION
R1	8m	M	R	B/S	1993
R2A	3y	M	R	B/S	1993
R2B	3y	M	L	B/S	1993
R3	5y	F	R	B/S	1993
R4A	1y9m	M	R	B/S	1994
R4B	1y10m	M	L	B/S	1994
R5	1y6m	F	L	B/S	1995
R6A	2y6m	M	R	B/?F	1995
R6B	2y6m	M	L	B/?F	1995

KEY:

M = Male
F = Female
y = Year/s
m = Months
R = Right eye enucleated
L = Left eye enucleated

B/S = Bilateral sporadic (from information in files at Baragwanath)
B/?F = Bilateral, possibly familial (from information in files at Baragwanath)

Table 3: Data on patients with unilateral RB - Age, sex, eye enucleated and year of enucleation

PATIENT NUMBER	AGE	SEX	EYE	ENUCLEATION
R7	3y	F	L	1993
R8	4y	M	R	1993
R9	1y8m	M	R	1993
R10	1y8m	M	R	1994
R11	2y3m	F	R	1995
R12	1y6m	F	L	1995
R13	4y	F	R	1995
R14	5y	M	R	1995
R15	3y6m	M	L	1995
R16	2y	F	L	1995

KEY:

M = Male
F = Female
y = Year/s
m = Months
R = Right eye enucleated
L = Left eye enucleated

Table 4: RB tumour type and histology

TUMOUR SPECIMEN No.	RB TYPE (UNILATERAL OR BILATERAL)	TUMOUR HISTOLOGY
R1	B	WELL DIFFERENTIATED - CONFINED TO THE EYE
R2A	B	POORLY DIFFERENTIATED - BEYOND THE EYE (INFILTRATED INTO THE MUSCLE)
R2B	B	POORLY DIFFERENTIATED - BEYOND THE EYE
R4A	B	INFILTRATED BEYOND THE EYE
R5	B	WELL DIFFERENTIATED - CONFINED TO THE EYE
R6A	B	MODERATE TO POORLY DIFFERENTIATED - CONFINED TO THE EYE
R7	U	POORLY DIFFERENTIATED - INFILTRATED INTO THE SOFT TISSUE BEYOND THE EYE
R9	U	MODERATELY DIFFERENTIATED - CONFINED TO THE EYE
R11	U	POORLY DIFFERENTIATED - CONFINED TO THE EYE
R12	U	MODERATE TO WELL DIFFERENTIATED - CONFINED TO THE EYE
R14	U	MODERATELY DIFFERENTIATED - CONFINED TO THE EYE
R15	U	MODERATE TO WELL DIFFERENTIATED - INFILTRATED INTO THE SOFT TISSUE
R16	U	POORLY DIFFERENTIATED - INFILTRATED UP TO THE RESECTION MARGIN OF THE OPTIC NERVE

KEY:
Well Differentiated = Early Stage Tumour
Poorly Differentiated = Late Stage Tumour
U = Unilateral tumour
B = Bilateral tumour

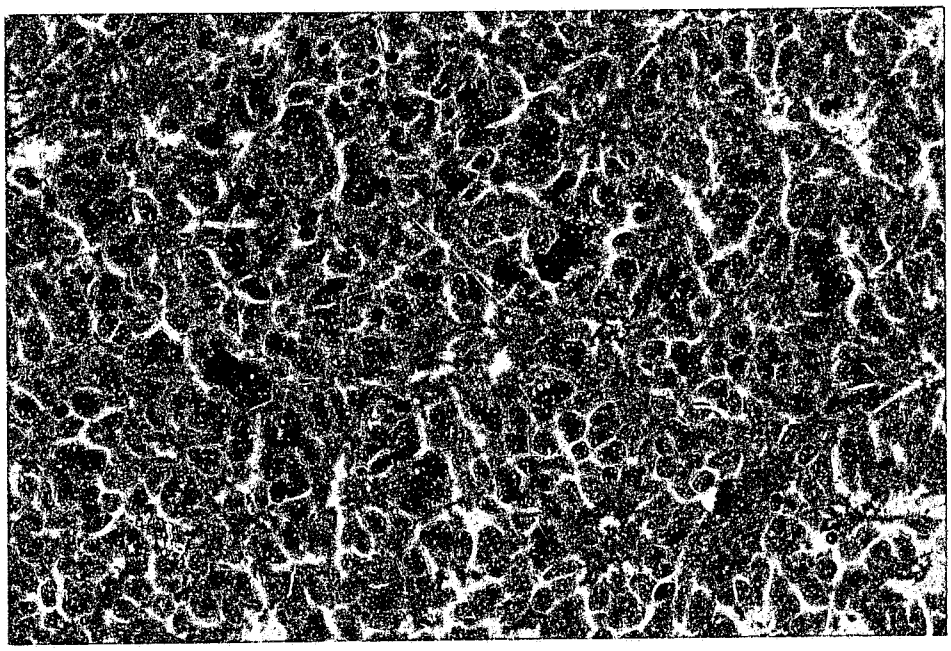


Figure 3.1: Photograph of a histological section of a well-differentiated (early stage) RB tumour (1000x magnification).

Note rosette formation (arrows) which are characteristic of a well differentiated tumour of this type.

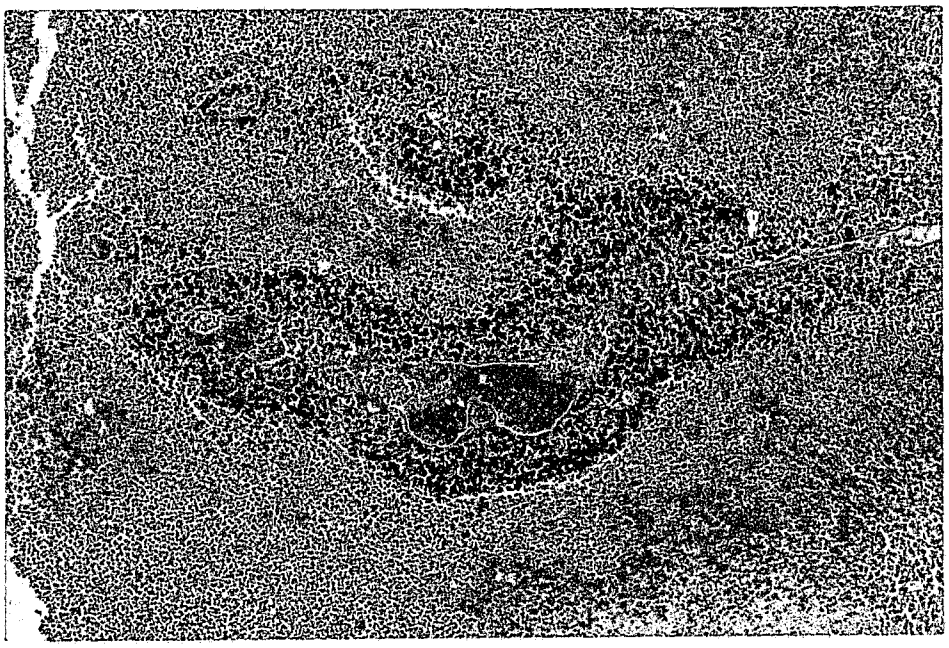


Figure 3.2: Photograph of a histological section of a poorly-differentiated (late stage) RB tumour (1000x magnification.

(Darker area = Tumour tissue; Lighter area = Necrotic tissue)

3.3 DNA EXTRACTION FROM PARAFFIN-EMBEDDED TUMOUR MATERIAL

DNA was extracted from all nineteen paraffin-embedded tumour specimens. The specimens had been fixed in formalin for some time before paraffin fixation. The use of formalin caused loss of DNA and in many cases, partially or totally degraded DNA samples were obtained. The varying quality of DNA obtained can be seen in Figure 3.3.

3.4 METHYLATION-SENSITIVE PCR ANALYSIS

3.4.1 PCR AMPLIFICATION

Two PCR systems incorporating primer pairs RBP1F and RBP1R, and RBP5F and RBP5R (sequences of primers can be found in section 2.2.6.1) in the Rb-1 promoter region required optimization. In order to optimize the PCR systems the following were varied: the amount of template DNA was increased, then decreased; the annealing and extension times were varied; the annealing temperature was decreased and increased and 5% DMSO was added to the reaction mix.

However, because the template DNA comprised of partially degraded DNA, weak PCR product bands could be observed on agarose gels (see Figure 3.4). The quality of these bands was not sufficient for the purposes of the assay. A *Sma* I and an *Fnu D* II site site would have been studied in this region of the Rb1 promoter.

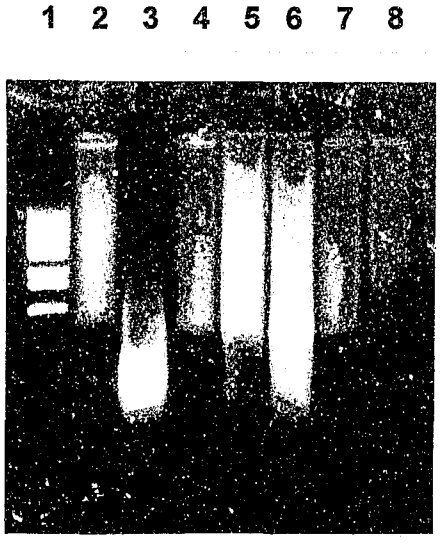


Figure 3.3 : Agarose gel showing examples of extracted tumour DNA

Lanes 2 - 8 contain tumour DNA.
Lane 1 contains 1 kb ladder.
The DNA in Lanes 2, 4, 7 & 8 is partially degraded.
DNA in Lane 3 is degraded
The DNA in Lanes 5 & 6 is of 300 bp or more.

Only samples in Lanes 2, 5, 6 & 7 could be further utilized.

(For fragment sizes of DNA molecular weight (1kb) marker- see appendix F)

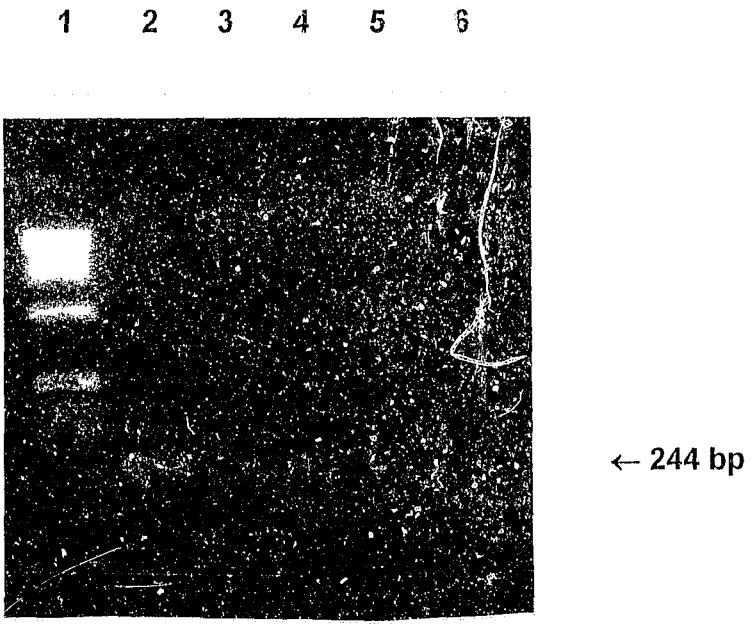


Figure 3.4: Agarose gel showing PCR product of the PCR system utilizing primer pair RBP1F and RBP1R

Lane 1 contains 1 kb ladder. Lanes 2, 3 , 4 and 5 each contain a very weak band of amplified RB tumour DNA of 244 bp. Lane 6 is blank and is a control containing no DNA to ensure that no contamination is present in the PCR system.

(For fragment sizes of DNA molecular weight (1kb) marker- see appendix F)

3.4.2 NESTED PCR

The use of nested PCR increased both the sensitivity and specificity of the PCR when poor quality DNA is used. The use of **Expand High Fidelity PCR System** in the first round of PCR resulted in an increased fidelity of DNA synthesis due to the inherent 3' - 5' exonuclease proofreading activity of *Pwo* DNA polymerase.

The first round of PCR utilizing primers RBP1F and RBP5R (their sequences can be seen in figure 2.2), was carried out according to the instructions for the **Expand** System, supplied by the manufacturers, (method as seen in section 2.2.6.3), so optimization was not required.

The second round PCR using primers RBP5F and RBP5R (their sequences can be seen in figure 2.2), and the product of the first round as template, required optimization. Although the annealing temperature had been determined in the initial PCR system, the denaturation, annealing, elongation times and the number of cycles had to be varied in order to achieve the correct cycling parameters for this system. 5% DMSO was

also added to the reaction mix and this improved the quality of DNA bands observed on agarose gel (method as seen in section 2.2.6.3).

Restriction enzyme treated samples of fetal retina DNA did not yield bands when visualized on 2,5% agarose gels. Untreated (no restriction enzyme) samples of fetal retina DNA yielded single bands of the expected size (296 bp)(For examples of both types of PCR product, see Figure 3.5).

Untreated (no restriction enzyme) DNA samples gave single bands of the expected size (296 bp) for each tumour sample amplified. DNA samples which had previously been digested with restriction enzymes and whose CpG sites were methylated, also gave single bands of the expected size (296 bp) for each tumour. Tumour DNA CpG sites when unmethylated, were cleaved by restriction enzymes and so these tumour samples yielded short sequences of DNA which could not be amplified by PCR and hence these samples yielded no band when run on 2,5% agarose gels. (For examples of all 3 types of specimens, see Figure 3.5).

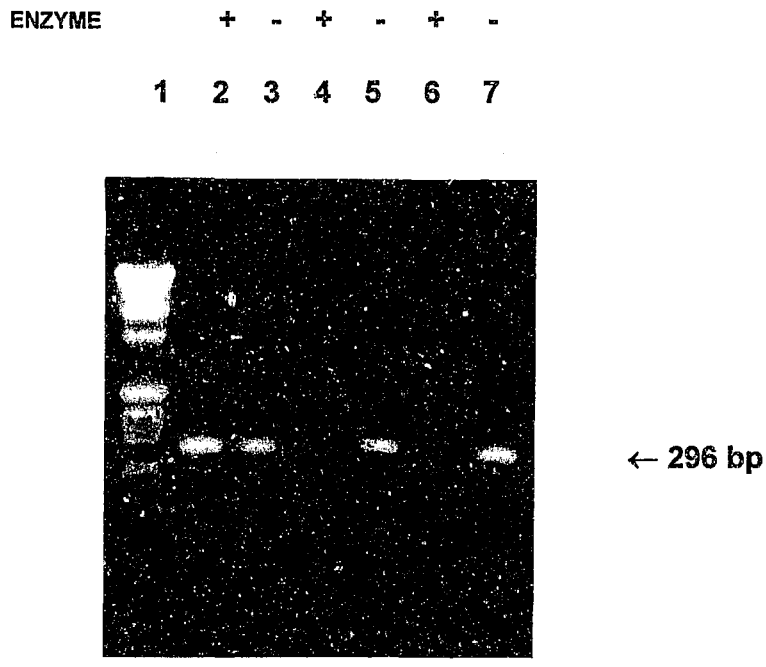


Figure 3.5: Agarose gel showing DNA digested with methylation sensitive restriction enzyme (*BssH* II) followed by PCR amplification

Lane 1 contains 1 kb ladder. Lanes 2,3,4 and 5 contain tumour DNA, and lanes 6 and 7 contain fetal retina DNA.

DNA was digested with *BssH* II, PCR amplified and run on an agarose gel. The presence of a 296 bp band in lane 2 indicates lack of cutting, hence this site is methylated in this tumour sample. Absence of a band in lanes 4 and 6 indicates that these sites are unmethylated. DNA in lanes 3, 5 and 7 was not digested.

KEY:
+ = RE digested
- = Untreated (no RE)

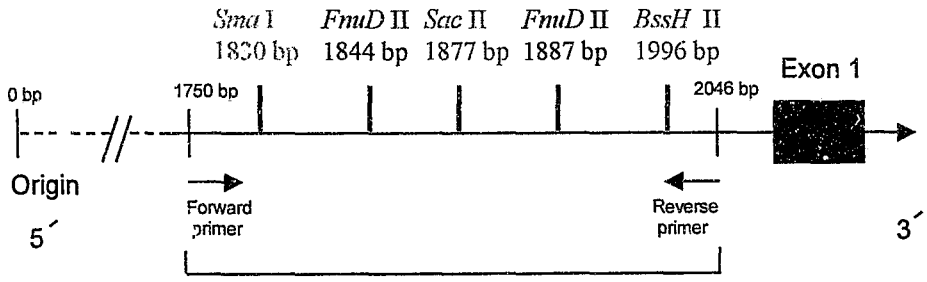
(For fragment sizes of DNA molecular weight (1kb) marker- see appendix F)

3.5 DETERMINATION OF THE DNA METHYLATION STATUS OF RB TUMOURS AND CONTROL

Within the 296 bp region flanked by primers RBP5F and RBP5R, five methylation-sensitive restriction enzyme sites were used to determine the methylation status of the thirteen RB tumour specimens and the fetal retina control.

Three bilateral and two unilateral tumours had all 5 sites methylated. PCR product of the correct size could be seen on agarose gels for all 5 sites. One unilateral tumour had 4 sites methylated. One unilateral and four bilateral tumours had 3 sites methylated. One unilateral and one bilateral tumour had only 2 sites methylated. No one methylation-sensitive restriction enzyme cutting site was always methylated in all tumour samples observed. All 5 sites were unmethylated in the fetal retina control. No PCR product was seen on agarose gel for the 5 restriction enzyme sites. (See Figure 3.6 for graphic representation of methylation status of each sample).

Due to primer design, it was not possible to distinguish between the two *FnuD* II sites ie: if either one or both of the sites was unmethylated, the result would show non-methylation. Only when both sites were methylated could a PCR product be seen, indicating conclusively that the sites were methylated.



Specimens:

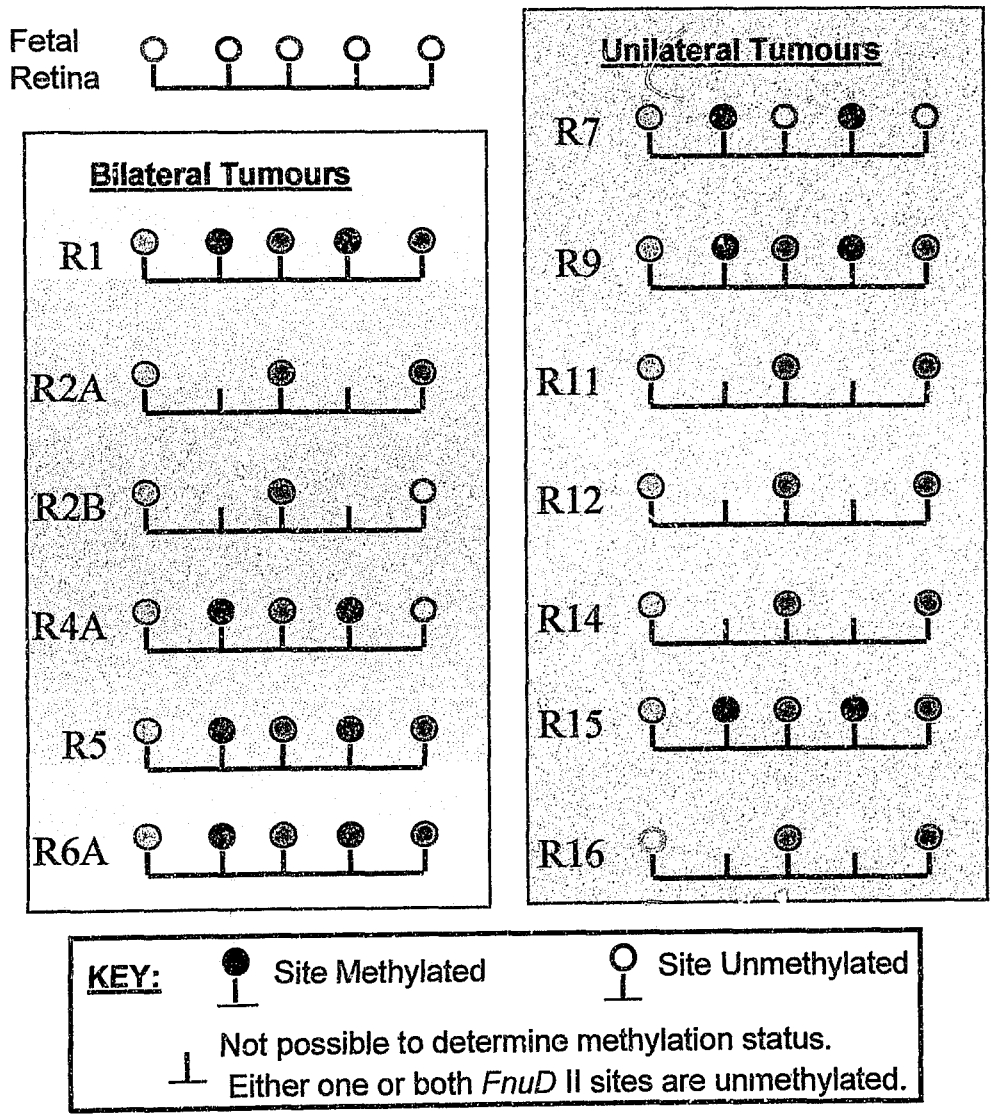


Figure 3.6 : DNA methylation status of RB tumours and normal control retina.

4. DISCUSSION

The mutation of tumour - suppressor genes contributes to tumour growth by inactivating proteins that normally act to limit cell proliferation, as is the case in retinoblastoma. Alterations of the methylation patterns of DNA are common in cancer cells and could comprise a subset of causal events in the carcinogenesis process. This study served to show the differences between the methylation patterns of the Rb-1 gene in RB tumours as opposed to normal retinal tissue. The ideal normal control would have been to obtain normal tissue adjacent to the tumour tissue for each case. But, because the specimens were not obtained from fresh tissue, this was not possible. Although the study was limited in sample size, it was able to define more accurately than previous studies, the role that DNA methylation plays in the inactivation of the Rb-1 tumour - suppressor gene. If time had permitted, a larger number of restriction enzyme sites in the Rb-1 promoter region would have been analyzed.

4.1 SUBJECTS

Of the subjects chosen for the study, there were an equal number of males and females. In previous studies, no significant differences were found in the numbers of males and females who developed unilateral sporadic RB (6,55). However, a significant bias in bilateral sporadic cases towards males was observed (6,55). This bias was also found in the present study, with four of the six bilateral sporadic cases being male. This may be because far more cell divisions occur between embryonic development and meiosis in males than in females resulting in increased *de novo* germline mutations in the sperm. Alternatively, there may be a less efficient repair mechanism in sperm precursors and embryonic cells leading to more affected male offspring (1).

The ages of the subjects ranged from 8 months to 5 years at the time of enucleation. The age range was found to be similar in both the unilateral and bilateral groups. Both groups (except for one bilateral tumour) were sporadic cases of RB. Usually, hereditary cases present

earlier because one mutated Rb-1 allele is inherited and only the second, somatic mutation has to occur in the developing retinoblast (1,55).

4.2 TUMOUR HISTOLOGY

Histologically, retinoblastoma can present differentiation zones known as rosettes, which are formations of tumour cells. The amount and size of these regions is related to the degree of malignancy. Small tumours tend to have a greater number of well formed rosettes - they are well differentiated (69). A poorly differentiated tumour has a worse prognosis for survival and has often metastasized. Thorough observation of both slides and histology reports revealed that both unilateral and bilateral tumours could be either poorly differentiated or well differentiated. The stage of differentiation is related to tumour progression. From these data, it could be concluded that both tumour types could have been in either the early stage or the late stage of differentiation. Only the severity to which the tumour had progressed when enucleation was carried out determined the stage of its differentiation.

4.3 CYTOGENETIC ANALYSIS

Deletions of 13q14 are found only in approximately 10% of RB tumours (55). Other cytogenetic defects detected in tumour cells are usually secondary alterations found in advanced stages of tumorigenesis (55). The most common ones found in RB are tetrasomy 6p and trisomy 1q (55,69).

Chromosome analysis was previously carried out on blood lymphocyte cultures from all patients in this study (70). All karyotypes were found to be cytogenetically normal ie: 46,XX or 46,XY, using both conventional Giemsa banding and fluorescence *in situ* hybridization (FISH). Since no deletions were observed cytogenetically in all cases in this study (70), this was not the cause of tumour formation in these patients. It was therefore necessary to use molecular genetic methods to try to analyze the mechanism of carcinogenesis in these cases. A methylation-sensitive PCR assay was used to determine the methylation status of the Rb-1 promoter region in each case. This could help to elucidate the role that DNA methylation played in affecting the functioning of the promoter region of the Rb-1 tumour suppressor gene and silencing its transcription in these patients.

4.4 DNA EXTRACTION FROM PARAFFIN-EMBEDDED TUMOURS

A wide range of different tissue types are stored in pathology archives. The most commonly stored is routinely processed, formalin-fixed, paraffin-embedded tissue. The ability to extract DNA from such material would allow future analysis at various stages of a disease (52). Formalin is the most commonly used fixative in routine histopathological preparation. It is a stable, non-coagulative fixative, usable with almost any tissue. It acts by forming crosslinks between protein molecules which forms a gel maintaining the relationship of the intracellular components of the tissue. Tumour samples were fixed in formalin before paraffin-fixation. Formalin is not a good fixative of DNA, as DNA in its native state does not react with formalin (52). For a reaction to occur between the DNA and formalin, it is necessary to destroy hydrogen bonds that hold together the two strands of DNA, for example by heating. This would allow the reaction of formalin with the amino groups on the bases, exposed by the uncoiling of the double-stranded DNA molecule. The bound formalin would however oppose any renaturation of the DNA on cooling (52). The poor fixation of DNA by formalin at room temperature caused the loss of DNA into the fixative solution by leaching. The length of time that a sample

remained in solution increased the amount of DNA leached. This led to large reduction in yields and quality of DNA extracted from paraffin-embedded tissue (52). To obtain optimal quality, high molecular weight DNA, the DNA should be extracted from fresh tumour tissue and then stored. However, PCR is a very sensitive technique, requiring only a small amount of template DNA. This technique could therefore amplify the semi-degraded DNA which could subsequently be analyzed.

4.5 METHYLATION - SENSITIVE PCR ANALYSIS

The methylation status of tumours in this study could only be assessed using PCR amplification, after the DNA from each specimen had been digested, separately, using various rare-cutting methylation-sensitive restriction enzymes. These enzymes only cut DNA at their recognition sites if the cytosines within these sites are unmethylated. The DNA could then be amplified using PCR and the methylation status analyzed by the presence or absence of a band on agarose gel. As a control, for each tumour specimen which was RE digested, the same tumour specimen was subjected to the identical conditions and to all the components of the digest, only addition of the RE was

omitted. These control samples were PCR amplified at the same time as the RE digested samples in order to deduce whether digestion had occurred in the samples where RE had been added.

When dealing with poor quality, degraded DNA, the use of nested PCR increases both the sensitivity and specificity of DNA amplification. The process utilizes two consecutive PCR amplifications of 20 - 25 cycles each. The first round of PCR contains an external pair of primers and the second round of PCR contains one of the primers from the first round of PCR and a single nested primer, which is internal to the first primer pair. The larger fragment produced by the first reaction is used as template for the second PCR. The result may be an increase in the sensitivity of up to a thousand times compared to 40 - 50 cycles of standard PCR (52). This may be due to the more effective denaturation of smaller size template added to the second round PCR reaction, from the first round of amplification, as opposed to high molecular weight DNA. In the system utilized, sensitivity was greatly increased. So, this may be the best method of amplification for DNA from degraded, poor quality archival paraffin-embedded tissue (52).

4.6 DNA METHYLATION STATUS OF RB TUMOURS AND NORMAL CONTROL RETINA

DNA from the normal fetal retina was found to be unmethylated at all of the methylation-sensitive restriction enzyme CpG sites analyzed. This result was as expected of a CpG island of a tumour-suppressor gene in normal tissue which is highly conserved and protected from methylation (21,22,35,65). These findings with regard to CpG islands in normal tissue were also observed in previous studies, which showed that approximately 1% of CpG's in the genome are clustered in unmethylated areas or islands. The sequences flanking the islands are heavily methylated (27,35). Other studies have shown specifically that the Rb-1 promoter is completely unmethylated in normal tissue specimens (56). The finding that all sites analyzed were unmethylated in the promoter region of the Rb-1 gene in the normal fetal retina tissue was therefore consistent with the findings in other studies. The normal control, being unmethylated at all of the analyzed sites, was a consistent indicator that normal tissue is always unmethylated. It also showed that complete RE digestion had occurred for each of the different REs used. Although the normal retina was a constant and reliable control in this study it was not the ideal control. The ideal

controls would have been if it had been possible to PCR amplify DNA extracted from retina tissue of unaffected (normal) areas of the same enucleated eye as that from which tumour material had been taken from each patient. DNA had been extracted from fresh retinal tissue which had been stored in liquid nitrogen. The quality of DNA extracted from this tissue was therefore better than that extracted from formalin-fixed archival tumour specimens. The quality of DNA could also have been improved if the DNA had been extracted from fresh tumour tissue directly after enucleation. Only one fetal retina could be obtained, so there was only a single normal control.

All tumour specimens analyzed were methylated at two or more methylation-sensitive restriction enzyme CpG sites analyzed. All of the sites observed were sometimes methylated in any tumour, indicating that no one site in the region analyzed is more important than any other in the initiation and/or progression of RB. Both unilateral and bilateral RB tumours exhibited similar methylation patterns. The methylation patterns of early and late stage RB tumours were also found to be similar. This indicated that methylation must have occurred early on in tumour formation. In previous studies of

DNA methylation in RB tumours, both Sakai *et al* (28) and Ohtani - Fujita *et al* (33) observed hypermethylation in 16% of unilateral sporadic cases and Greger *et al* found 13% of sporadic unilateral tumours to be hypermethylated (29). Most recently, Stirzaker *et al* observed extensive DNA methylation in the promoter region of all unilateral RB samples with no observable mutations in their study. Their study did not include any bilateral RB tumour specimens (56).

The hypermethylation of the 5′ CpG island in all RB tumours in this study could be responsible for gene inactivation and initiation and/or progression of tumorigenesis in these individuals. Greger *et al* have noted that changes in the methylation pattern in Rb-1 play a role in the development of some RB tumours by interfering with gene expression and inactivating the tumour-suppressor gene (32). Methylation across the Rb-1 CpG island could also substantially influence the expression levels of Rb-1 by reducing promoter activity by up to 92% (33,56). Transcription factors were also found to be unable to bind to their recognition sequences when these sites were methylated in RB tumours (29,33). A selective advantage of proliferating cells with a high degree of methylation at the Rb-1 CpG

island supports an hypothesis that hypermethylation of Rb-1, stimulates tumour cell proliferation (29). This process is aided by a lack of tumour suppressor activity.

Previous RB studies have shown that promoter activity is reduced and that transcription factors are unable to bind to their recognition sequences when these sites are methylated. These findings are in accordance with other studies on other genes, previously carried out on the contribution of DNA methylation in the silencing of other tumour-suppressor genes. These include: p15, p16 (MTS1), p53, Von-Hippel-Lindau (VHL) syndrome gene, E-cadherin, Wilm's tumour (WT-1) gene, Neurofibromatosis type 1 (NF1) and type 2 (NF2) genes and adenomatous polyposis coli (APC) gene (22,31,32,33,34,48,56, 57,58,59,60). In the present study, a specific region of the Rb-1 was selected, and it was therefore possible to pinpoint specific sites in the promoter region that are methylated and therefore play a role in silencing of the Rb-1 gene.

Global methylation level changes and methylation modifications in individual genes have been observed in tumours involving

chromosomes 3p in VHL syndrome, 11p in Wilm's tumour and 17p in NF1 (22,3,57). DNA methylation has been found to influence transcription factor binding, by interfering with their ability to attach to their binding sites. This is the case in retinoblastoma and VHL syndrome (22,27,56,61). Methylation also directs chromatin into an inactive structure affecting the positioning of nucleosomes and so inhibits transcription (66). Methylation therefore affects DNA integrity and function and leads to malignant transformation (22,56,61).

Certain genes with a tissue-restricted pattern of expression (ie: tissue-specific genes) have shown a direct correlation between gene activity and hypomethylation of their promoter regions and this mechanism allows specific cellular differentiation and somatic organization to occur (21,22). These tissue-specific genes are unmodified in their cell type of expression but are heavily methylated in non-expressing cells (65). Hypermethylation of DNA in normally unmethylated CpG islands associated with gene promoter regions is generally able to repress gene expression. In tumour-suppressor genes, this may result in carcinogenesis (20,56,58,59,60,62,63).

Methylation of the VHL tumour-suppressor gene has been observed in tumours of individuals with VHL syndrome. In inherited cases, the first hits were classical germline mutations in one allele of the VHL gene. The second mutations were methylation of the other allele and resulted in somatic inactivation. DNA methylation in sporadic cases, leads to aberrant functioning of transcription factors and inactivated both alleles of the VHL gene (57,58,64). The same mechanisms could be responsible for the silencing of other tumour-suppressor genes as well.

Aberrant functioning of transcription factors or their inability to attach to their binding sites due to *de novo* DNA methylation of these sites may be the cause of tumorigenesis in RB in the absence of any predisposing mutation (22,56). Hypermethylation has been observed in both unilateral and bilateral tumours. All cases in this study (except for one which may be inherited) were sporadic, according to the data in the patient files. From the data analyzed in this study, it can be observed that DNA methylation may therefore be the mechanism responsible for silencing of the Rb-1 tumour-suppressor gene in these RB tumours.

5. CONCLUSION

This study has identified that:

(i) Extensive DNA hypermethylation occurred in the Rb-1 promoter region CpG island in RB tumours in the South African black population .

(ii) No methylation occurred in the Rb-1 region CpG island in normal retina tissue.

(iii) No one methylation-sensitive restriction enzyme CpG site analyzed appeared to be more critical in Rb-1 tumour-suppressor inactivation that any other.

(iv) Methylation pattern of tumour tissue was clearly different to that of normal tissue.

(v) There are no differences in the methylation patterns of well and poorly differentiated tumours.

(vi) Of the cases studied, a larger number of bilateral sporadic cases were male. Further studies with a larger sample size are necessary to evaluate the importance of this finding.

(vii) A higher number than reported bilateral sporadic cases were observed in the southern African negroid population sample analyzed. The sample size was small, so further studies with a larger sample size are necessary to evaluate the importance of this finding.

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APPENDIX A

SOLUTIONS:

2,5% AGAROSE GEL

Mix 2,5g HGT Agarose
100ml 1 x TBE

Stir and boil until mixture is completely dissolved giving translucent appearance. After cooling add 30mg Ethidium bromide.

CHLOROFORM

Chloroform used in protein removal consists of 24:1 chloroform:isoamyl alcohol. Chloroform allows for denaturation of protein and separation of the aqueous and organic phases. Isoamyl alcohol reduces foaming.

DNA MOLECULAR WEIGHT (1 kb) MARKER

Mix: 220μl (50μg) DNA Marker
250μl Ficoll dye
750μl TE (pH 7.5)

Vortex, aliquot into Eppendorfs and Store at 4°C.

DEOXYNUCLEOTIDES (dNTPs)

Dissolve each deoxynucleotide, dATP, dCTP, dGTP and dTTP in distilled water to a concentration of approximately 100mM. Adjust each deoxynucleotide solution to pH 7.0 with 1 M Tris-base. Absorbance of each aliquot is measured at the wavelengths shown below and the molarity calculated using the formula:

Molarity = Absorbance x Dilution factor / Extinction coefficient

BASE	WAVELENGTH(nm)	EXTINCTION COEFFICIENT
A	259	1.54×10^4
C	271	9.10×10^3
G	253	1.37×10^4
T	260	7.40×10^3

Dilute deoxynucleotides to 10mM, aliquot and store at -20°C.

dNTPs FOR PCR:

Mix 125μl each of 10mM stock of dATP, dCTP, dGTP and dTTP with 500μl of double distilled water.

0.5M ETHYLENEDIAMINE TETRA-ACETIC ACID (EDTA)

Dissolve: 18,61g $Na_2EDTA.2H_2O$

Adjust pH to 7.5/8.0 with NaOH, make up to 100ml with dH_2O, and autoclave.

ETHIDIUM BROMIDE (EtBr)

To prepare 10mg/ml stock, dilute 0,1g EtBr in 10ml sterile distilled H_2O, and shake overnight until dissolved. Store in a dark container at 4°C.

FICOLL DYE

Mix: 50% Sucrose = 10ml 0,5M stock

50mM EDTA = 10ml 0,5M stock pH 7,0

0,1% Bromophenol Blue (BPB) = 0,1g

10% Ficoll = 10g

Make up to 100ml with distilled H_2O. Allow to dissolve overnight and then filter through Whatman no. 1 filter paper.

1M MAGNESIUM CHLORIDE ($MgCl_2$)

Dissolve: 20,33g $MgCl_2.6H_2O$

Make up to 100ml with dH_2O, and autoclave.

1M MAGNESIUM SULPHATE ($MgSO_4$)

Dissolve: 24,68g $MgSO_4$

Make up to 100ml with dH_2O, and autoclave.

PHENOL

Melt phenol at 65°C. Extract several times with equal volume 0,1 M Tris pH 8.0, until the pH of the phenol phase is greater than 8.0. Add 8-hydroxyquinoline to a final concentration of 0,1%. Extract once more with Tris until the pH of the aqueous phase reaches 8.0. Store at 4°C.

3M SODIUM ACETATE (NaAc)

Dissolve: 40,81g $NaAc.3H_2O$

Adjust the pH to 4.6 with glacial acetic acid, make up to 100ml with dH_2O, and autoclave.

10% SODIUM DODECYL SULPHATE (SDS)

Autoclave 100ml dH_2O.

10g SDS

90ml sterile dH_2O

Heat to disslove, adjust pH to 7.2 with concentrated HCl and make up to 100ml with sterile dH

APPENDIX B:

ETHICS CLEARANCE FROM THE COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Israelstam

CLEARANCE CERTIFICATE PROTOCOL NUMBER M 950614

PROJECT Investigation of the role of Methylation and deletions in the aetiology of retinoblastoma (RB)

INVESTIGATORS Ms M B J Israelstam

DEPARTMENT Human Genetics, SAIMR

DATE CONSIDERED 950630

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 950710

CHAIRMAN. .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr TJL De Ravel
Dept of Human Genetics, SAIMR

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE..... .....SIGNATURE .....

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division
of Deputy Registrar (Research)

M E M O R A N D U M

TO: Ms M B J Israelstam
c/o School of Pathology, Human Genetics, SAIMR

FROM: Mrs Pam Kissane
For: Deputy Registrar (Research)
Tel: 716-3556 Fax: 339-5708

DATE: 6 September 1995

REF: R14/49

Protocol No 950614

The amendment/extension to the above protocol, as submitted in your letter of 28 August 1995, has been approved by the Acting Chairman of the Committee for Research on Human Subjects (Medical), Professor W J Kalk.

/pb

APPENDIX C

SEQUENCE OF THE RB GENE:

Bases 1 - 2700 (Exon 1 begins at 2060)

ORIGIN

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1     ggatccagattcttttgaaattcctcctgcaccaatatcagcatttctaccttctctgta
61    ggttggcttgcctcacgttacaatggctgcagcaatcagaagtgtcacatcctcagtaat
121   acttaatattattactattatttaataatatttagaactgtgccatccctgtttcaattt
181   atcaggctcccagcagactactccttatctttcaaatgtcaaaactgcatcctgagctct
241   tgcctaaactaatctggggtgaggtgaatggaagtagcactttaattgtattcattcttt
301   gtagctggacctgggcctgggctatctcctgacatttgcccacaagaaagatttctgaag
361   ttaggtaggaatggctgttgagtaggccagtgcttgccaaacctttacacatcctcacat
421   atgtcataatatgcagataaaaagataatcc cttatacaacttgctgggataaactcagg
481   aggcttacagcatgacctgcttgaaggttcttcctgccttagaccttgctcagctgctcc
541   aggatgaggggatttacatcacagcacaactgtattttattcacagcataaaccatct  c
601   ttccttctcagttgacgagttcagatgggcaataacagtgtctgccaaagagaaaaaaaa
661   atgtattcaaactagataatctattggtacaaataccgagacacagaagtgataacagct
721   ttaagccaatgtttgatggtggtagtcccagcaagctcttttctgatgtctttgtgcctt
781   tgcacatgctccttctctgtcactgttttcttcatcaaacataatataatggacaagtgg
841   aatcaaatagaattgagttcaaattctctgctacccatcggccctggtattggacaaatt
901   aactcctctgagcctgtttcctcatctgcaacgtagactagctaatactacccattggaa
961   agcgttgtttcttagctaatgcatgcaaggcttaaaacctagatgacgggttgataggtg
1021  cagcaaacctccatggcatacgtatgcctatgtaacaaacctacacgttctgcacttgta
1081  tcccggaacttaaagtaaaaaaaaaaaaaaaaaaaaaaagaaagaaagaaaaagaaaaaa
1141  aaggctgtttctggggattaaataagacaattatgtaaggtggccagcacagttcctggt
1201  acatagtaaatgtcaggcctgcctgacagacttctattcagcagc  ctgctcccctgaa
1261  aatcttcctcagacgtttccacggtgcttcccgttcttacaccac  caatcctttatta
1321  cactactatccgttcattccccacagctccctccctcctttccctaaccagtgatccca
1381  aaaggccagcaagtgtctaacattttctatcttctaagtgactggtaaagttccgcacct
1441  atcagcgctccaagtttgtttttgttttggccgactttgcaaaacggattgggcgggatg
1501  agaggtgggggcgccgccaaggagggagagtggcgctcccgccgagggtgcactagcca
1561  gatattccctgcgggcccgagagtcttccctatcagaccccgggatagggatgaggccc
1621  acagtcacccaccagactctttgtatagccccgttaagtgcaccccggcctggaggggt
1681  ggttctgggtagaagcacgtccgggccgcgccggatgcctcctggaaggcgcctggaccc
1741  acgccaggtttcccagtttaattcctcatgacttagcgtcccagcccgcgcaccgaccag
1801  cgccccagttccccacagacgccggcgggcccgggagcctcgcggacgtgacgccgcggg
1861  cggaagtgacgttttcccgcggttggacgcggcgctcagttgccgggcgggggagggcgc
1921  gtccggtttttctcaggggacgttgaaattatttttgtaacgggagtcgggagaggacgg
1981  ggcgtgccccgacgtgcgcgcgcgtcgtcctccccggcgctcctccacagctcgctggct
2041  cccgccgcggaaaggcgtcatgccgcccaaaaccccccgaaaaacggccgccaccgccgc
2101  cgctgccgccgcggaacccccggcaccgccgccgccgccccctcctgaggaggacccaga
2161  gcaggacagcggcccggaggacctgcctctcgtcaggtgagcgagcagagccgccgtcgc
2221  ctcacgcgggaagggcgccccgggtgtgcgtagggcgggcgcaaggcggctcggcgggga
2281  cccgtcctcgccaggggccgggtcccggcgggaggaggcgccctccctgccccccgccac
2341  ggcggagcgtctgcagaatggtgacaggattctgggttcttgggcgaggggtctcggctt
2401  caacttgacaggtgtcgggcgggtggggctagggtcctgagcgaagtgacaggtgcagtt
2461  ccctcttgtgaggctcggaggcagagggtcgttgcgagcgtccatcagacgcaaaaaatg
2521  aaaaataaaaatacaaaaatggtgtctgtgggagagtttttcaccggagaattggagtac
2581  tccggtggtcgtctgactttctgttttggttcacgcgatgcaacagttgggaagtatttt
2641  cttccgggcgtgcactgcatctgaagtccatttgtgggagaggccgaccagaaagccttg
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APPENDIX D

OLIGONUCLEOTIDE PRIMER REHYDRATION:

To calculate the amount of double distilled water (ddH_2O) required to rehydrate primer DNA supplied:

Work out the **EXTINCTION CO-EFFICIENT (EC)** for each primer ie: for every:

A x 15,4
C x 7,3
G x 8,8
T x 11,7

Add all values together to give the EC.

$$[\text{Concentration}] = \frac{OD}{EC} \times 10\,000 \text{ (Dilution factor)}$$

= No. μl ddH_2O required

= [Stock] = 100pMol /μl = 100μM

FOR PRIMER RBP1F:

5 x A = 77
4 x C = 29,2
10 x G = 88
1 x T = 11,7

EC = 205,9 & THE OD IS 16,0

$$[] = \frac{16,0}{205,9} = 0,0777076 \times 10\,000$$

= **777,08μl**

FOR PRIMER RBP1R:

(6A + 4C + 6G + 4T) =

(92,4 + 29,2 + 52,8 + 46,8) = EC = 221,2

OD = 18,1, [] = $\frac{18,1}{221,2}$ = 0,0818264 x 10 000

= **818,26μl**

FOR PRIMER RBP5F:

(4A + 6C + 1G + 9T) =

(61,6 + 43,8 + 8,8 + 105,3) = EC = 219,5

OD = 23,6 , [] = $\frac{23,6}{219,5}$ = 0,1075170 x 10 000

= **1075,17μl**

FOR PRIMER RBP5R:

(3A + 5C + 10G + 2T) =

(46,2 + 36,5 + 88 + 23,4) = EC = 194,1

OD = 31,7, [] = $\frac{31,7}{194,1}$ = 0,1633178 x 10 000

= **1633,18μl**

APPENDIX E

QIAmp TISSUE EXTRACTION PROTOCOL

1. Cut 25mg tissue and put into a microcentrifuge tube with 180μl of buffer ATL (provided in kit).
2. Add 20μl Proteinase K, mix and incubate at 55°C overnight.
3. Add 20μl RNAse (20mg/ml), mix and incubate at room temperature for 2 minutes. Then add buffer AL (provided in kit), mix well and incubate at 70°C for 10 minutes.
4. Add 210μl of ethanol and mix well.
5. Place a QIAmp spin column in a 2ml collection tube. Pour the mixture into the column, close the cap and spin in a microcentrifuge at 6000 xg (8000rpm) for 1 minute.
6. Put the column into a clean 2ml collection tube and discard the tube with the filtrate.
7. Carefully open the spin column and add 500μl of buffer AW (provided in kit). Centrifuge at 6000 xg (8000rpm) for 1 minute. Put the column into a clean 2ml collection tube and discard the tube with the filtrate.
8. Carefully open the spin column and add 500μl of buffer AW (provided in kit). Centrifuge at 6000 xg (8000rpm) for 1 minute and at full speed for 2 minutes.

9. Put the column into a clean 2ml collection tube and discard the tube with the filtrate.

10. Carefully open the spin column, elute DNA twice with 200μl of TE buffer of distilled water, preheated to 70°C. Incubate for 1 minute at room temperature and centrifuge at 6000 xg (8000rpm) for 1 minute.

NOTE:

(a) 5 minute incubation at 70°C after adding 200μl of TE buffer may increase the yield of DNA.

(b) A third elution with the same 400μl of buffer containing DNA, reheated to 70°C may increase the DNA yield by up to 15%.

APPENDIX F

FRAGMENT SIZES OF DNA MOLECULAR WEIGHT (1kb) MARKER