Cytogenetic, Mutation Detection, Methylation Status, Fragment Analysis of RB1 Gene and Phenotype Correlation in Retinoblastoma Patients from India

THESIS

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By

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CERTIFICATE

This is to certify that the thesis entitled **"Cytogenetic, Mutation Detection, Methylation Status, Fragment Analysis of RB1 Gene and Phenotype Correlation in Retinoblastoma Patients from India"** submitted by **Biju Joseph ID No. 1999PHXF412** for the award of Ph.D. Degree of the Institute, embodies the original work done by him under my supervision.

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Abstract of thesis

Cytogenetic, Mutation Detection, Methylation Status, Fragment Analysis of RB1 Gene and Phenotype Correlation in Retinoblastoma Patients from India

Retinoblastoma is an intra-ocular malignancy in children less than 5 years of age and has an incidence of 1 in 20,000 live births. Multiple genetic and molecular events result in two hits of the RB1 gene derailing the normal mitotic process. Detecting these events is a complex process that needs dedicated personnel and laboratory. Diagnostic testing of the proband and closely related young siblings could avoid frequent eye examination under anesthesia necessary in clinical management. In the current study, tumor DNA was subjected for mPCR, followed by fluorescent sequencing of exons 1-27 of RB1 gene on ABI 310 sequencer. Methylation analysis of RB1 promoter was done by methylation-specific PCR and silver staining. Log transformation statistical method was used to derive meaningful peripheral blood karyotype results. The retinoblastoma genetic testing was evaluated for cost saving when compared to clinical management. A detailed genotype-phenotype correlation was done for 11 retinoblastoma patients with RB1 mutations. Eight mPCR amplifying 18 exons of RB1 gene resulted in 33.3% saving of tumor DNA, 46% of PCR thermal cycler time and 30% of reagents compared to uniplex PCR. Eleven mutations including four novel were identified with seven (CGA) arginine, two serine, one splice and an 8 bp deletion mutations and all resulted in stop codons. Fragment analysis was established as a rapid mutation screening method for the 8-bp deletion mutation. Out of 45 patients, three showed hypermethylation (6.6%) of RB1 promoter. Deletion of 13q14 region was detected in 11.8% of the 59 patients who underwent karyotyping. Analysis showed significant cost-saving in genetic testing compared to clinical diagnosis. The diagnostic model offers 22-fold cheaper genetic testing for retinoblastoma patients compared to the cost of Richter *et al*, (2003). Genotype-phenotype correlation established a severe form of retinoblastoma in particularly when it happens in the N terminus of the RB protein. Collectively this study has established an effective molecular genetic diagnostics for retinoblastoma patients at the Institution and such a service with genetic counseling is not available anywhere else in south Asia.

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ABBREVIATIONS/ SYMBOLS

Ab	Antibody
BL	Bilateral
bp	Base Pairs
BLAST	Basic Local Alignment Standard Tool
BRCA1	Breast Cancer Related, A1
CDH1	Cadherin 1
CpG	Cytosine Phosphorylated Guanine
CT	Computerised Tomography
DAPK	Death Associated Protein Kinase
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	Dimethyl Sulfoxide
dNTP	deoxy Nucleotide Triphosphate
EMQN	European Molecular Genetics Quality Network
EUA	Examination Under Anesthesia
FISH	Fluorescent In Situ Hybridization
FNAB	Fine Needle Aspiration Biopsy
GDB	Genome Data Bank
GTG	Giemsa Trypsinisation Giemsa
HIC1	Hypermethylated In Cancer 1
kb	Kilo Bases
LOH	Loss Of Heterozygosity
MLH1	Mut L homologue1
mM	milli Molar
MGMT	Methyl Guanine Methyl Transferase
mPCR	Multiplex Polymerase Chain Reaction
MS-PCR	Methylation Specific Polymerase Chain Reaction
MSH2	Mut S Homologue 2
$_{P}15$	Inhibitor Of Cyclin Dependent Kinase 4b
$_{P}16$	Inhibitor Of Cyclin Dependent Kinase 4a
PAGE	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PRPF8	Pre-mRNA Processing Factor 8 homologue
pRB	Retinoblastoma-associated protein
QM PCR	Quantitative Multiplex PCR
RB	Retinoblastoma
RB1	Retinoblastoma Susceptibility Gene
RPE65	Retinal Pigment Epithelium 65
RPMI	Rosewell Parker Memorial Institute Medium
SSCP	Single Strand Conformation Polymorphism
UL	Unilateral
VHL	Von Hippel Lindau

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Overview of retinoblastoma

Retinoblastoma is a malignant tumor arising from undifferentiated retinal progenitor cells of eye with an incidence of 1: 20,000 live births. The disease usually occurs in less than five years of age group. Clinical diagnosis, requiring frequent eye examination for the proband, if delayed, results in enucleation of eye and/or poor prognosis. Successfully treated children require frequent follow-up examinations for early detection of new or recurrent tumors. Identifying retinoblastoma patients with germline RB1 mutations is important due to the increased risk for second primary cancers later in life. Gallie *et al*, (1995) used genetic testing followed by premature delivery and early treatment to reduce visual morbidity in retinoblastoma. Richter *et al*, (2003) have highlighted benefits of earlier treatment, lower risk, avoiding unwanted ophthalmic surveillance and informed family-planning decisions due to genetic diagnosis in retinoblastoma.

Retinoblastoma occurs due to functional inactivation of both alleles of RB1 gene in retinal cells. This could be due to chromosomal errors (deletion and rearrangements involving 13q14 region), gene mutations and hypermethylation of CpG islands in RB1 promoter. The present study is to evaluate the benefits of a genetic diagnostic model compared to conventional clinical screening for retinoblastoma. The approach comprised of multiplex PCR (Chapter 1), mutational screening and fragment analysis of RB1 gene (Chapter 2), methylation analysis of RB1 promoter (Chapter 3), karyotype analysis (Chapter 4), cost analysis of screening (Chapter 5), diagnostic model (Chapter 6) and genotype-phenotype correlation analysis (Chapter 7).

Chapter 1

Multiplex PCR for RB1 gene

1.1 Aim

- To establish multiplex PCR for 27 exons of RB1 gene

1.2 Review of Literature

Generally PCR is employed to amplify single target sequence with a pair of oligonucleotide primers. These strategies, even though useful for mutational screening of small genes or genes with mutational hotspots, will not be cost or labour-efficient for large or multiple genes with many target sequences. In addition, such an approach is limited by the DNA quantity; especially from irrecoverable samples like tumor. Considerable saving of reagents, time and effort can be achieved by simultaneously amplifying multiple sequences in a single reaction, a process referred to as multiplex PCR. Chamberlain *et al*, (1998) pioneered mPCR for the amplification of dystrophin gene in Duchene muscular dystrophy. mPCR was subsequently applied to situations, varying from molecular detection of multiple organisms from limited amount of specimen in a diagnostic setting, to amplification of large number of exons in a single reaction required for subsequent analysis, as in a mutational screening program or for genotyping (Cerro *et al*, 2002; Monstein and Ellnebo-Svedlund, 2002; Obata-Yasuoka *et al*, 2002; Ohtaki *et al*, 2002). mPCR amplification of short exon fragments corresponding to 19 MLH1 and 16 MSH2 exons in four separate tubes by fluorescent primers has been used for detection of exon deletions and duplications in colorectal cancer (Charbonnier *et al*, 2000). mPCR applied to 14 exons of RPE65 gene in patients with retinal degenerative diseases enabled rapid mutational screening (Joseph *et al*, 2002).

One of the major goals in the treatment of patients with retinoblastoma is to improve the clinical usefulness of genetic testing for patients and their families. Despite the use of molecular techniques, establishing a single genetic test is not possible because of the large size and distribution of all the types of mutations throughout the RB1 gene with no mutation hotspots (Lohmann *et al*, 1992). Li and Vijg (1996) used pre-amplification by long-distance PCR followed by multiplex co-amplification of 24 exons for RB1 mutational screening. Orsouw *et al*, (1996) applied PCR multiplexing to amplify all the RB1 coding regions except exons 1, 15 & 16, prior to mutational screening by two-dimensional electrophoresis. Du and Gallie (1999) detected mutations in 50% of retinoblastoma patients by quantitative fluorescent mPCR for RB1 gene. Simpson *et al*, (2000) used mPCR for identification of homozygous deletion of exons 20-24 of RB1 gene from pituitary tumor DNA. A quantitative mPCR technique combined with high-resolution fragment analysis has been shown to detect 37% of RB1 mutations (Lohmann *et al*, 2002). Richter *et al*, (2003) developed quantitative multiplex and allele specific multiplex PCR for four recurrent mutations, R251X, R358X, R445X and R579X. In this way, the direct cost of mutation screening of RB1 gene was reduced by one-third and the time for analysis was reduced to less than three weeks (Richter *et al*, 2003).

Apart from retinoblastoma, mutational inactivation of RB1 gene is associated with a limited group of non-familial tumors, including small cell lung cancer, sarcomas and melanomas (Simpson *et al*, 2000). In hereditary retinoblastoma, lipomas could be considered as a clinical marker for susceptibility to sarcomas (Rydholm and Berg, 1983). Heritable retinoblastoma patients have a life long risk of 20% for second malignancies, osteogenic sarcoma during childhood and breast or bladder cancer later (Hurwitz *et al*, 2003) and could be dependent on the RB1 mutations (Li *et al*, 1997). The lung cancer incidence among hereditary retinoblastoma patients is higher compared to non-hereditary retinoblastoma cases (Kleinerman *et al*, 2000).

On an average every year 60 new retinoblastoma patients undergo treatment at the department of vitreo-retina, Sankara Nethralaya. Nearly half of these were one-child families. A molecular diagnostic method for RB1 gene will be useful for these families in the decision to have another child or not. In the present study mPCR for 27 RB1 exons is described.

1.3 Methodology

In the laboratory initially PCR for the exons 1-27 of RB1 gene were standardized in individual 20 µL reactions containing 100-150 ng DNA, 4-6 pM of primers, 40 nmoles of each dNTPs, 1X PCR buffer and 0.6 units of *Taq* DNA polymerase. For multiplexing, the exons were arranged in ascending order of product sizes in an excel worksheet. The exons were segregated into different clusters so that exons within each cluster were sharply demarcated from other clusters in product sizes. These were further rearranged so that annealing temperature of each exon of a cluster will be around the mean annealing temperature of the exons (mean ± 2) within the cluster and also distinct product sizes to be separated in agarose electrophoresis. In this way, maximum numbers of exons were included in each cluster. The annealing temperatures and product sizes of the RB1 exons are given in Table 1.

Exon fragments of each multiplex group were co-amplified from 150 ng of DNA extracted from peripheral blood using flanking primer sequences from the GDB human genome database (www.gdb.org) and synthesized at Bangalore Genei Ltd, India. PCR was carried out in PE 2700 thermal cycler in 20 µL reactions with 6 pM of each primer, 0.9 units of *Taq* DNA polymerase and reagents from Bangalore Genei Ltd, India. Denaturation for 45 seconds and 60 seconds of annealing and extension was used for all the mPCRs. Annealing temperature and number of thermal cycles were varied till amplified products with good yield could be obtained consistently.

Exons that could not be multiplexed due to the closeness of the product sizes or lack of close by annealing temperature were amplified in individual reactions, and if possible, in a single PCR machine run. The amplified products were electrophoresed in 2% agarose with ethidium bromide stain and visualized in *Amersham Pharmacia* gel documentation system. The mPCR products were ethanol precipitated overnight at -20^{0}C, washed with 70% alcohol, air-dried and dissolved in 20 µL of autoclaved milli Q water. These products were used for sequencing PCR with fluorescent dNTPs. The primer giving better sequence data, especially when the exonic sequences commence within the initial 50 bases of the sequence output, is used for cycle sequencing. The time and cost saving by mPCR was calculated in comparison with uniplex PCR.

Table 1: All RB1 exons, annealing temperature and product sizes

Exon	Sense primer/Anti sense primer	Annealing temperature	Product size
1	5'GCGAATTCGTGCGCGCG CGTCGTCCTCC3' 5'GCGAATTCGGCCCCTGG CGAGGACGGGTC3'	62^{0}C	318 bp
2*	5'TTGATTTATAAGTATAGCC3' 5'TTTTGTATAGTGATTTCGC3'	49^{0}C	153 bp
3*	5'AGTTTTAACATAGTATCCAG3' 5'TTTCCTTTTATGGCAAGAGGC3'	54^{0}C	125 bp
4*	5'GAATTGAAATATCTATGATT3' 5'CTAATTGTGAACAATGACAT3'	45^{0}C	262 bp
5*	5'TACTATGACTTCTAAATTA3' 5'CTAACTATCAAGATGTTTGA3'	49^{0}C	176 bp
6*	5'TGGAAAACTTTCTTTCAGTG3' 5'GAATTTAGTCCAAAGGAATG3'	49^{0}C	207 bp
7	5'CCTGCGATTTTCTCTCATAC3' 5'GCAACTGCTGAATGAGAAAG3'	53^{0}C	238 bp
8*	5'ATTTTATATGATGGATGTAC3' 5'ATCTAAATCTACTTTAACTG3'	50^{0}C	159 bp
9*	5'AGTCAAGAGATTAGATTTT3' 5'CAATTATCCTCCCTCCACAG3'	52^{0}C	202 bp
10*	5'GAGATGTAAAGGATAATTGT3' 5'AGCTAAAGACTATATAATCT3'	47^{0}C	233 bp
11	5'GATGCATAAAGCAAATTG3' 5'CTGAAACACTATAAAGCCAT3'	49^{0}C	205 bp

Exon	Sense primer/Anti sense primer	Annealing temperature	Product size
12*	5'CTCCCTTCATTGCTTAACAC3' 5'TTTCTTTGCCAAGATATTAC3'	45^0C	185 bp
13*	5'CTTATGTTCAGTAGTTGTG3' 5'GCTATGTGTTCCTTTATTACC3'	51^0C	539 bp
14	5'AAACAGTGAGACTCCATCTC3' 5'AGGATGATCTTGATGCCTTG3'	57^0C	265 bp
15&16	5'CCTTATCTTTCCAATTCTAT3' 5'TCCTGGATAATTGAGCCTTG3'	59^0C	360 bp
17*	5'TTCTTTGTCTGATAATAAC3' 5'CTCTCACTAACAATAATTTGTT3'	47^0C	351 bp
18*	5'AAATTATGCTTACTAATGTG3' 5'TCTTTATAGAATGTTACATT3'	46^0C	248 bp
19*	5'TGTACAACCTTGAAGTGTAT3' 5'TCAGCCTAGTTTCAGAGTC3'	46^0C	175 bp
20*	5'GGGGAAAGAAAAGAGTGG3' 5'AGGAGAGAAGGTGAAGTG3'	53^0C	200 bp
21	5'ATTCTGACTACTTTTACATC3' 5'TTATGTTATGGATATGGAT3'	40^0C	195 bp
22*	5'ATATGTGCTTCTTACCAGT3' 5'TTGGTGGACCCATTACATTA3'	49^0C	170 bp
23*	5'TCTAATGTAATGGGTCCACC3' 5'TCAAAATAATCCCCCTCTCA3'	53^0C	280 bp
24*	5'GAATGATGTATTTATGCTCA3' 5'TTCTTTTATACTTACAATGC3'	46^0C	165 bp
25*	5'CTAACTATGAAACACTGGCA3' 5'CAGATGACCATCTCAGCTAC3'	56^0C	203 bp
26	5'TCCATTTATAAATACACATG3' 5'TAACGAAAAGACTTCTTGCA3'	47^0C	168 bp
27	5'TACCCAGTACCATCACT3' 5'TCAAGAGGTGTACACAGTG3'	53^0C	347 bp

*- indicate exons amplified by mPCR and the rest were amplified in uniplex reactions.

1.4 Results

The eight multiplexed exon groups along with their product size and annealing conditions are shown in Table 2. Figure 1 shows the electrophoresed multiplex PCR products. Exons 9 & 25 and 5 & 8 were amplified in duplex sets. The remaining nine exons were amplified in six individual reactions (Table 3).

Table 2: Multiplexed RB1 exon groups, annealing temperature and product sizes

Multiplex Group	Exons	Annealing temperature and cycles	Product sizes (bp)
I	4 ;12	44^0C X 10 + 45^0C X 23	262 ; 185
II	6 ;22	48^0C X 10 + 49^0C X 23	238 ; 170
III	10; 19	46^0C X 10 + 47^0C X 23	233 ; 175
IV	13;20;23	50^0C X 8 + 51^0C X 8 + 52^0C X 8 + 50^0C X 5 + 51^0C X5 + 52^0C X5	539 ; 200 ; 280
V	17;18;24	45^0C X10 + 46^0C X23	351; 248 ;165
VI	5; 8	43^0CX10 + 44^0CX10 + 45^0C X10	177; 243
VII	9; 25	52^0C X20 + 53^0C X13	202; 248
VIII	2; 3	47^0CX5 + 48^0CX15 + 49^0C X10	150; 229

Table 3: Exons amplified in individual PCR, annealing temperature and product sizes

	Exons	Annealing temperature (cycles)	Product size (bp)
I	21	40^0C (35)	195
II	26	47^0C(35)	167
III	11	49^0C(35)	205
IV	14	59^0C(35)	265
	15&16		360
V	7	53^0C(35)	238
	27		347
VI	1	62^0C(35)	318

Table 4: Time and cost savings in multiplex PCR for RB1 gene

PCR	Total time for 27 exons (hours)	Total cost for 27 exons (in rupees)	Total savings (%) Time in hours / Cost in rupees
Uniplex PCR	78 (26 X 3)	532.48	36 (46 %) / 162.24 (30.4 %)
mPCR	42 (8X3+ 6X3)	370.24	
Savings	36	162.24	

In the present study, time for amplifying 27 exons in individual reactions was 78 hours (26 X 3 = 78 hours). Amplification of 18 exons in eight multiplexed reactions saved 30 hours (18X3 - 8X3). Individually amplifying exons 7 and 27 at 53^{0}C, and exons 14, 15 and 16 at 59^{0}C in two separate runs saved three hours each. In this way a total of 36 hours of PCR run time (46%) was saved by mPCR amplification of 27 exons (Table 4). Each individual and multiplex PCR cost Rs. 20.48 and 25.80 respectively. Cost of amplifying 27 exons is Rs. 532.48 (26X 20.48) by uniplex PCR and Rs. 370.24 (8X25.80 + 8X20.48) by mPCR. A total of Rs.162.24 (30.4%) was saved by mPCR (Table 4). The number of mutations identified in the current study is given in Table 5. The eight multiplex reactions saved 600 ng (33.3%) of tumor DNA with each uniplex and multiplex reaction using 100 and 150 ng of DNA.

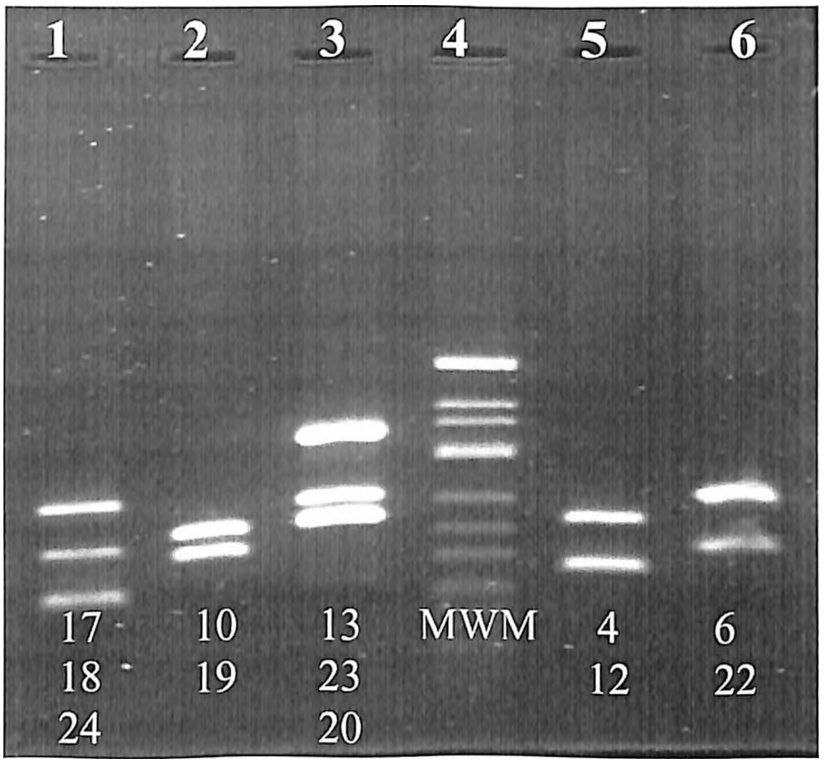

Figure 1 : mPCR products of RB1 gene after 2% agarose electrophoresis. 1 - 6 indicate the gel lanes; Lane 1: exons 17 (351 bp), 18 (248 bp) and 24 (165 bp); Lane 2: exons 10 (233 bp) and 19 (175 bp); Lane 3: exons 13 (539 bp), 20 (200 bp) and 23 (280 bp); Lane 4: Molecular weight marker (Hinf I digest); Lane 5: exons 4 (262 bp) and 12 (185 bp); Lane 6: exons 6 (238 bp) and 22 (170 bp).

Table 5: Retinoblastoma patients screened by mPCR

	Laterality	Patients screened	Number of mutations
I	Bilateral	17	8
II	Unilateral	8	3

1.5 Discussion

Amplifying large genes exon by exon occupies considerable amount of time; limiting the efficiency of molecular diagnostic screening in genetic disorders. The mPCR amplification has become a powerful tool for implementation of cost effective mutation detection strategies for large genes like dystrophin (Chamberlain *et al*, 1988) MLH1 and MSH2 (Charbonnier *et al*, 2000), RPE65 (Joseph *et al*, 2002) and RB1 (Du and Gallie, 1999).

In mPCR, designing a set of conditions that amplify a large number of fragments without spurious amplification is very important. This could be further limited by the subsequent technique that was to be applied like denaturing gradient electrophoresis, SSCP or heteroduplex analysis. SSCP analysis requires that the primers should result in amplified product size 200 bp or less. In DGGE application, the primers should have an annealing temperature near 50^0C. Orsouw *et al*, (1996) successfully combined two-dimensional electrophoresis and PCR multiplexing for RB1 gene mutation detection. mPCR followed by heteroduplex analysis and sequencing has been used as an efficient screening method for retinoblastoma (Lohmann *et al*, 1997).

DNA based diagnostic services are essential for retinoblastoma patients and their families in taking important decision regarding reproductive options for the parents and for the proband. Multiplexing is a necessity for mutational screening for the 27 exons of RB1 gene due to the large size and lack of mutation hotspots

(Scheffer *et al*, 2000). PCR multiplexing offered more versatility for subsequent genetic analysis by simultaneous amplification of multiple exons. The timesaving by mPCR is very important since the results could help the ophthalmologist in deciding further management of retinoblastoma patients and families. Ophthalmologist will be able to take more informed decisions, based on the genetic results, whether the next child or a close relative should have constant ophthalmic surveillance or not. The timesavings are crucial when prenatal diagnosis or testing a newborn child for RB1 gene mutations is indicated. The results of genetic testing could have important influence in the decision to proceed with pregnancy or not. Avoiding unwanted ophthalmic surveillance has been shown to result in considerable economic benefits and timesavings (Noorani *et al*, 1996). Richter *et al*, (2003) has shown that a child proved to be carrier of RB1 germline mutations with high penetrance could be delivered earlier and treated so that the vision could be saved in the eye.

The PCR multiplexing saved 36 hours by amplifying 27 exons in 14 PCR thermal cycler runs. In the SN ONGC laboratory of Genetics and Molecular Biology, two PCR thermal cyclers were available during the study period for carrying out three research projects. Assuming 48 hours of work per week and six PCRs were done per day, savings of PCR thermal cycler run time alone (36 hours) was equivalent to five working days (75% of labour time). An earlier result, especially if negative, will reduce unwanted stress for the families besides helping the clinician in patient management. These indirect benefits will have enormous economic impact in the retinoblastoma families by avoiding longer stay at the hospital and avoiding unnecessary anesthetic examinations besides manpower savings. In addition to rapid mutation screening, saving in 33.3% of tumor DNA (33%) was very crucial considering the irreplaceable nature. mPCR was also useful in assessing suspected exonic deletions. Failure to amplify the suspected exon alone in an mPCR indicated the exonic deletion.

In the present study mPCR followed by ethanol purification and cycle sequencing was used for rapid mutation screening of RB1 gene. Eight mPCR amplifying altogether 18 exons were used and the remaining exons were amplified in individual reactions. The mPCR saved reagents since many exons are amplified in the same reaction tube. Ethanol purified mPCRed products were directly sequenced without using agarose gel elution. In addition to savings of time, avoiding gel elution procedures helped in getting uniform peaks in sequence data. In gel elution procedures, there is chance of loosing the PCR products, especially for a beginner, that will become evident only after subsequent fluorescent sequencing reaction resulting in wastage of costly chemicals and run time of the sequencer.

1.6 Conclusions

The recognition of specific mutations in RB1 gene could unravel the underlying molecular basis of the disease, which could facilitate more effective genetic counseling. Different studies employed techniques like pre-amplification by long PCR prior to mPCR and SSCP, two-dimensional and denaturing gradient electrophoresis after mPCR. In the present study mPCR followed by direct sequencing was used for mutation screening of RB1 gene. This might be the ideal strategy for the RB1 gene with its large size and mutations distributed throughout the exons. mPCR for RB1 sequencing offered significant savings of tumor DNA, equipment time and reagents compared to conventional methods and overall reduction in turnaround time for a sample. These indirect savings have significant impact on the health expenditure for the retinoblastoma families.

1.7 References

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Chapter 2

Mutational screening and fragment analysis of RB1 gene

2.1 Aims

- Mutational characterization of RB1 gene in retinoblastoma patients.
- Establish fragment analysis as a rapid diagnostic tool in mutational screening.

2.2 Review of literature

Functional loss of both alleles of RB1 gene, in developing retinal cells, causes retinoblastoma (MIM 180200). Biallelic inactivation of RB1 gene could occur due to mutations or deletions. In hereditary retinoblastoma (bilateral and unilateral familial), the germline mutation arises as a *de novo* event or inherited from one of the parents. Subsequently the second mutation happens in one or several retinal cells leading respectively to unifocal or multifocal tumors. In unilateral non-familial retinoblastoma both mutations occur in retinal cells. Heterozygous RB1 germline mutation carriers have a relative risk of 400,000-fold for retinoblastoma and a 500-fold for other tumors like sarcoma (Gallie *et al*, 1999).

The family of tumor suppressor proteins includes RB1, RB2/ p130 and p107 with sequence homology in the pocket region (Brown *et al*, 1999). These tumor suppressors encode transcription factors that regulate activity of other cellular genes. RB1 is an ubiquitously expressed gene of 27 exons spanning over 180 Kbp in 13q14 region (Figure 2) (Lee *et al*, 1987). pRB is a nuclear phosphoprotein with 928 aminoacids and 110 kDa molecular weight. Retinoblastoma-associated protein has an N terminus (aminoacids 1–392); A domain (aminoacids 393–571); Spacer domain (aminoacids 572-645); B domain (aminoacids 646–772); a C pocket (aminoacids 773–869) and a Carboxyl terminus (aminoacids 870–928). (DiCiommo *et al*, 2000; Qi *et al*, 2005). Large pocket of pRB (comprises of A domain, spacer, B and C domains)

is responsible for binding of viral oncoproteins (Lee *et al*, 1998). Most of the RB1 gene mutations affect the 'large pocket' of the protein (Harbour, 1998). The N-terminus domain is also involved in growth suppression and contains six phosphorylation sites (Brown *et al*, 1999; Goodrich, 2003). Aminoacids, 140–202, are required for interaction between pRB and transcription factors SP1 and SP3 (Udvadia *et al*, 1995). A domain, spacer and B domain, collectively called as 'small pocket' binds to E2F (Chellappan *et al*, 1991) and are necessary for cell growth, differentiation and transcriptional regulation. The B domain requires the A-B interface for stable folding (Lee *et al*, 1998). The C-terminus, containing important phosphorylation sites, confers growth suppression through RB1/E2F interaction (Hiebert *et al*, 1992; Welch and Wang, 1993).

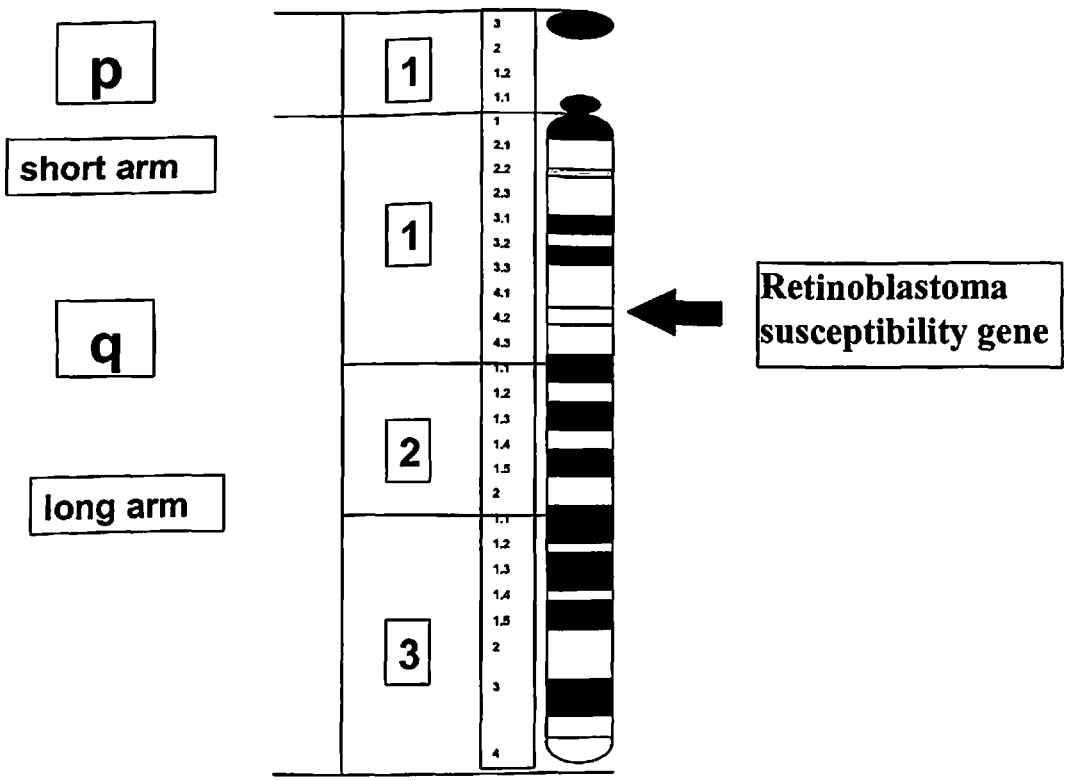


Figure 2: Human chromosome 13 showing location of the RB1 gene

Of all exons, exon 27 is the largest, with 1889 bases and exon 5 is the smallest with 31 bases in RB1 gene. No mutations so far have been reported in the RB1 exons 26 and 27. CGA codon mutations in RB1 exons 8, 10-11, 14-15, 17-18 and 23 accounted for 88% of recurrent single base substitution mutations in RB1 gene (Richter *et al*, 2003). Exons 8, 14 and 17 have two CGA codons and the other exons have one CGA codon each.

RB1 gene mutational screening is essential for confident presymptomatic prediction, genetic counseling and early management of hereditary retinoblastoma (Zhang and Minoda, 1995). A single diagnostic test could not detect all the different types of mutations distributed throughout the RB1 gene. The RB1 mutation spectrum that could be detected by conventional karyotyping, sequencing and quantitative multiplex PCR are given in figure 3. According to Harbour (1998), the minimal variability in the spectrum of RB1 mutations between countries would facilitate internationally valid genetic tests. Cowell and Cragg, (1996) emphasized the importance of genetic screening and counseling of early onset unilateral retinoblastoma patients due to probability of constitutional mutations.

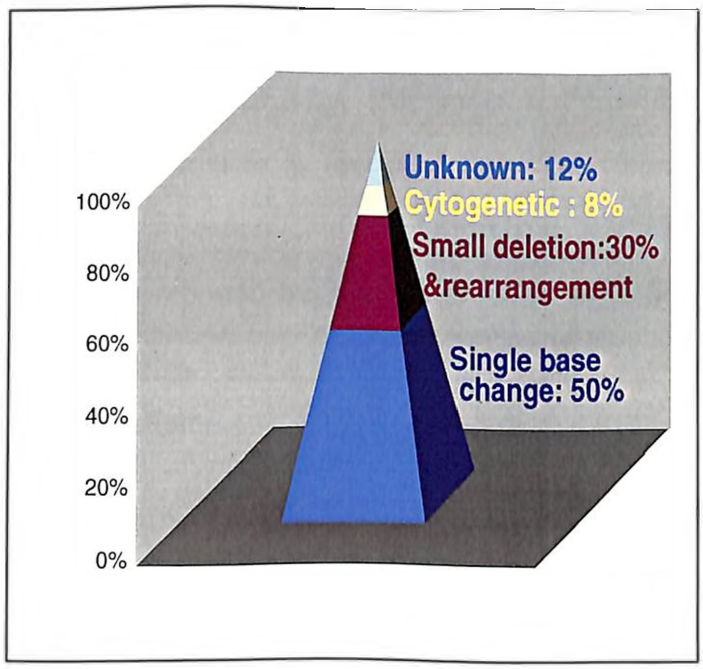


Figure 3 : RB1 mutation spectrum in retinoblastoma
(Lohmann *et al*, 2002 and other studies collectively)

Harbour (1998) reported 43% nonsense, 12% intronic mutations, and 9% of missense and small inframe deletions. Nearly reporting the same pattern, Lohmann *et al*, (1997) reported 42.9% nonsense mutations, 11.1% splice mutations, 6.5% missense and inframe deletions and 1.9% complex mutations. However, Alonso *et al*, (2001) reported higher frequency of splice mutations (31%) and frameshift mutations (41%) compared to nonsense mutations (27%). Richter *et al*, (2003) showed nearly 40% of nonsense mutations in both bilateral and unilateral patients. Significantly, Richter *et al*, (2003) detected 34.6% of large and exonic deletions by QM-PCR technique; not described in previous studies (Table 6).

Ata-ur-Rasheed *et al*, (2002) detected seven RB1 mutations by sequencing in Indian retinoblastoma patients. Kiran *et al*, (2003) detected mutations in 46% (22/47) of Indian retinoblastoma patients by SSCP and sequencing. Kumaramanickavel *et al*, (2003) reported a novel 8 bp exon 4 deletion and CGA to stop codon mutations in exons 8 and 14 from Indian retinoblastoma patients.

Retinoblastoma families with autosomal dominant inheritance demonstrate almost complete penetrance and high expressivity. But some families display reduced penetrance and expressivity referred to as low penetrance retinoblastoma. In these families majority of mutation carriers are unaffected or only unilaterally affected. The low penetrance RB1 mutations reported are Asn480del (exon 16), R661W (exon 20), deletion of 40 aminoacids in exon 4 and deletion spanning exons 24 and 25 (Bremner *et al*, 1997) and promoter mutations.

Table 6: Compilation of RB1 mutation screening studies

Number of patients	Mutations							Study
	Nonsense	Frameshift	Splice	Intron	Missense / small inframe deletion	Large and exonic deletions	Complex (Deletion and insertions)	
192	43%	35%	-	12%	9%	-	-	Harbour, (1998)
368	42.9%	36.4%	11.1%	-	6.5%	-	1.9%	Lohmann, (1999)
43	27%	41%	31%	-	-	-	-	Alonso *et al*, (2001)
199 bilateral	42%	24%	16%	-	6%	13%	-	Richter *et al*, (2003)
236 unilateral	40%	17.3%	9.3%	-	4.2%	21.6%	-	

The European Molecular Genetics Quality Network formed guidelines for reporting of RB1 mutations (Lohmann *et al*, 2002). The reference sequence L11910 should be used in the report for nomenclature of mutations. If the mutation is known to be associated with incomplete penetrance, it should be indicated in the results. The report should include expected recurrence risk to siblings and own offspring of a patient. It should be also mentioned if more precise risk figures could be obtained by genetic testing for relatives.

Mutational inactivation of RB1 gene is associated with small cell lung cancer, sarcomas and carcinomas (Simpson *et al*, 2000). Heritable retinoblastoma patients have 20% life long risk for osteogenic sarcoma during childhood and breast or bladder cancer later in life (Hurwitz *et al*, 2003). Being a tumor suppresser, inactivation of RB1 gene leads to an imbalance in the cell cycle regulatory mechanisms making the cell susceptible to uncontrolled proliferation.

Fragment analysis using locus-specific microsatellites helped Kondo *et al*, (2003) to detect rapidly 5 bp duplication in PRPF8 gene with its altered mobility. Richter *et al*, (2003) established fluorescent fragment amplification in six multiplex PCRs and analysis on *OpenGene* automated system to detect exonic deletions or insertions in retinoblastoma patients. Bi-directional sequencing was used for rapid detection of the changes in aberrantly migrating bands (Richter *et al*, 2003).

Nork *et al*, (1994) employed immunocytochemical techniques to identify pRB in formalin-fixed, paraffin-embedded retinoblastoma. The study concluded that pRB could be identified in paraffin-embedded tissues by immunohistochemical methods (Nork *et al*, 1994).

2.3 Methodology

The study was done with ethical approval by institutional review board/ethical committee. After informed consent during genetic counseling, mutational screening by exon by exon sequencing from tumor DNA was done for 17 bilateral and eight unilateral retinoblastoma patients who came from different states of India and neighboring Sri Lanka and Bangladesh. DNA from tumor tissue and peripheral blood of retinoblastoma probands was extracted by phenol-chloroform method (Appendices I & II). Peripheral blood DNA was screened for mutations identified in tumor to rule out germline origin. Immunohistochemical analysis of retinoblastoma-associated protein expression on formalin-paraffin tumor sections was done to find the association with RB1 mutations. In addition, protein modeling for the RB1 mutations using ExPaSy bioinformatic tools was done to find out any disruption of the three-dimensional structure of pRB. Aminoacid conservation analysis using BLAST search was performed to understand the importance of the wild type aminoacid at the nonsense mutant sites in pRB function.

2.3.1 Sequencing

DNA sequencing involves amplification of specific sequence from DNA, electrophoresis of the amplified product in 2% agarose gel, cycle sequencing, purification of extension product, capillary electrophoresis in ABI PRISM 310 automated sequencer and sequence analysis.

2.3.2 PCR and agarose electrophoresis

Requirements: DNA (50 ng/μL); diluted from stock DNA with TE buffer. 10X PCR buffer (Bangalore Genei, Ltd, India) (100 mM Tris pH 9.0, 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin). *Taq* DNA polymerase (3 U/μL) (Bangalore Genei, Ltd, India). dNTPs: Each dNTP 10 μM/100 μL (40 nM dNTP per reaction) (Bangalore Genei, Ltd, India). Forward and reverse primers of RB1 exons 1-27 (Bangalore Genei, Ltd, India). The primer sequences of the 27 exons of the RB1 gene are given in chapter 1 Table 1.

10X TBE buffer

Tris	-	500 gm
Boric acid	-	27.5 gm
EDTA	-	3.72 gm
Distilled water	-	500 ml

Tracking dye

Bromo phenol blue	-	0.1 gm/100 ml 1X TBE buffer
Sucrose	-	4 gm/100 ml water

Equal volumes of 0.1% bromo phenol blue and 4% sucrose solutions were mixed.

A 20 μL reaction using 100-150 ng of the genomic DNA, 0.2 μL of primers (20 pM/μL), 2 μL dNTPs (40 nM), 2 μL PCR buffer and 0.2 μL of *Taq* DNA polymerase (3 U/μL) was set up. Initial denaturation was done at 94^0C for 5 minutes. Denaturation at 94^0C for 45 seconds, annealing temperature (varied depending on the exon) for 60 seconds and extension at 72^0C for 90 seconds was used for 30-35 cycles of amplification. The amplified products were used for agarose electrophoresis.

The agarose gel trough was cleaned with ethanol and the ends were sealed with cellophane tape. Combs were placed in the respective positions. Molten agarose (0.5 gm agarose in 25 ml of 1XTBE Buffer) mixed with 8 μL of ethidium bromide

(1mg/ml) was poured into the gel trough and allowed to solidify in dark. After solidification of the gel, the cellophane tapes and combs were removed and the trough was placed in electrophoresis tank containing 250 ml of 1XTBE buffer. Ten μL of each amplified product mixed with 5 μL of tracking dye was loaded into the wells. One μL of Hinf I digest, diluted with 4 μL of 1X TBE buffer, was used as molecular weight marker. The electrophoresis was carried out at 100 Volts for 45 minutes. UV sample tray and orange filter were used to visualize the ethidium bromide stained gel. The gel image was captured in Pharmacia Biotech gel documentation system using *Liscap* software and analyzed using *Imagemaster Totallab.*

2.3.3 Cycle sequencing and purification

In Big Dye terminator cycle sequencing, primers are annealed to single stranded DNA and extended by a thermo stable DNA polymerase with incorporation of 5' fluorescent-labeled dideoxynucleotide triphosphates (chain terminators). Each of the four extension reactions (A, G, T and C) generate a nested set of labeled products, beginning with sequencing primer and ending at dideoxynucleotide triphosphate of A, G, T or C. DNA sequencer detects fluorescence from the four different dyes when excited by an argon ion laser to identify the A,G,T and C extension reactions in capillary electrophoresis.

Cycle sequencing PCR, set up as in Table 7, used initial denaturation at 96°C for 60 seconds; 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds and extension at 60°C for 4 minutes. The cycle sequenced products were purified to remove unincorporated dye terminators. To 20 μL of the cycle sequenced product, 80 μL of filtered autoclaved milli Q water was added, mixed well and transferred to 0.5 ml microfuge vial. Ten μL of 3 M sodium acetate (pH 4.8) and

250 µL of 95% ethanol were added; vortexed and microfuged for 25 minutes. After discarding the supernatant, washing of the product was done thrice by adding 250 µL of 70% ethanol each time and microfuging for 5 minutes. The vial was covered with paraffin film, perforated and dried at 37°C overnight. Twenty µL of template suppression reagent was added to each vial of the extension product; vortexed and microfuged for 30 seconds. The sample was denatured at 90°C for 3 minutes and kept in ice till loading in the sequencer. The sample details were entered into a sample sheet and saved (48 sample tubes tray). The sample sheet was opened in the injection list window and capillary electrophoresis was done for 36 minutes at 50^0C using POP6 rapid (1mL) E run module.

Table 7: Cycle sequencing reaction protocol

Reagents	Volume (µL)
PCR product	1.0
Ready reaction mix	2.0
5Xsequencing buffer	3.0
Forward/ reverse Primer (3.2 pM/µL)	9.5
Autoclaved milliQ water	4.5
Total	20

2.3.4 Sequence analysis

Sequencing analysis 3.3 was used for analysing the raw data. Raw sequence data was added to sample manager window. After selecting Base caller ABI-CE1, start icon was clicked. Successful analysis was indicated by green color checked box under

parameter menu. The sequence data was evaluated for quality and the average signal intensity of the four dyes in the electrophoretogram. The total average signal intensity of all the four dyes was checked to ensure that the value is below the upper threshold 4000 RFU (Above this value indicate signals saturating the laser detector, leading to poor sequences).

Wild type sequence: RB1 exon (Genbank accession number L11910) with 10 bases on either side of the exonic sequences was pasted in a notepad with 10-font and arial style in running letters. The sequences were saved into a floppy and stored in RB1 folder in Seq data Bank in Macintosh computer. These were used as wild type for comparative analysis of patient sequences using sequencing navigator 1.0.1. Wild type RB1 sequence of the exon was imported from sequence data bank into a new layout using import sequence command. The sequence of the sample was imported into the layout. Comparative alignment was done using aligning option. Create shadow and compare sequence commands were selected from sequence menu. Mismatches were indicated by * symbol in between the compared sequences. Electrophoretogram was opened in another window to compare with the navigator window. Manual editing was done wherever necessary. Mutations were confirmed by sequencing from an independent PCR with the alternate primer (forward/reverse) for the exon. Mutations were named by conventional nomenclature to clearly identify the effect on expected protein product. The genomic RB1 sequence L11910 was used for annotation of the nucleotide changes. Thirty-five unrelated controls were also sequenced for the exons in which mutations were detected.

2.3.5 Fragment analysis

Fragment analysis, using 6-FAM labeled fluorescent RB1 exon 4 primers, was done for the 8 bp RB1 exon 4 deletion mutation in the bilateral patient (N15). The exon 4

of RB1 gene was amplified in the tumor sample of the bilateral patient with 8 bp deletion using custom synthesized 6 FAM dye labeled exon 4 primer (PE Applied Biosystems, USA). Five μL amplification reaction containing 0.1 μL each of fluorescent FAM labeled forward primer and unlabelled reverse primer, 0.05 μL of *Taq* DNA polymerase (3 U/μL), 0.5 μL of dNTPs and 0.5 μL of 10X PCR buffer was done at 46^0C with initial denaturation at 95^0C for 12 minutes, 10 cycles consisting of denaturation at 94^0C for 15 seconds; annealing at 46^0C for 15 seconds; extension at 72^0C for 30 seconds and 30 cycles consisting of denaturation at 89^0C for 15 seconds; annealing at 46^0C for 15 seconds; extension at 72^0C for 30 seconds. A normal blood DNA was also amplified in a similar way. Five μL of the amplified product was added to injection mix containing 0.5 μL of GS500 ROX size standard and 12 μL deionized formamide; denatured at 95^0C for 5 minutes. Three runs were performed - the tumor DNA amplified product alone, the tumor DNA amplified product pooled with the normal blood DNA (in 1:1 ratio) and the normal blood DNA alone in ABI 310 automated sequencer using filter set C for 24 minutes at 50^0C. Data was analyzed using Genescsan analysis and the three runs - the tumor with deletion, the pooled product and the control - were observed.

2.3.6 Immunohistochemistry for pRB expression on paraffin sections

Formalin fixed tissue sections on silane-coated slides was cut at 5-μm thickness for overnight incubation at room temperature with two specific anti-RB primary antibodies, IF8 and C15 (Santa Cruz Biotechnology, Santa Cruz, USA). After washing with tris buffer for 5 minutes, the slides were incubated with LSAB Link secondary antibody for 1 hour. The slides were incubated with Di-Amino Benzidine for 5 minutes, washed and counterstained with Harris haematoxylin. Nuclear staining was considered as indication of pRB expression.

2.3.7 **Aminoacid conservation analysis** (Stephen *et al*, 1997)

Aminoacid conservation analysis across species was done for the eight nonsense mutations using BLAST server for similarity search using short peptide sequences and maintained at National Center for Biological Information website. The wild type aminoacid, at each of the mutant site, with 12 flanking aminoacids on either side was submitted at the server. The aminoacids were searched for similarity against various species reference sequences in multiple peptide sequence databases including GenBank CDS translations, Protein Data Bank and SwissProt by the BLAST tool. Default settings were used and the results were generated as HTML format by following the instructions on the website. The results were evaluated to assess the conservation of the wild type aminoacid against few species.

2.3.8 **Homology modeling for RB1 mutants**

Protein modeling for the RB1 mutations was done on Swiss-PDB server (appendix VI).

2.4 **Results**

2.4.1 **Sequencing of RB1 exons**

Sequencing of RB1 exons were standardized by cycle sequencing using 2.0 μL RR mix and electrophoresis on ABI 310 sequencer. DMSO (2.5%) was used as additive for RB1 exon 1 sequencing.

2.4.2 **RB1 mutations identified**

Eleven mutations were detected in 10 (eight bilateral and two unilateral) of the 25 unrelated retinoblastoma patients (Table 8). The 10 single base substitutions consisted of an intron 11 splice mutation, six CGA codon [exons 8 (two sites), 11, 14, 18 and 23

(two patients)] and two non-CGA exon mutations [exons 7 and 23]. In addition an 8 bp deletion in exon 4 was detected in a bilateral patient. None of these changes were observed as sequence variations in 35 healthy controls.

Six of the bilateral retinoblastoma patients (patients N29, N19, N34, N26, N37 and S_I61) had single base substitutions; one had an intron 11 splice mutation (N8) and the last bilateral patient an 8 bp deletion in exon 4 (N15). Among bilateral patients, two had germline mutations, S215X in exon 7(N29) and R787X in exon 23(N37) and the rest six had somatic mutations in intron 11 and the exons 4, 8, 11, 18 and 23. Two somatic single base substitutions were detected in the exons 8 and 14 in the unilateral patient, N12 and the second unilateral patient (Q56) had a C to A substitution in exon 23.

In a bilateral retinoblastoma patient (N29), a C to G change in exon 7 (S215X) was detected in tumor and blood DNA (Figure 4). C to T mutation resulting in R255X in exon 8 (Figure 5) and R358X in exon 11(Figure 6) was detected in two bilateral patients, N19 and N34 respectively. Figure 7 shows the three-dimensional view of wild type pRB generated by ExPaSy server. The bilateral patient, N26, had a C to T mutation (exon 18) resulting in R579X (Figure 8). Arg787stop mutation was detected in two unrelated bilateral patients, S_I 61 (Figure 9) and N37 (Figure 10) with the latter having the mutation in blood also.

Table 8: Novel and recurrent RB1 gene mutations detected in the study

S. No.	ID (Age in months\sex)	Phenotype Sporadic	Site	Domain	Normal/Mutant	Position	Effect	Reported in Lohmann RB database
A. *CpG substitutions (C>T)*								
1	N19(36/ F)	bilateral	E08	N terminus	AGGCGAGGT/AGGTGAGGT	g.59695	R255X	11
2	N34(24/M)	bilateral	E11	N terminus	CCACGAAAA/ CCATGAAAA	g.65386	R358X	12
3	N26(10/M)	bilateral	E 18	Spacer	GACCGAGAA/ GACTGAGAA	g.150037	R579X	15
4	$S_1$61(1.3/M)	bilateral	E23	C terminus	CCTCGAAGC /CCTTGAAGA	g.162237	R787X	11
5	N37(11/M)	bilateral	E23	C terminus	CCTCGAAGC /CCTTGAAGA	g.162237	R787X	11
6	N12(60/M)	unilateral	E08	N terminus	CCTCGAACA/ CCTTGAACA	g.59683	R251X	4
7			E14	A-B pocket	TAGCGA TAC /TAGTGA TAC	g.76430	R445X	5
B. *Non CpG substitutions*								
8	N29(14/ F)	bilateral	E07	N terminus	ATTTCATTT/ ATTTGATTT	g.56889	S215X	Nil
9	Q56(30/ M)	unilateral	E23	C terminus	TTGTCACCA/TTG TAACCA	g.162217	S780X	Nil
C. *Splice changes*								
10	N8(5 / M)	bilateral	In11	N terminus	tctagG /tctaaG	g.70241	380X	Nil
D. *Small deletions*								
11	N15(21/M)	bilateral	E 04	N terminus	AACTTACTAAAAGA /AACAGA	g.41945-41952	136X	Nil

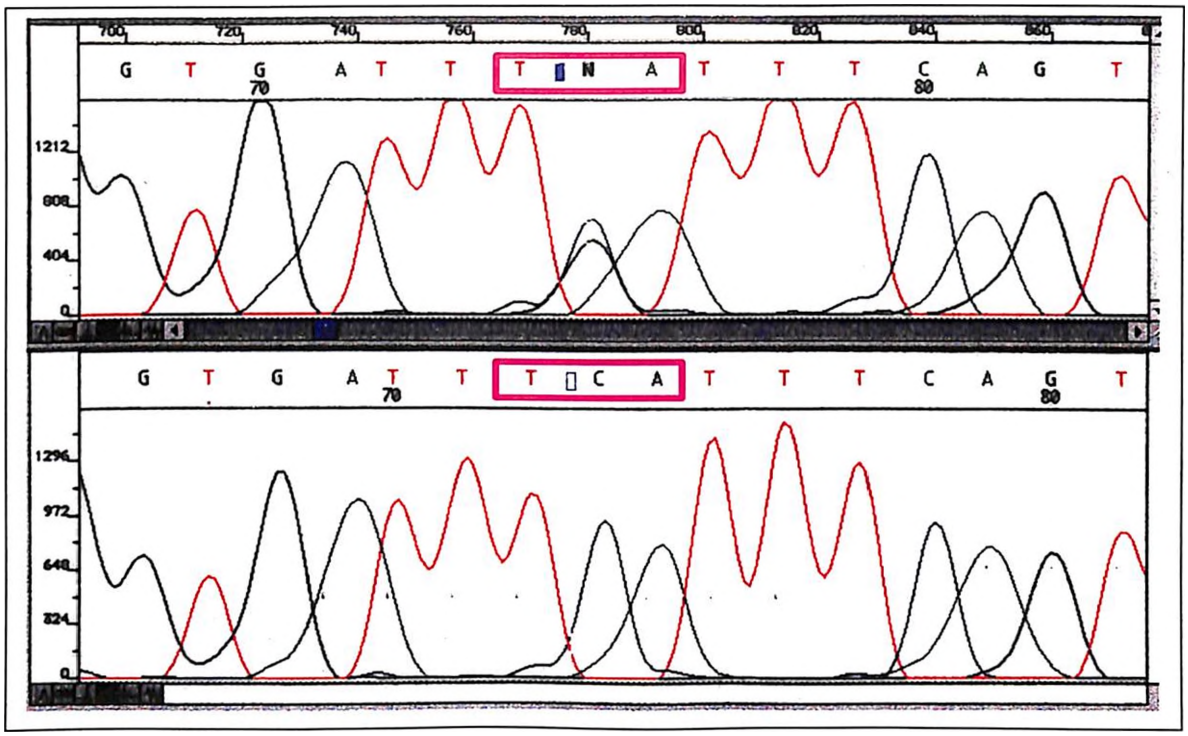


Figure 4 : Mutant (Top) and wild type (bottom) sequence of exon 7 of RB1 gene from genomic DNA of BLRB patient (N29) with forward primer showing Ser215stop

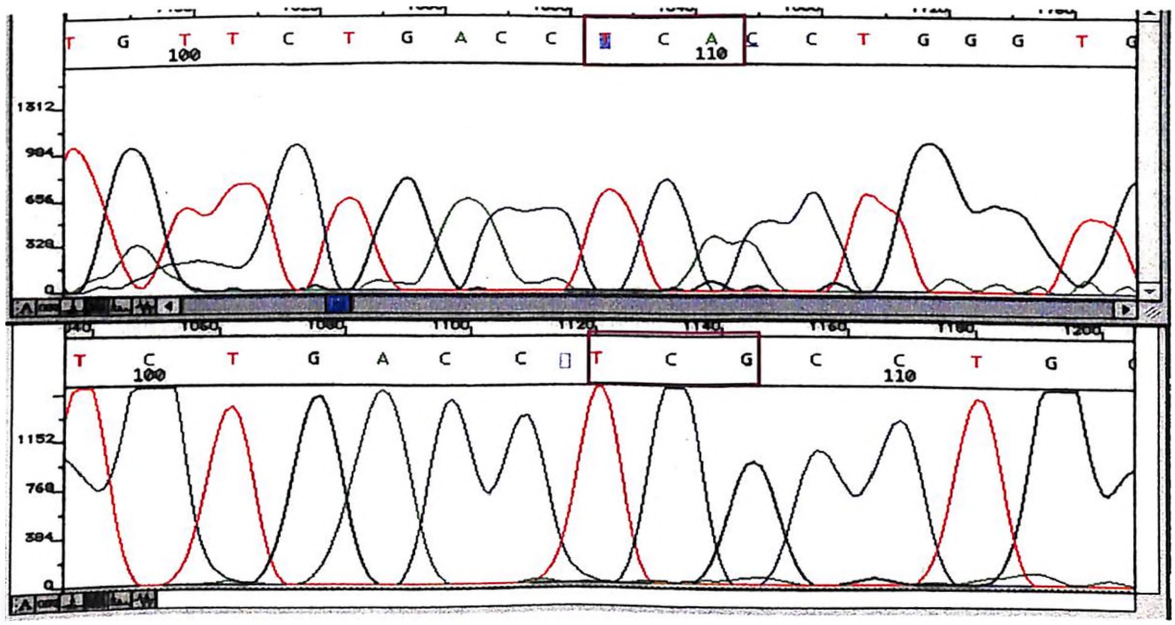


Figure 5 : Mutant (Top) and wild type (bottom) sequence of exon 8 of RB1 gene from tumor DNA of BLRB patient (N19) with reverse primer showing Arg255stop

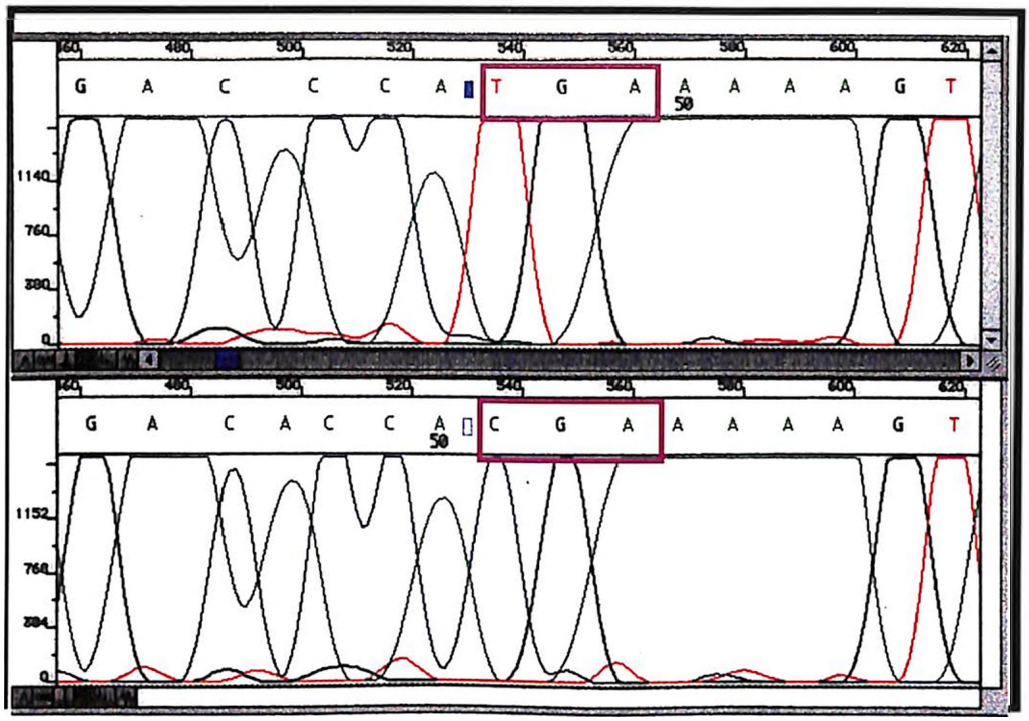


Figure 6 : Mutant (Top) and wild type (bottom) sequence of exon 11 of RB1 gene from tumor DNA of BLRB patient (N34) with forward primer showing Arg358stop

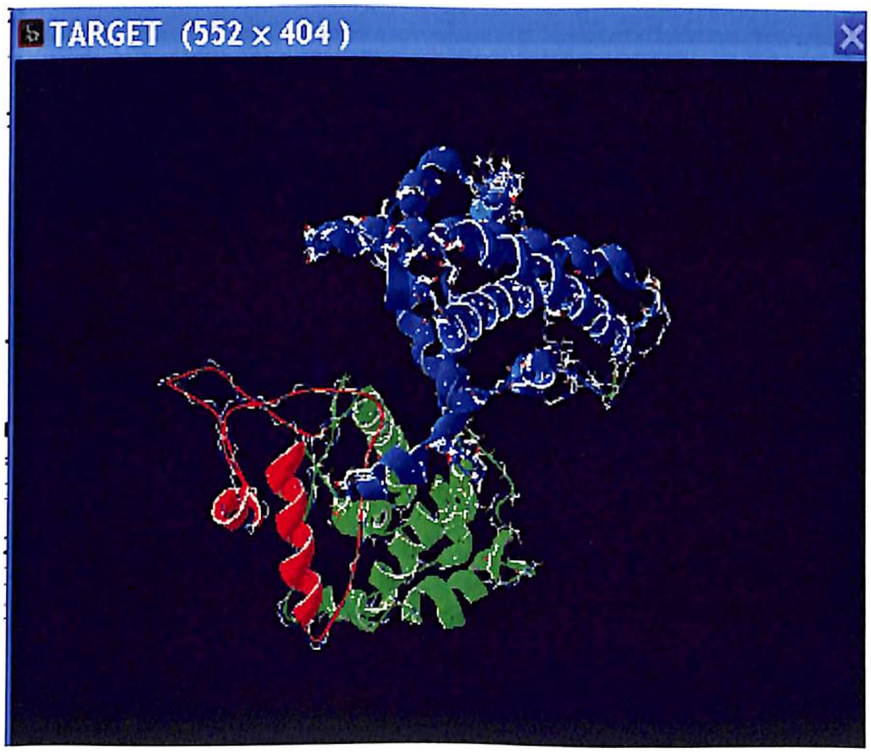
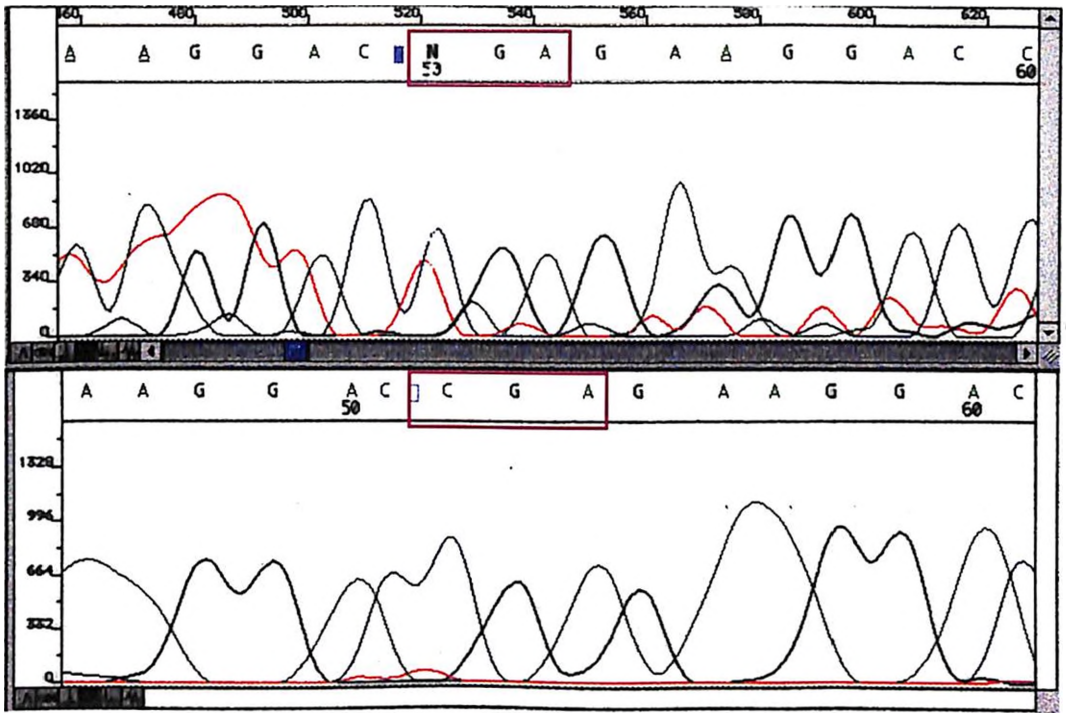


Figure 7 : The three-dimensional view of wild type pRB generated by ExPaSy server



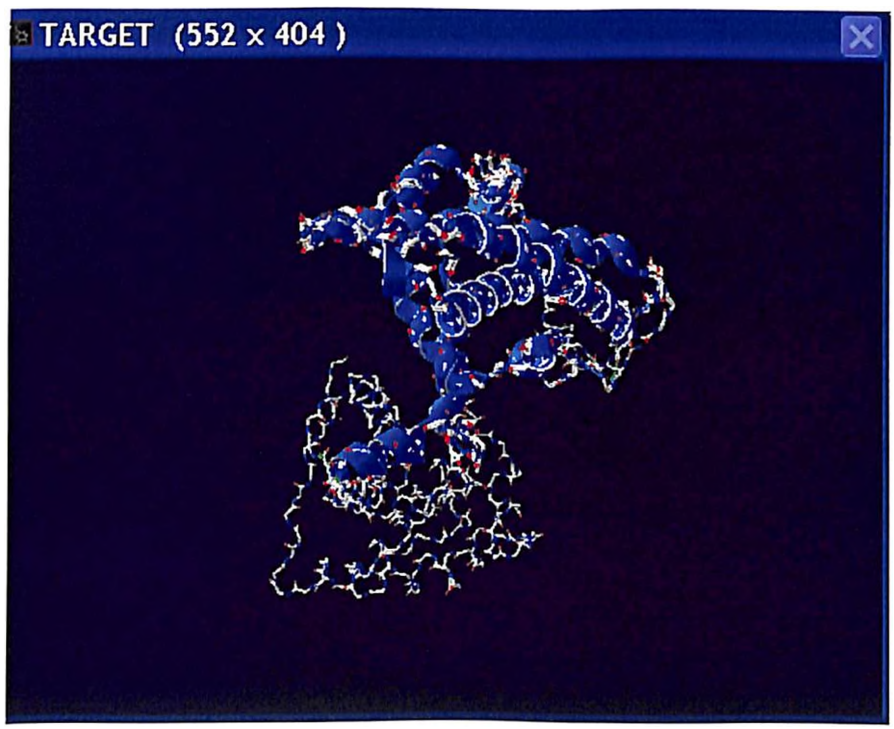


Figure 8 : Mutant (Top) and wild type (bottom) sequence of exon 18 of RB1 gene from tumor DNA of BLRB patient (N26) with forward primer showing Arg579stop. The three-dimensional view of the mutant protein generated by ExPaSy server is also shown.

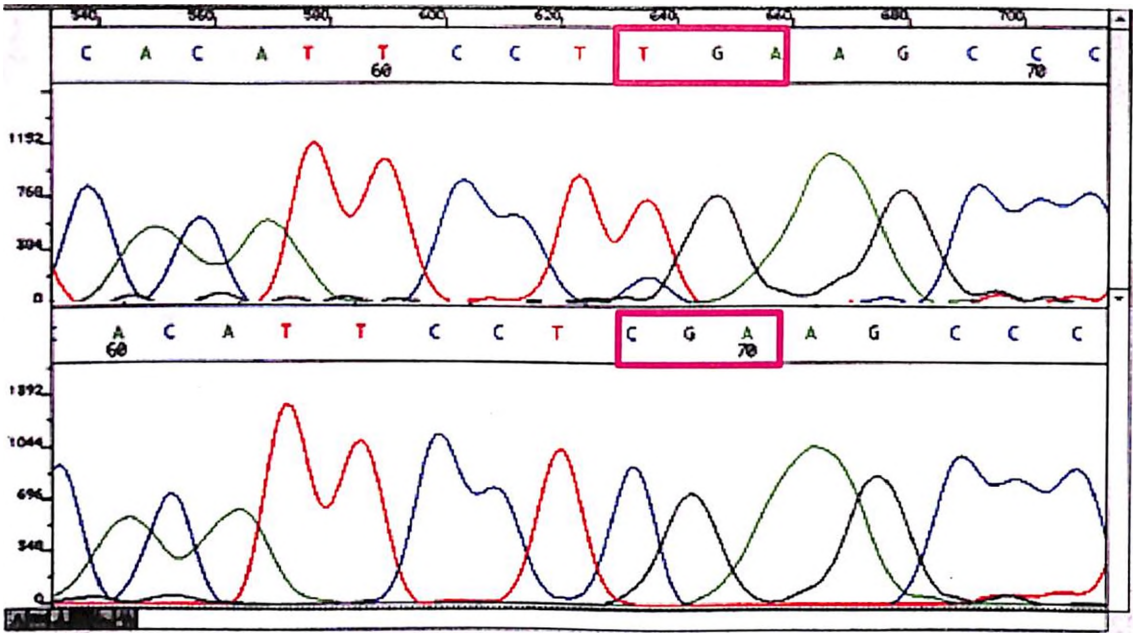


Figure 9 : Mutant (Top) and wild type (bottom) sequence of exon 23 of RB1 gene from tumor DNA of BLRB patient (S_t61) with forward primer showing Arg787stop. The three-dimensional view of the mutant protein is also shown.

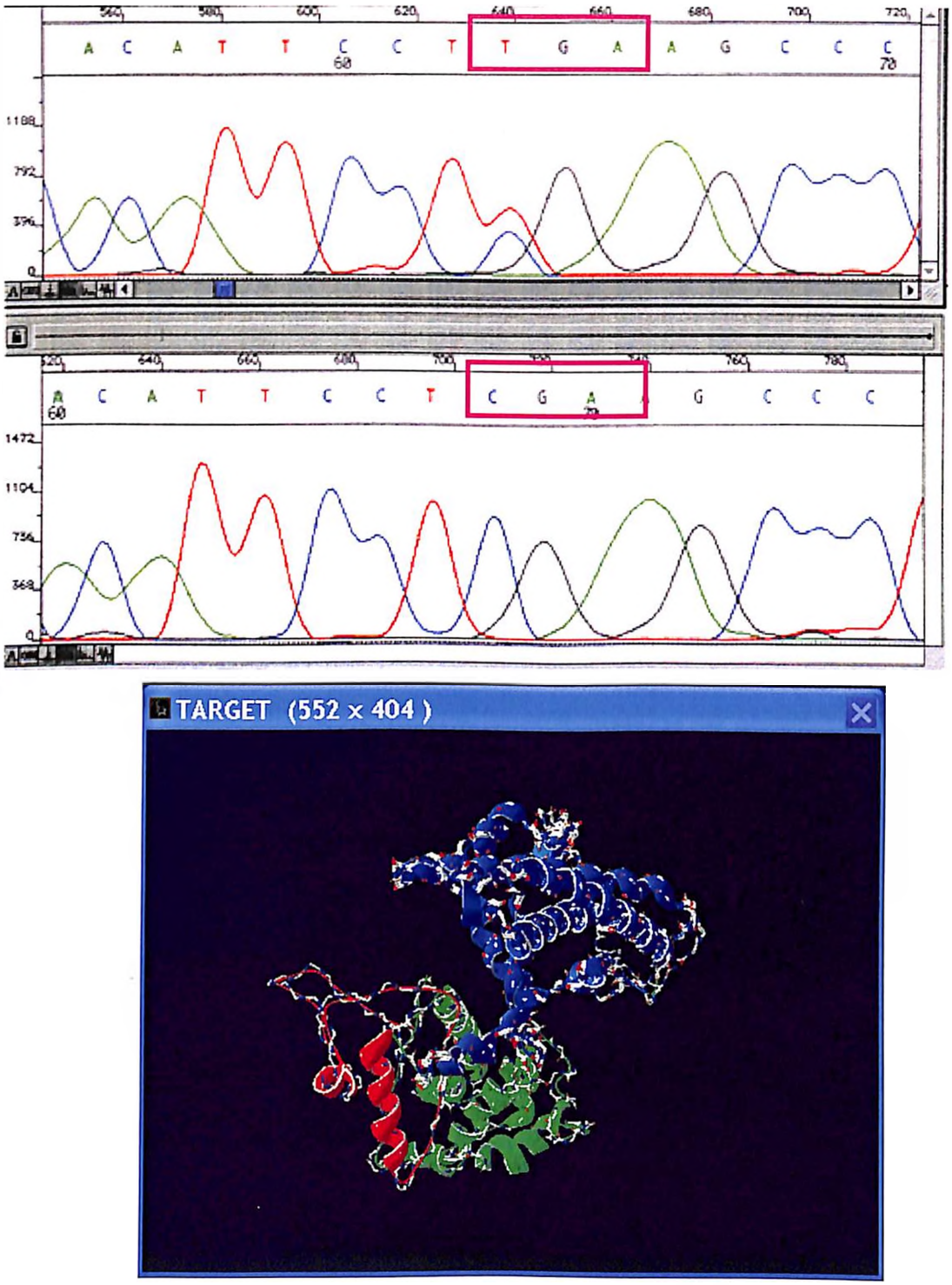


Figure 10 : Mutant (Top) and wild type (bottom) sequence of exon 23 of RB1 gene from genomic DNA of BLRB patient (N37) with forward primer showing Arg787stop. The three-dimensional view of the mutant protein is shown.

CGA codon mutations in exons 8 (R251X) and 14 (R445X) was detected in a unilateral patient (N12) (Figures 11 and 12). A homozygous C to A substitution in exon 23 changed serine to a stop codon in the unilateral patient (Q56) (Figure 13). In the bilateral retinoblastoma patient (N8), a G to A splice acceptor mutation in intron 11 was identified in tumor DNA (Figure 14). A novel 8 bp deletion mutation (frameshift of aminoacids NLLK to NRNX) in RB1 exon 4 in a bilateral patient resulted in a stop codon (136X) (Figure 15).

Sequencing has been standardized for RB1 exons 1-27 using DNA from formalin-paraffin sections of retinoblastoma tumor tissue. RB1 exon 5 sequence from formalin-paraffin DNA is shown (Figure 16).

Out of the 10 tumors, nine did not exhibit nuclear staining with monoclonal IF8 Ab for pRB (Table 9). The bilateral patient (N8) with intron 11 splice site mutation showed pRB expression in 100% of cells (Figure 17). With C15 polyclonal Ab, five tumors N15, N29, N34, N8 and N37 did not exhibit nuclear staining (Table 9). Five tumors N12, N19, N26, Q56 and S_I61 exhibited nuclear staining in varying percentage of cells (20%, 70%, 30%, 40% and 15% respectively) (Figure 17).

Email was received for receipt of aminoacid sequences for modeling. The aminoacids, submitted with N terminus region included, did not generate models for wild type or the pRB mutants. In the second step, omitting the N terminus aminoacids generated models for the mutants R445X, R579X, S780X and R787X and the pocket domain of pRB. The results were received through email (www.expasy.org). The images are shown along with the mutant sequences in figures 7,8,9,10,12 and 13.

Analysis showed conservation of the wild type aminoacid at the eight single-base substitution mutation sites in the study (Table 10). Aminoacid positions conserved across species are underlined. The aminoacid R358, (A/B domain) was not conserved in Canis familiaris (domestic dog), R579 (Spacer) was not conserved in Rattus norvegicus (domestic rat) and Mus musculus (House mouse). R787 (C terminus) was not conserved in Oryzias latipes (Japanese kill fish).

2.4.3. Fragment analysis

Fragment analysis using FAM labeled RB1 exon 4 primer demonstrated mobility shift for the 8 bp deletion tumor sample of the patient compared to control. RB1 exon 4 primer labeled with 6-FAM dye gave 262 bp product with the control and 254 bp product for the tumor sample with 8 bp deletion. In the pooled run, the tumor-amplified product was ahead of the control due to the shorter length of the product size (Figure 18).

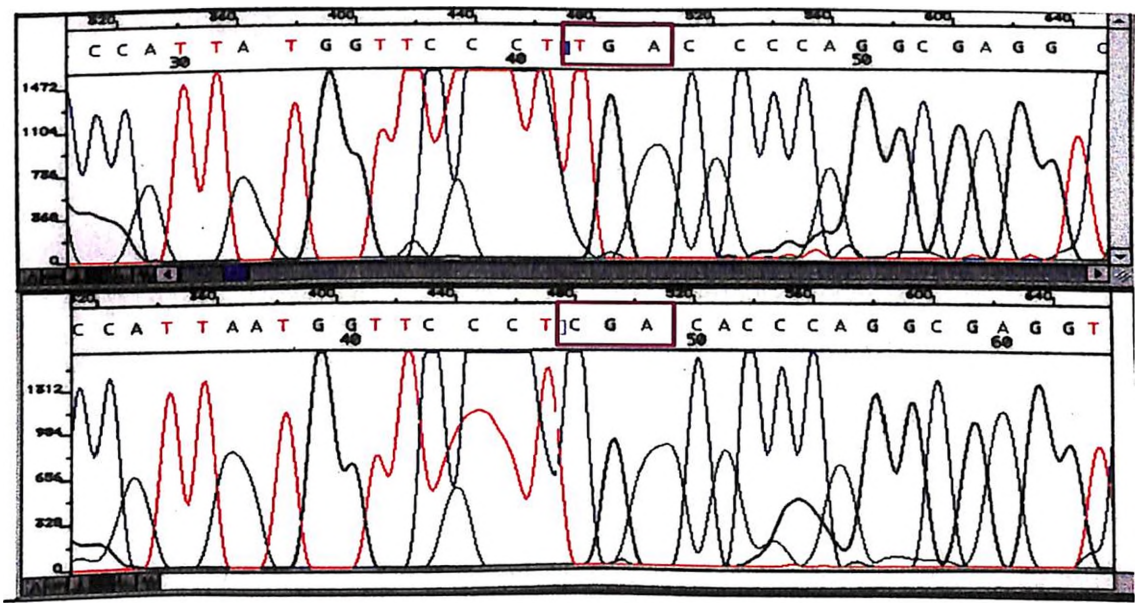
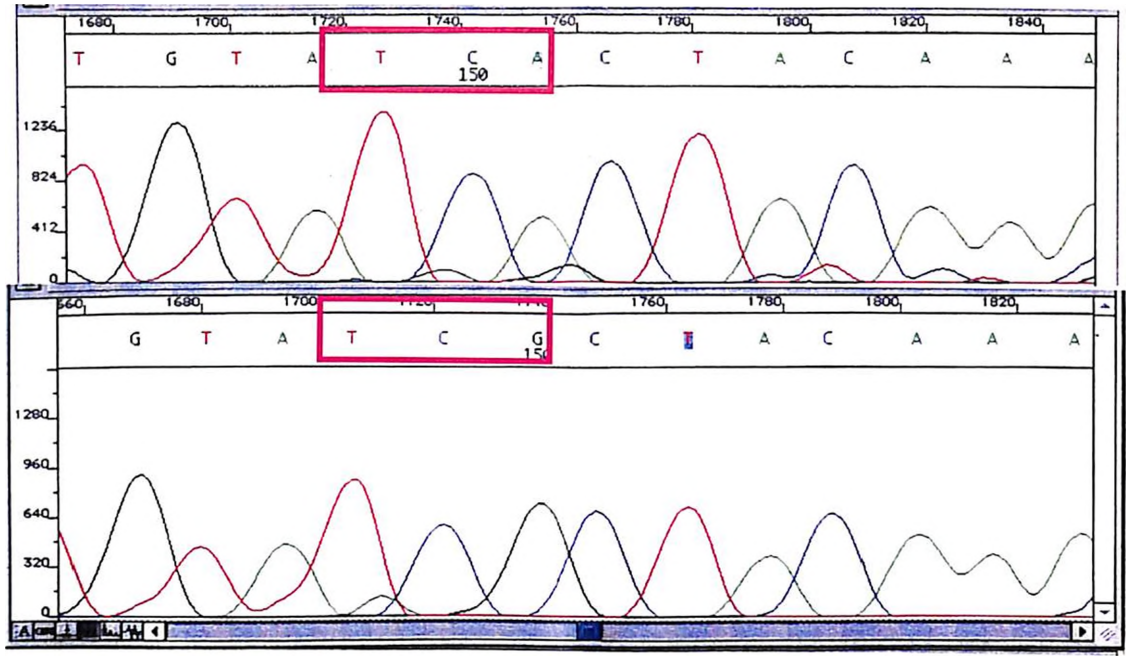


Figure 11: Mutant (Top) and wild type (bottom) sequence of exon 8 of RB1 gene from tumor DNA of ULRB patient (N12) with forward primer showing Arg251stop



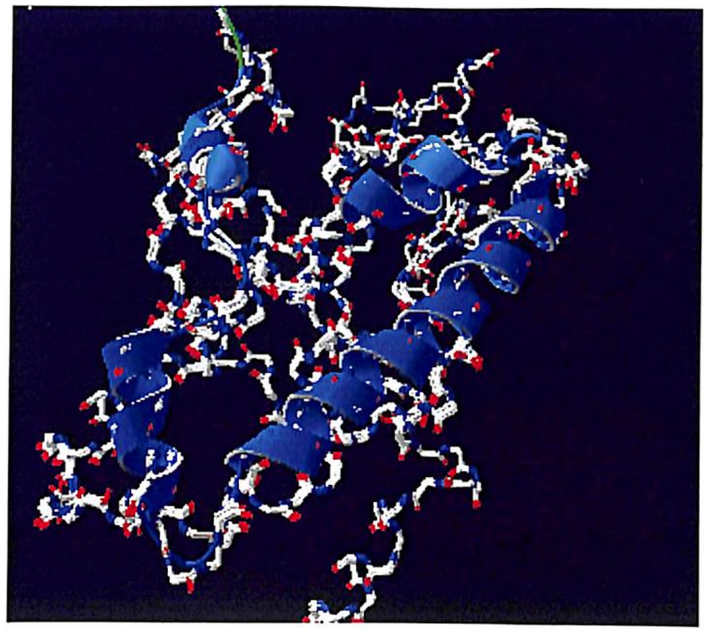
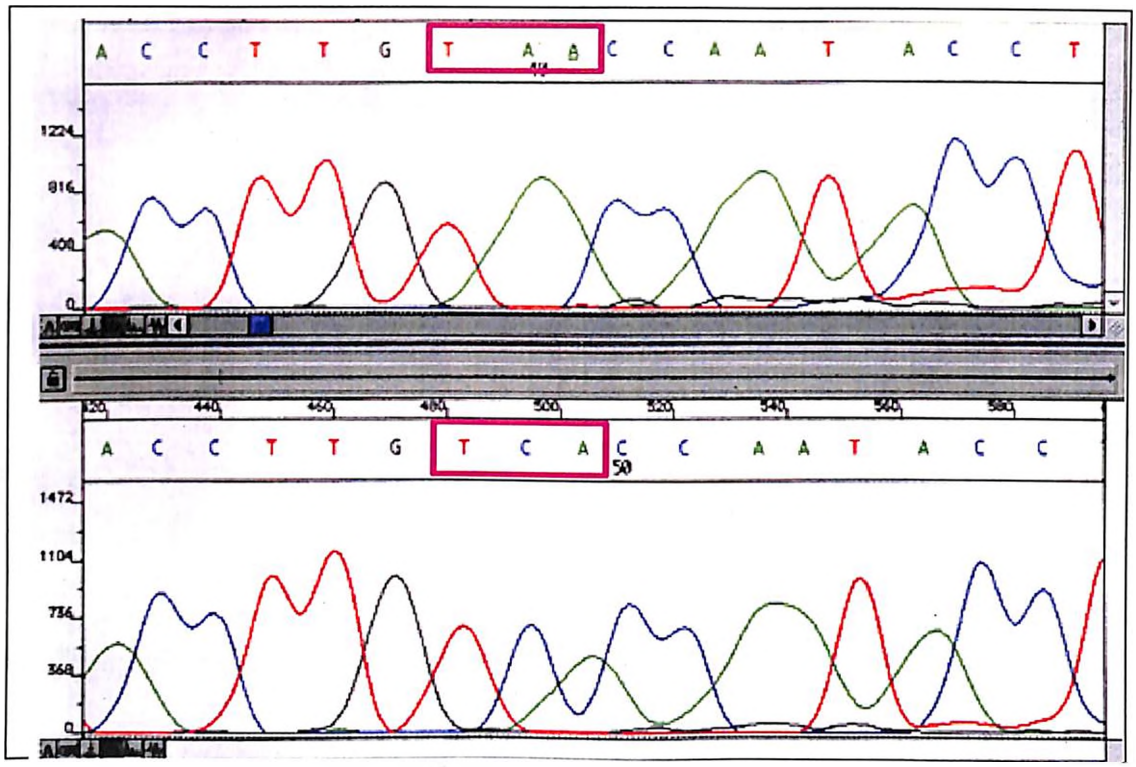


Figure 12 : Mutant (Top) and wild type (bottom) sequence of exon 14 of RB1 gene from tumor DNA of ULRB patient (N12) with reverse primer showing Arg445stop. The three-dimensional view of the mutant protein is also shown.



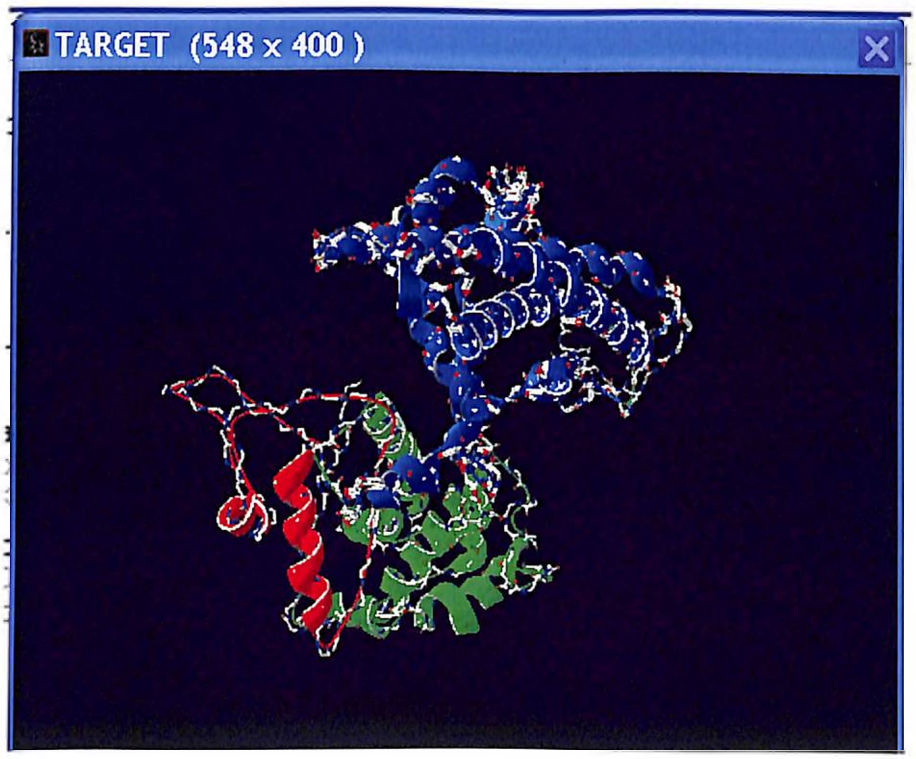


Figure 13 : Mutant (Top) and wild type (bottom) sequence of exon 23 of RB1 gene from tumor DNA of ULRB patient (Q56) with reverse primer showing Ser780stop. The three-dimensional view of the mutant protein is also shown.

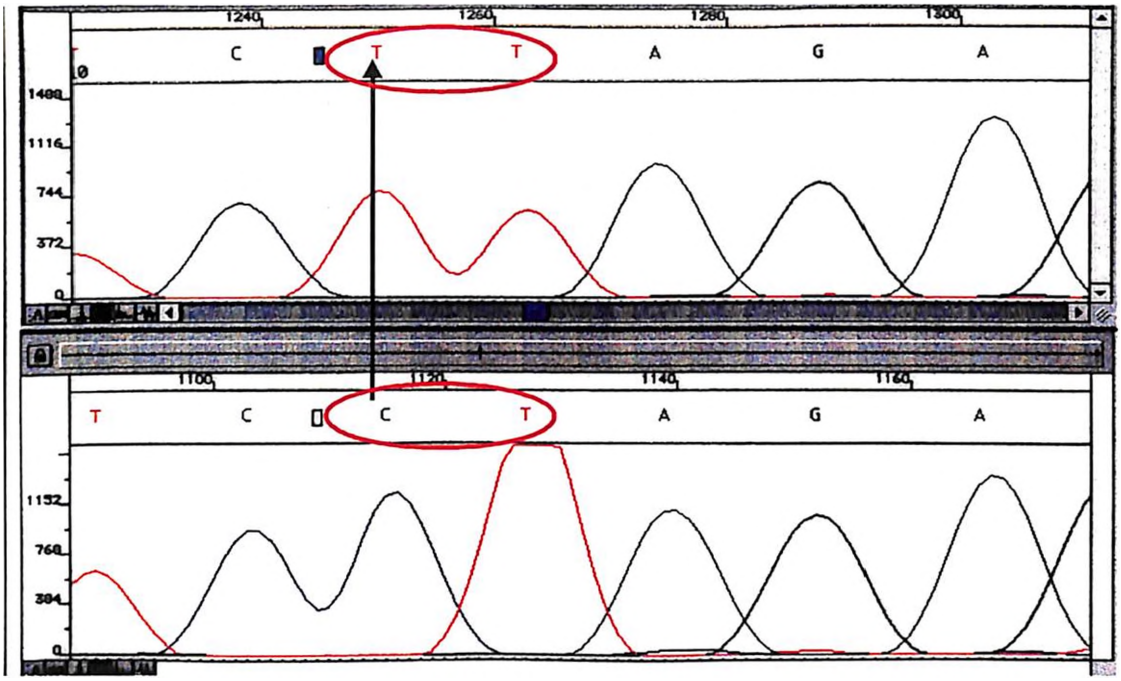


Figure 14 : Mutant (Top) and wild type (bottom) sequence of intron-exon splice acceptor site in exon 11 of RB1 gene from tumor DNA of BLRB patient (N8) with reverse primer

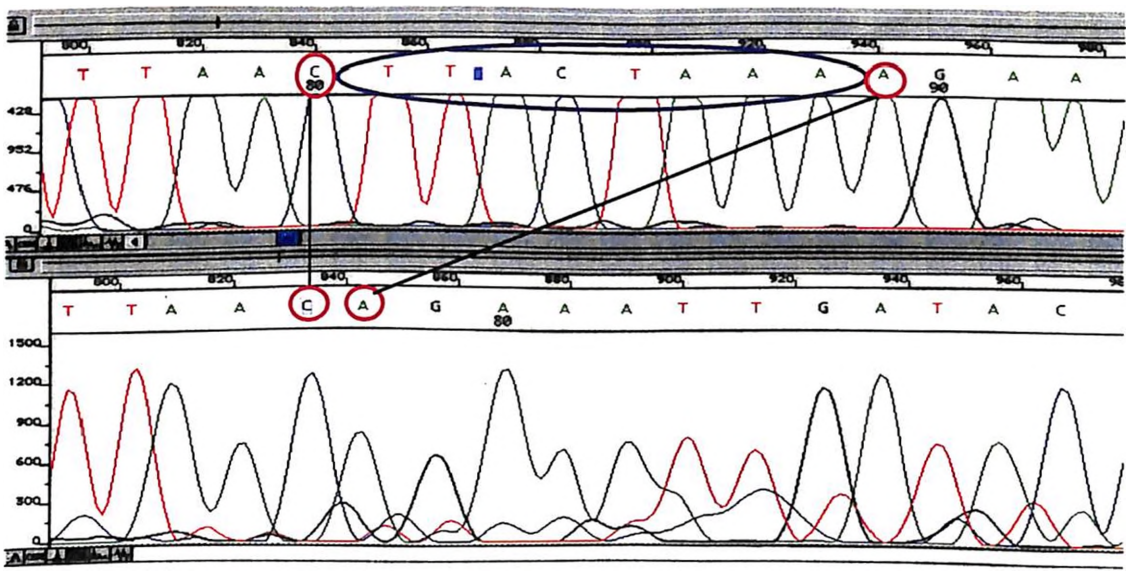


Figure 15: Wild type (top) and mutant (bottom) sequence with forward primer of exon 4 of RB1 gene from tumor DNA of BLRB patient (N15) with 8 bp deletion

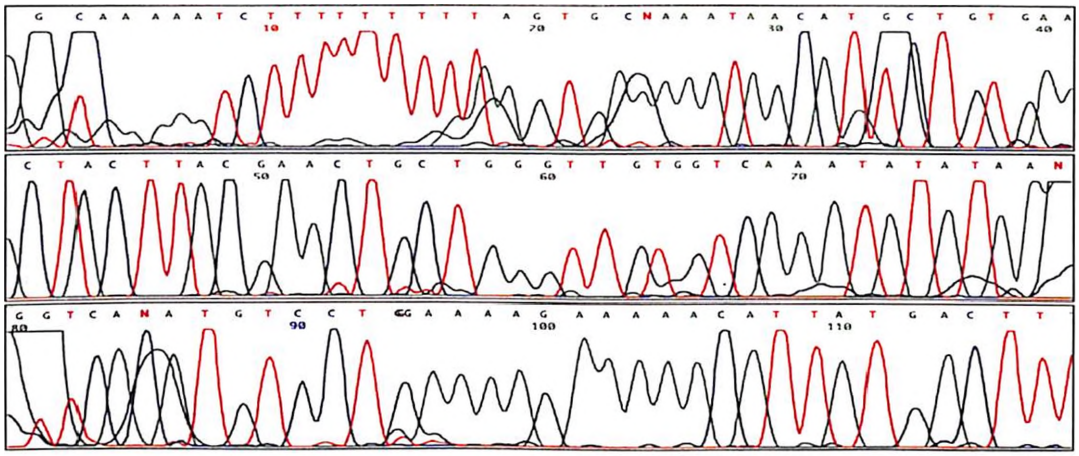


Figure 16 : RB1 exon 5 sequence from formalin-paraffin tumor extracted DNA

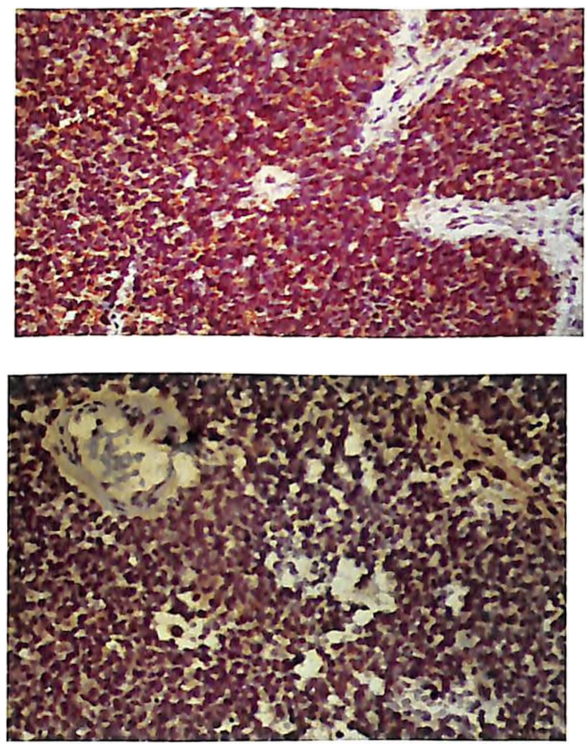


Figure 17 : Microphotograph showing 100% tumor cells positive for retinoblastoma protein expression with IF8 monoclonal Ab(top) and 40% tumor cells positive for retinoblastoma protein expression with C15 polyclonal Ab (bottom) (250X and DAB with haematoxylin counterstain)

Table 9: pRB expression results by immunohistochemistry for IF8 and C15 antibodies

NMD / Expression	Exp. / Obs.	Exon 4 deletion (N15)	S215X (N29)	*R251X (R445X) (N12)	R255X (N19)	R358X (N34)	Intron 11 splice (N8)	*R445X (R251X) (N12)	R579X (N26)	S780X (Q56)	R787X ($S_1$61)	R787X (N37)
Possibility of NMD		+	+	+	+	+	+	+	+	+	+	+
IF8 expression (%)	**Exp.**	INA	INA	INA	INA	INA	INA	INA	INA	INA	INA	INA
	Obs.	0	0	0	0	0	100	0	0	0	0	0
C15 expression (%)	**Exp.**	0	0	0	0	0	0	0	0	0	0	0
	Obs.	0	0	20	70	0	0	20	30	40	15	0

NMD- nonsense mediated decay;+- NMD could happen; Exp.- expected; Obs- observed; * - One patient had two mutations. INA- Information not available

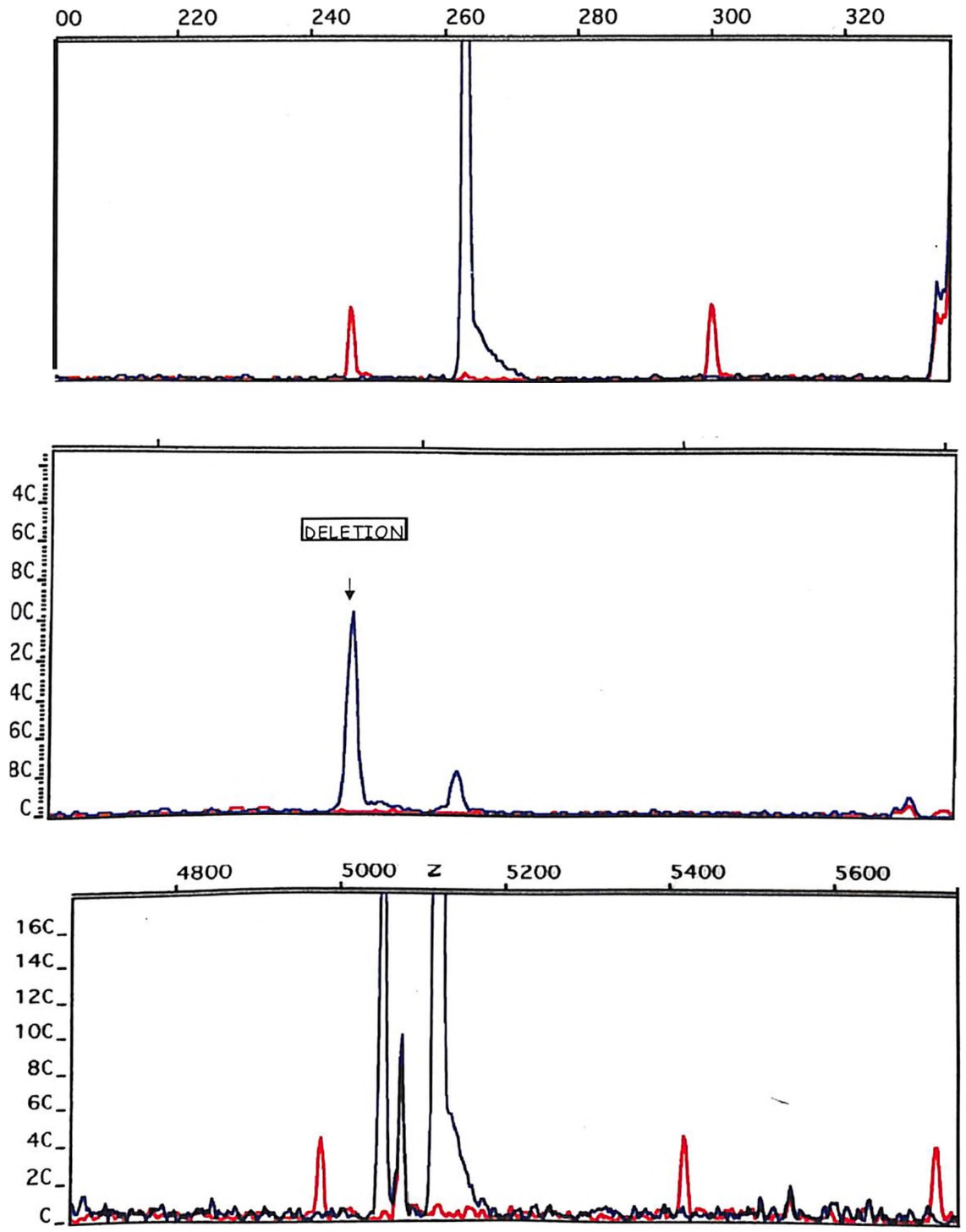


Figure 18 : Fragment analysis showing mobility shift of the 8 bp deletion in exon 4 of RB1 gene on ABI 310 sequencer, Electrophoretic run of control without deletion, deletion sample, pooled sample and control are shown

Table 10: Summary of aminoacid conservation at the mutant sites

1. S215

ID	Sequences	Position	Species
Query 1	GEVLQMEDDLVI S F QLMLCVLDYFI	25	pRB
Sbjct 157	GEVLQMEDDLVI S FQLMLCVLDYFI	181	Pan troglodytes
Sbjct 172	GEVLQMEDDLVI S FQLMLCVLDYFI	196	Canis familiaris
Sbjct 67	GQVLQMEDDLVI S FQLMLCVLDYFI	91	Bos taurus
Sbjct 175	GEVVQMEDDLVI S FQLMLCVLDYFI	199	Rattus norvegicus
Sbjct 197	GEVLQMEDDLVI S FQLMLCVVDYFI	221	Mus musculus
Sbjct 191	GKVLQMEDDLVI S FQLLLCVLDYFI	215	Gallus gallus

The numbers on either side indicates the position in the retinoblastoma related protein from the databases. Query refers to the submitted sequence. Sbject refers to the database sequence. The conserved aminoacids are underlined.

2. R251, 3. R255

ID	Sequences	Position	Species
Query 1	K T A V I P I N G S P R T P R R G Q N R S A R I A	25	pRB
Sbjct 194	K T A V I P I N G S P R T P R R G Q N R S A R I A	218	Pan troglodytes
Sbjct 234	K T A A I P I N G S P R T P R R G Q N R S A R I A	258	Mus musculus
Sbjct 209	K T A V I P F N G S P R T P R R G Q N R S A R I A	233	Canis familiaris
Sbjct 212	K T A A T P I N G S P R T P R R G Q N R S A R I A	236	Rattus norvegicus

4. R358

ID	Sequences	Position	Species
Query 1	D S I D S F E T Q R T P R K S N L D E E V N V I P	25	pRB
Sbjct 300	D S I D S F E T Q R T P R K S N L D E E V N V I P	324	Pan troglodytes
Sbjct 318	D T I D S F E T E R T P R K S N P D E E A N M V T P	343	Rattus norvegicus
Sbjct 340	D P I D S F E T E R T P R K N N P D E E A N V V	363	Mus musculus
Sbjct 315	D P T D S F E M Q R T P Q K S N S D E E V N V I	338	Canis familiaris

5. R445

ID	Sequences	Position	Species
Query 1	AVGQGCVEIGSQ R YKLGVRLYYRVM	25	pRB
Sbjct 387	AVGQGCVEIGSQ R YKLGVRLYYRVM	411	Pan troglodytes
Sbjct 50	AVGQGCMEIGSQ R YKLGVRLYYRVM	74	Canis familiaris
Sbjct 297	AVGQGCMEIGSQ R YKLGVRLYYRVM	321	Bos taurus
Sbjct 402	AVGQGCMEIGSQ R YKLGVRLYYRVM	426	Canis familiaris
Sbjct 405	AVGQGCIDIGAQ R YKLGVRLYYRVM	429	Rattus norvegicus

6. R579

ID	Sequences	Position	Species
Query 1	SPLFDLIKQSKD R EGPTDHLESACP	25	pRB
Sbjct 521	SPLFDLIKQSKD R EGPTDHLESACP	545	Pan troglodytes
Sbjct 184	SPLFDLIKQAKD R EGPADHLESAC	207	Canis familiaris
Sbjct 539	SPLFDLIKQSKD G EGP-DHLESAC	561	Rattus norvegicus
Sbjct 431	SPLFDLIKQAKD R EGPVDHFEPAC	454	Bos taurus
Sbjct 561	SPLFDLIKQSKD G EGP-DNLEPACP	584	Mus musculus

7. S780

ID	Sequences	Position	Species
Query 1	ILQYASTRPPTL S PIPHIPRSPYKF	25	pRB
Sbjct 722	ILQYASTRPPTL S PIPHIPRSPYKF	746	Pan troglodytes
Sbjct 803	ILQYASTRPPTL S PIPHIPRSPYKF	827	Rattus norvegicus
Sbjct 761	ILQYASTRPPTL S PIPHIPRSPYKF	785	Mus musculus
Sbjct 385	ILQYASTRPPTL S PIPHIPRSPYKF	409	Canis familiaris
Sbjct 760	ILQYASNRPPTL S PIPHIPRSPYQF	784	Gallus gallus
Sbjct 632	ILQYASTRPPTL S PIPHIPRSPYKF	656	Bos taurus

8. R787

ID	Sequences	Position	Species
Query 1	RPPTLSPIPHIP R SPYKFPSSPLRI	25	pRB
Sbjct 729	RPPTLSPIPHIP R SPYKFPSSPLRI	753	Pan troglodytes
Sbjct 392	RPPTLSPIPHIP R SPYKFSSSPLRI	416	Canis familiaris
Sbjct 746	RPPTLSPIPHIP R SPYKFSSSPLRI	770	Rattus norvegicus
Sbjct 768	RPPTLSPIPHIP R SPYKFSSSPLRI	792	Mus musculus
Sbjct 639	RPPTLSPIPHIP R SPYKFSSSPLRI	663	Bos taurus
Sbjct 765	RPPTLSPIPQIP C SPYKFPNSPLRV	789	Oryzias latipes

2.5 Discussion

Sequencing was standardized for RB1 exons 1-27 in 20 µL reaction with 2.0 µL RR Mix under standard cycle sequencing conditions with 12.8 pM of primer. Cycle sequencing reaction using 1.0 µl RR Mix was used for smaller exons of RB1 gene (less than 200 bp size). Sequencing of RB1 exon 1 was achieved by supplementing 2.5% DMSO in cycle sequencing reaction. The RB1 mutation screening was first done in tumor DNA of retinoblastoma patients and then screening was performed in peripheral blood DNA for the same mutation. This method was undertaken so that exon screening was not duplicated in blood and tumor, so that time and money was saved.

Sequence data after 11 repeats of 'T' in intron 15 was ambiguous and hence bi-directional sequencing was used for RB1 exon 15&16. Each reaction will give good sequence for the ambiguous region up to the homopolymer region. Sequencing of RB1 exon 22, which has a homopolymer region of 34 T in the beginning of exon, was done with reverse primer. In this way, the exonic region will be upstream of the repeat region and hence readable. Ata-ur-Rasheed *et al*, (2002) digested exon 22 PCR product with DdeI and the fragment containing the exon was purified after agarose gel electrophoresis before sequencing.

Mutations were identified in N terminus (exon 4, 7, 8 and 11), introns (intron 11), A/B domain (exon 14), spacer (exon 18) and C terminal region (exon 23). This is consistent with the distribution of mutations throughout the length of RB1 gene (Harbour, 1998). All mutations resulted in premature termination of proteins. Seven of the eleven mutations identified were in CGA codons. Six single base substitutions (R251X, R255X, R358X, R445X, R579X and R787X) detected in the study were previously reported as recurrent mutations (Richter *et al*, 2003).

In one unilateral patient, two CGA codon mutations resulted in arginine to stop codon in exons 8 (R251X) and 14 (R445X). The N terminal domain, where RB1 exon 8 is located, is important in pRB-mediated tumor suppression (Goodrich, 2003). Exon 14 is present in the A domain of pRB where transcription factor E2F has a binding site.

In two unrelated bilateral retinoblastoma patients (N37, $S_1$61), heterozygous C to T mutation was detected in exon 23 resulting in arginine to stop codon (R787X). In the patient, N37, the mutation was detected in peripheral blood as well (Genbank accession no. AY642691) and was somatic in origin (Genbank accession no. AY642692) in the other ($S_1$61). This mutation could have abolished binding of E2F and *c-abl* tyrosine kinase.

C to T mutation in exon 18 in a bilateral retinoblastoma patient (N26) resulted in R579X. The mutation could have resulted in a defect in the spacer of the pRB protein affecting the interaction of A and B domains of retinoblastoma protein.

In two bilateral retinoblastoma patients (N19 and N34), C to T transition was noted in exon 8 (R255X) and in exon 11 (R358X) respectively. Both exons are in the N terminus domain of pRB. The N terminus domain is important in pRB-mediated tumor suppression (Goodrich, 2003).

In a bilateral retinoblastoma patient (N29), a C to G substitution in exon 7 in peripheral blood DNA was detected resulting in S215X (Genbank accession no. AY 643840). In this patient, at first the mutation was ignored as an artifact because cycle sequencing from tumor DNA using reverse primer did not confirm the exon 7 mutation. Since the mutation was detected in peripheral blood DNA of the patient, sequencing from tumor DNA was repeated. It was realized that detecting the mutation in tumor DNA is dependent on the tumor DNA clone. It is possible that at least some RB1 mutations might have been missed in the study due to tumor heterogeneity

resulting in lack of correlation. This aspect is relevant in the current study since mutation screening was done in tumor DNA and then blood DNA was checked for the mutation.

In a unilateral patient (Q56), a homozygous C to A single base substitution in the tumor DNA in exon 23 changed S780 to a stop codon. S780X is present in the middle of a 13–residue carboxy terminal of pRB (Aminoacids 775–787). Cyclin D1\cdk4 complex preferentially phosphorylate S780. The negative charge on phosphorylated C-domain is important in interaction with LXCXE binding lysine residues in B domain. This mutation in C terminal region of pRB could have also abolished binding of E2F and *c-abl* tyrosine kinase to pRB (Genbank accession no. AY642690).

In a bilateral retinoblastoma patient (N8), a homozygous G to A mutation was identified in the splice acceptor site of intron 11 in tumor DNA. Most mutations that affect splicing are associated with bilateral retinoblastoma (Lohmann, 1999). This could have resulted in skipping of exon 12 and premature truncation in the protein.

In the present study the novel 8 bp deletion in RB1 exon 4 in a bilateral patient (N15) resulted in frameshift (aminoacids NLLK changed to NRNX) and created a stop mutation in the same exon (136X). RB1 exon 4 is present in the N terminal domain of pRB. Deletion of 40 aminoacids from the N-terminal domain (in RB1 exon 4) of pRB has been associated with low penetrance retinoblastoma (Dryja *et al*, 1993). Amino terminus even though not completely essential for pRB function and tumor suppression, might be important in the interaction of pRB with the large pocket region (Dryja *et al*, 1993). Complete N terminal deletion might not have any effect on pRB function (probably leading to apoptosis) while internal small mutations have dominant inhibitory effect on large pocket function (Goodrich, 2003). The Genbank

accession numbers for the 8 bp deletion, S215X, S780X and R787X mutations are given in appendix VII.

Fragment analysis showed mobility shift for the 8 bp deletion mutation in exon 4 compared to normal control (Figure 18). This technique could be used as a rapid diagnostic tool in screening the close blood relatives for the mutation. Automated sequencing to screen for the mutation (in proband) in close blood relatives requires much more time compared to fragment analysis. The overall genetic analysis cost for the family could be reduced by rapid fluorescent fragment analysis for the deletion mutations in close blood-relatives. Fragment analysis helped in detecting the two at risk individuals in a family and thereby eliminating unwanted clinical screening for retinal tumors for 23 relatives (Bremner *et al*, 1997). Richter *et al*, (2003) established fragment analysis for RB1 gene to identify aberrantly migrating bands indicating exonic deletions or insertions. Fragment analysis has advantages over sequencing in being more reproducible, less susceptible to changes in electrophoretic conditions, lesser turn around time and less ambiguous in interpretation, which was useful for analysis. Once DNA is available the test could be completed in less than three hours enabling rapid screening within the shortest possible time. This could help in deciding the treatment course for the patient and family. This aspect of genetic diagnosis is very relevant in India where the patients have to travel long distances for availing treatment and genetic testing. However the patient with 8 bp deletion did not have family history nor genomic deletion and hence this diagnostic method was not used in this family.

Immunohistochemical detection of retinoblastoma protein in formalin-paraffin tumor sections has advantages of convenience, economy and compatibility with routine histopathological analysis (Shi *et al*, 1996). The epitopes for IF8 Ab, raised against the RB product-beta galactosidase fusion protein is not known and hence the pRB

expression analysis with IF8 Ab could not be inferred with the truncation mutations according to the hybridizing site. IF8 monoclonal Ab did not show pRB expression in nine out of ten tumors (Table 9) and showed positive correlation with nonsense mediated decay for the same nine tumors (Table 9). Nonsense mediated mRNA decay could occur when premature truncation of the gene occur more than 50 nucleotides away from the next exon-intron junction (Frischmeyer and Dietz, 1999). However, it was not clear, why a bilateral patient having the splice site mutation and possibility of nonsense mediated decay had protein expression in 100% of tumor cells with IF8 anitbody. The polyclonal C15 Ab hybridizes at the 'C' terminus of pRB. Since all the RB1 mutants in the study had truncation before C terminus, no pRB expression was expected with C15 antibody. However, five of the tumors did not show pRB expression with C15 Ab; but the remaining five had expression ranging from 0-70 %. The variation in expression could be due to the difference in the number of epitopes and the polyclonal nature of the antibody besides we did not find any correlation of this antibody with nonsense mediated decay (Table 9).

The BLAST analysis showed conservation of the wild type aminoacid at all the mutant sites (Table 10) in various species. This shows that pRB and related proteins might play vital function not only in humans; but also in other species like domestic cow, house mouse and domestic rat.

Aminoacid sequences from L11910 were used for the three-dimensional model prediction for the RB1 truncating mutants in A/B and C terminus, for which templates are available. Retinoblastoma–associated protein is not crystallized in complete form till now. The models were based on the templates In4mA, 109 kG, 109 kA, 109 kE and 109 k at ExPaSy server, derived from crystals generated when bound to fragments of E2F or HPV DNA in large pocket of pRB. In step 1, inclusion of N terminus aminoacids, 378 in number, did not generate models for wild type or mutants due to

lack of homology. This could be expected considering N terminus region constituting 40% of the aminoacids of pRB. Omission of aminoacids in N terminus region resulted in model prediction for R445X, R579X, S780X, R787X and wild type pRB since above 25% homology was obtained. The results indicate that protein modeling could be performed only to restricted parts of the pRB.

2.6 Conclusions

Altogether, 11 mutations were identified including four novel mutations. RB1 mutations were frequent in N terminus domain (six of eleven) of the protein. The CGA arginine codon was involved in 7 out of 10 single base substitutions. Lack of RB1 mutations in nine bilateral and six unilateral retinoblastoma patients could be due to missed mutations from clonal nature of the tumor, mutations belonging to a class not detected by the techniques used (i.e. copy number changes or somatic mosaicism), intronic or other regulatory element including promoter mutations leading to hyperphosphorylation and functional inactivation of RB1 gene (Goodrich, 2003). RB1 gene could have also got inactivated by mechanisms other than mutations like promoter hypermethylation (Joseph *et al*, 2004). Retinoblastoma protein expression showed positive correlation with possibility of nonsense mediated mRNA decay for nine samples for IF8 Ab. Conservation analysis showed the importance of the mutated aminoacids in pRB function. Homology modeling revealed that modeling could be done only for restricted parts of pRB.

2.7 References

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Chapter 3

Methylation analysis of RB1 promoter

3.1 Aim

- To estimate the RB1 promoter hypermethylation frequency in retinoblastoma tumor DNA.

3.2 Review of Literature

Retinoblastoma, a childhood malignancy of the retina, is developed through different genetic pathways. Inactivation of RB1 gene is usually caused by mutations affecting the coding region but the mutations confined to promoter region have also been reported. In addition to genetic changes hypermethylation of CpG island in the promoter region also results in the silencing of RB1 gene and other tumor suppressor genes like BRCA1, p15 and p16 (Costello *et al*, 2000; Dobrovic and Simpfendorfer, 1997; Gregor *et al*, 1989; Gregor *et al*, 1994; Herman *et al*, 1996; Ohtani-Fujita *et al*, 1993). Hypermethylation of the promoter region of tumor suppressor genes leads to the silencing of the gene and transcriptional repression which has been postulated as one of the potential mechanisms for oncogenesis (Yan *et al*, 2001). CpG island hypermethylation is shown to result inactivation of more than 90 tumor-related genes in many cancers. Hypermethylation is infrequent and occurs in approximately 1% of CpG islands in the breast tumor genome (Costello *et al*, 2000). Yan *et al*, (2001) applied CpG island microarray technique to study the methylation profile in breast and ovarian cancer. The results of the study supported the notion that hypermethylation of critical CpG island loci has a role in development of cancer (Yan *et al*, 2001). Promoter hypermethylation of RB1 gene has been described in 25% of glioblastomas (Nakamura *et al*, 2001). Salem *et al*, (2000) has described progressive increase in *de novo* methylation of CpG islands in bladder cancer.

Hypermethylation of VHL CpG island and lack of gene expression is proved in clear cell renal carcinoma (Herman *et al*, 1994). Inactivation of p16 gene, due to hypermethylation, is established in brain, breast, colon, head and neck and non-small-lung cancers (Baylin *et al*, 1998). Methylation induced inactivation of p15 gene occurs in haematopoietic neoplasms (Herman *et al*, 1997). Methylation induced silencing of the BRCA1 gene is responsible for 10-15% of sporadic breast cancer (Esteller *et al*, 2000). Promoter CpG island methylation is shown to inactivate both alleles of MLH1 gene (Baylin and Herman, 2000). Absence of MGMT gene expression due to promoter hypermethylation is seen in cancers like colon, lung and lymphoid tumors (Esteller *et al*, 2000). Choy *et al*, (2002) has shown that MGMT expression was frequently absent in retinoblastoma tissues due to methylation of its promoter region. Edmunds *et al*, (2002) showed methylation of $p16^{INK4A}$ locus in uveal melanomas. Frequent promoter hypermethylation of CDHI, DAPK, RARB and HICI genes in patients with carcinoma of cervix had influence on the clinical outcome (Narayan *et al*, 2003).

A 600 bp CpG island consisting of the essential promoter is present at the 5' end of RB1 gene (Simpson *et al*, 2000). Aberrant methylation of the CpG island within the RB1 promoter region has been described in pituitary adenomas and unilateral retinoblastoma (Sakai *et al*, 1991; Simpson *et al*, 2000; Stirzaker *et al*, 1997). Methylation has been shown to result in inactivation of tumor suppressor genes, DNA repair genes and metastasis inhibitor genes (Wong, 2001). *In vitro* and *in vivo* studies have suggested that methylation of the RB1 promoter dramatically reduces gene activity (Ohtani-Fujita *et al*, 1993). Studies using methylation-sensitive restriction enzyme digestion techniques have shown that methylation of RB1 alleles are associated with reduced level of RB1 transcript in sporadic retinoblastoma (Gregor *et al*, 1994). Stirzaker *et al*, (1997) has shown that methylation was not confined to

single CpG island dinucleotides but extended to essentially all CpG dinucleotides spanning the RB1 CpG island, including the core promoter region. In a bilateral retinoblastoma patient with X:13 translocation silencing of RB1 gene by spreading of the X inactivation has been proved (Jones *et al*, 1997). In the present study the methylation status of the CpG island within the promoter region of RB1 gene has been evaluated to define the molecular mechanism responsible for retinoblastoma.

3.3 Methodology

Patients with retinoblastoma were enrolled in the study after being referred by the clinician or following enucleation. Tumor samples were obtained from the theatre after enucleation. Patient's tumor DNA was studied following ethical approval by the hospital institutional review board. Twenty-two bilateral and 23 unilateral patients were included in the study.

The tumor tissue was treated with digestion buffer containing 1M Tris, Triton- X 100 and Proteinase K (15mg/ml) and kept for complete digestion at 37°C. After digestion, DNA was extracted by standard phenol chloroform method (Wolff and Gemmil, 1998), quantified spectrophotometrically and used for MS-PCR.

3.3.1 Methylation specific PCR (Frommer *et al*, 1992)

CpG dinucleotides within the promoter region were examined for methylation using the MS-PCR (Herman *et al*, 1996). The MS-PCR technique uses bisulfite modification of DNA, under conditions whereby cytosine is converted to uracil, and 5-methyl cytosine remains non reactive (Simpson *et al*, 2000; Frommer *et al*, 1992).

Bisulfite-induced modification of DNA was done by 16-hour protocol. DNA (100 ng) was diluted to 50 μL with distilled water and denatured by addition of 5.5 μL of 2 M NaOH and incubated at 37°C for 10 minutes (to create single stranded DNA). Salmon sperm DNA was added to the DNA to act as a carrier prior to denaturation. Then 30 μL of 10 mM hydroquinone and 520 μL of 3 M sodium bisulfite were added. The mixture was incubated at 50°C for 16 hours. Bisulfite modified DNA was purified by a manual method and also by using Promega Wizard DNA Clean-Up system (Promega, USA). In the manual method, DNA was ethanol precipitated and washed with 70% alcohol, dried and resuspended in 20 μL of water. The procedures according to the manufacturer were followed for the kit method. To the eluted DNA 5.5 μL of 3 M NaOH added, incubated at room temperature for 5 minutes. One μL of 1/10 diluted salmon sperm DNA, 33 μL of 10 M ammonium acetate and three volumes of ethanol were added. The purified DNA was precipitated by overnight incubation at -20°C, washed with 70% ethanol, dried and resuspended in 20 μL of water. A 25 μL reaction was set up using 2 μL of bisulfite modified DNA, 0.3 μL of primers (300 ng/μL), 2.0 μL of dNTPs (25 mM), 2.0 μL PCR buffer and *Taq* DNA polymerase (1.25 U/μL). The sequences of both methylated and unmethylated set of primers along with the amplified product lengths are given in Table 11. A hot start PCR was done using initial denaturation at 95^0C for 5 minutes, 40 cycles consisting of denaturation at 95^0C for 30 seconds, annealing at 56^0C for 30 seconds and extension at 72^0C for 30 seconds and final extension at 72^0C for 4 minutes. The amplified products were electrophoresed using 12% polyacrylamide gel and silver staining was done. The gel was captured and analyzed using VDS Image master Gel documentation system (Pharmacia biotech.) Positive control is an *in vitro* methylated DNA, amplified with both methylated and unmethylated set of oligonucleotides following sodium bisulfite modification.

Table 11: Primer sequences for MS-PCR, their annealing temperature and the product size

Promoter	Primer sequence	Annealing Temperature	Product
M F	5' ACGTCGAAACACGCCCCG3'	56^0C	168 bp
M R	5' GGGAGTTTCGCGGACGTGAC3'		
UM F	5' GGGAGTTTTGTGGATGTGAT 3'		
UM R	5'ACATCAAAACACACCCCA 3'		

M – Methylated; UM – Unmethylated; F- Forward; R- Reverse

3.4 Results

The silver stained poly acrylamide gel is shown in Figure 19.There were 28 males and 17 females (Table 12). In the study of 45 samples, three (6.6%) were hypermethylated (one unilateral and two bilateral); 13 samples were partially methylated (29%) and the remaining 29 samples were unmethylated. Among bilateral patients, fourteen were unmethylated, two were hypermethylated and six had partial methylation while fifteen, one and seven unilateral patients respectively had unmethylated, hypermethylated and partially methylated RB1 CpG island. Table 13 shows the characteristics of the three patients with hypermethylation of the RB1 promoter region.

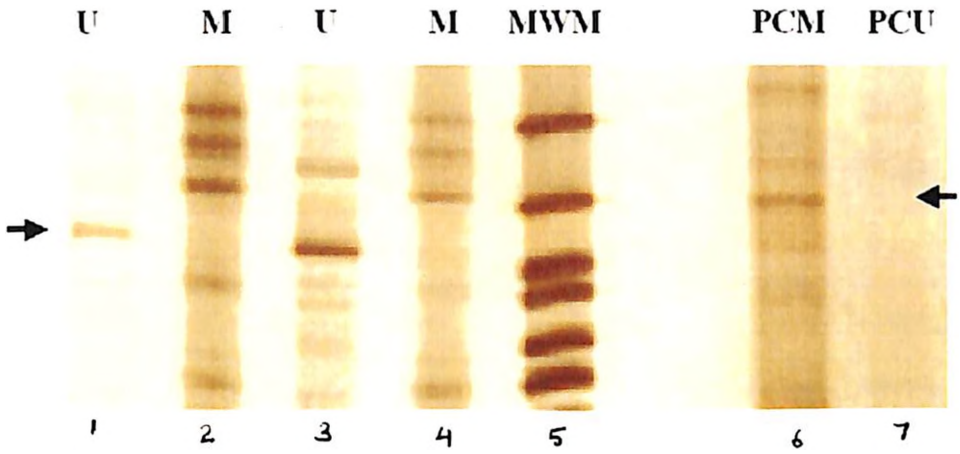

Figure 19 : Silver stained Polyacrylamide gel from methylation analysis of RB1 gene promoter M- methylated; U- un methylated; MWM - pBR322 DNA/ Hinf I Digest; PCM-control for methylation; PCU- control for un methylation

Table 12: Sex distribution against the methylation results for the 45 retinoblastoma patients

Sex	Laterality	Hypermethylated	Partially methylated	Unmethylated	Total
Males	Bilateral	1	2	13	16
	Unilateral	1	4	7	12
Females	Bilateral	1	4	1	6
	Unilateral	0	3	8	11
Total		3	13	29	45

Table 13: Characteristics of the three patients with hypermethylation of the RB1 promoter region

Category	Patient 1	Patient 2	Patient 3
Age / Sex	10 months /F	15 months /M	33 months /M
Laterality	bilateral	bilateral	unilateral
Age of onset	5 months	1 months	6 months
Karyotype	13q14 deletion	Normal	Normal
Histopathology	Well differentiated retinoblastoma	Well differentiated retinoblastoma	Undifferentiated retinoblastoma
Methylation status	complete	complete	complete

3.5 Discussion

Ohtani-Fujita *et al*, (1993) demonstrated dramatic reduction in pRB expression due to *in vitro* methylation of the RB1 promoter region and C to T transitions at CpG dinucleotides causing premature termination of protein synthesis. These CpG changes are the result of methylation of cytosine within these CpGs; hence the hypermethylation status of the CpG island contained within the promoter region of the RB1 gene was investigated.

Hypermethylation of RB1 promoter region was detected in 6.6% of samples. The results were similar to the results of Sakai *et al*,(1991) who reported 7% of hypermethylation; but is lower than 10% reported by Zeschnigk *et al*, (1997) and Choy *et al*, (2002). Lohmann *et al*, (1997) could not detect any hypermethylation.

Previous studies using methylation sensitive restriction enzyme digest techniques have shown that methylation of RB1 allele (promoter region) is associated with a reduced level of RB1 transcript in sporadic retinoblastoma (Gregor *et al*, 1994). These studies were extended by Stirzaker *et al*, (1997) who reported that methylation was not confined to single CpG dinucleotides but extended to essentially all CpG dinucleotides spanning the RB1 CpG islands including the promoter region in unilateral retinoblastoma. Using a model system, Ohtani-Fujita *et al*, (1993) have shown that transcription factors, important in activating the RB1 promoter is unable to bind when CpG dinucleotides within their recognition sequences are methylated.

Jones *et al*, (1997) suggested that complete gene silencing is dependent on the density and extensiveness of methylation that may vary with the developmental stage of the tumor. Partial methylation may result in the down-regulation of the RB1 gene and reduced pRb levels, whereas hypermethylation of the CpG island will lead to complete gene silencing.

Choy *et al*, (2002) studied methylation status of 23 retinoblastoma DNA extracted from archival or frozen tissues for the CpG islands immediately 5' to the transcription start site of the genes p14 ^{ARF}, p15 ^{INK4b}, p16 ^{INK4a}, VHL and MGMT. Complete methylation was seen in two samples for the MGMT. Partial methylation was seen in six samples shown by amplification both with methylated and non-methylated primer sets. The methylated samples had onset of disease before the age of two years and majority had poorly differentiated or undifferentiated tumor (Choy *et al*, 2002).

MS-PCR is a simple, rapid and inexpensive method to determine the methylation status of CpG islands. This approach allows the determination of methylation patterns from very minute quantity DNA samples, including those obtained from paraffin embedded samples and can be used in the study of abnormally methylated CpG islands in neoplasm. MS-PCR utilizes the sequence difference between methylated alleles and unmethylated alleles that occur after sodium bisulfite treatment (Herman *et al*, 1996).

It is highly unlikely that a patient with methylation will have germline mutations since methylation is an epigenetic phenomenon (Vogel, 1979). Methylation analysis will be useful in genetic counseling and will be of much relief for the family of the patient as it is non-hereditary. The clinical trials going on for inhibitors of methylation may offer new avenues of treatment options. Aza cytidine used for treatment of cancer is shown to have demethylating activity by inhibition of DNA methyl transferases. This drug has been shown to have potent antitumor activity against breast cancer *in vitro* (Bovenzi *et al*, 1999). 5-Aza cytidine is also shown to increase the activity of p16 or VHL genes in the cell lines in which these genes are hypermethylated and inactivated (Herman *et al*, 1994) and showed minimal to moderate antitumor activity in head and neck, and prostate cancer patients (Thibault *et al*, 1998). Procanamide, used in treatment of cardiac arrythmias, has been shown to reactivate the expression of p16 transcript from a methylation-silenced promoter in prostate cancer cells (Mareel *et al*, 1995).

3.6 Conclusions

This is the first study to demonstrate methylation of RB1 promoter region in Indian patients with retinoblastoma. In the survey of 45 patients with retinoblastoma, three showed hypermethylation constituting 6.6% of the changes that leads to complete silencing of RB1 gene. Lack of other methylation studies in retinoblastoma patients from India makes comparison of the results impossible. Evaluating the possibility of particular CpG sites being preferentially methylated with difference in methylation profile could predict the methylation phenotype in retinoblastoma. Further studies are warranted to find the details of phenotypic profile of the different methylation status using a higher sample size that can be applied in clinical practice for probable demethylation therapy.

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Chapter 4

Karyotype Analysis

4.1 Aim

- Chromosomal analysis by peripheral blood lymphocyte culture and GTG banding

4.2 Review of Literature

Retinoblastoma arises due to loss of both copies of RB1 gene in developing retinal cells. Genetic testing is crucial for accurate risk prediction for retinoblastoma in close relatives of proband. Of the genetic tests in retinoblastoma, karyotype analysis could be performed in a basic genetics laboratory and cheaper than the molecular genetic tests like mutation screening of RB1 gene and deletion analysis. In a developing country like India, cost influences the patient or parental decision to cooperate for genetic testing. Additional requirements like parental and close relatives' DNA samples make molecular deletion test more complicated.

Karyotyping from peripheral blood detects chromosomal abnormalities in 7.5-8.0% of bilateral and 1.0-4.9% of sporadic unilateral retinoblastoma patients (Ejima *et al*, 1988). The most frequent chromosomal abnormality in retinoblastoma is interstitial deletion of 13q14 region where the RB1 gene is located (Yunis and Ramsay, 1978). Patients with genomic 13q14 deletion could pass the susceptibility to 50% of their offspring. Amare *et al*, (2004) studied 36 consecutive retinoblastoma patients from India for constitutional RB1 deletion by conventional karyotyping and *fluorescent in situ hybridization* and fragile site expression in chromosomes. Constitutional RB1 deletion was detected in 12.3% of retinoblastoma patients. Nearly 50% of patients (with 13q14 deletion) in the study of Amare *et al*, (2004) showed mosaicism for 13q14 deletion. This shows the necessity of paying attention to the number of metaphases with 13q14 deletion in conventional karyotyping to derive meaningful

results. Amare *et al*, (2004) also proved higher frequency of q14 fragile site expression, in retinoblastoma patients with constitutional 13q14 deletion, indicative of inherent genomic instability.

Gandhewar *et al*, (2004) reported limited value of conventional cytogenetic analysis in retinoblastoma. The variability in trypsin digestion and the relative positioning of the chromosomes (lying bent or straight) in the metaphase influences the decision of 13q14 deletion. Lack of complete understanding of the processes during chromosome preparations act as a constraint in improving the accuracy of karyotype analysis (Claussen *et al*, 2002). However chromosomal region specific protein swelling is hypothesized to be responsible for Giemsa banding (Claussen *et al*, 2002). FISH on metaphase preparations also require extended chromosomes. The microscope cost, the limited number of samples that could be analyzed and requirement of expertise restrict routine application of FISH. FISH analysis with RB1 probes is considered as an adjunct to conventional cytogenetic studies in hematological malignancies with 13q14 abnormalities (Juneau *et al*, 1998).

Statistics has been widely used in medical research to convert non-normally distributed data to normal distribution before applying tests of significance. Transformation of non-normally distributed data to Gaussian distribution has been used in G-protein research (Freeman and Spina, 2004). An exact permutation test based on Baumgarter-Weiss-Schindler statistic B is suggested for asymmetric or heavily tailed distributions of microarray data (Neuhauser and Senske, 2004). Statistical methods in analysis of skewed data containing zeros in psychiatric clinical studies resulted in more meaningful conclusions (Delucchi and Bostrom, 2004). Transformations applied to skewed data ensured approximately normal distribution to derive reference intervals for biochemical tests in pediatric patients (Brinkworth *et al*, 2004). Statistical application in studies of retinoblastoma established the 'two- hit'

hypothesis, which explained the mechanism of tumorigenesis in hereditary and non-hereditary retinoblastoma (Knudson, AG (Jr.), 1971). Fijal *et al*, (2002) used an algorithm created by statistical modeling to analyze the phenotypic effects of the p53 gene mutation in colorectal tumors. Betinsky *et al*, (2003) used a statistical approach to analyze results of a molecular neuro-genetic study (with partially biased sample selection) to retain important samples for analysis. In the present study, transformation was applied to 13q14 deletion findings in retinoblastoma to derive more meaningful results.

4.3 Methodology

Patients with retinoblastoma were enrolled following enucleation surgery or referred by an ophthalmologist for genetic testing/counseling. The study was done with prior ethical approval by the hospital board/ethics committee. A pretest informed consent was obtained from the parents of the proband after detailed pedigree analysis and counseling. A post-test counseling was done after the karyotype reports were ready. Blood for karyotype analysis was collected from one regressed, 33 bilateral and 25 unilateral retinoblastoma probands during enucleating surgery or at a subsequent visit to the hospital.

Peripheral blood culture was done by 72-hour technique using Rosewell Parker Memorial Institute medium (RPMI 1640). Metaphase slides were prepared by standard methods followed by GTG banding. The methodology for peripheral blood culture and Giemsa banding are given in appendix VII.

The metaphases were first scanned under low power magnification to select well-spread and optimally stained metaphases with elongated chromosomes. Twenty-five good quality metaphases were karyotyped for each patient under 100X objective and additional metaphases, if necessary. Chromosome identification and nomenclature was done according to the International system for Human Cytogenetic Nomenclature (Mitelman, 1994).

Chromosomes were identified based on the length, arm ratio and banding. Initially all D group (chromosome 13 containing group) chromosomes were identified and then segregated into pairs based on the banding. Banding was given preference compared to size of chromosomes in segregating the chromosomes into pairs within each group. The chromosome 13 pair was examined in detail to assess the length of the bands and sub-bands and compared. If there is distinct size difference and band appearance in q14 region between the two homologous chromosomes, it was reported as 13q14 deletion. A diagram was drawn indicating each chromosome as in a line with the correct relative position, size and orientation in the metaphase. In equivocal cases photograph of the pair of 13 chromosomes were taken and compared.

Considering the wide variation observed in staining of chromosomal segments, a statistical approach was taken to estimate 13q14 deletion. In most of the patients (with 13q14 deletion), the deletion was apparent only in a percentage of metaphases (Table 14). The 13q14 deletion frequency (percentage of metaphases) in each case was entered into an excel worksheet for the 59 patients (Table 14). In the 19 cases without apparent 13q14 deletion, for statistical analysis the percentage of 13q14 deletion was taken as 0%. Outlier test was used to find whether the 13q14 deletion (percentages) data followed normal distribution. Since the data was not normally distributed, log transformation was done to convert the 13q14 deletion frequency to normal distribution. The log transformation values were entered and the 13q14 deletion cutoff value at 10, 20, 25, 50, 75 and 90 percentiles were calculated by SPSS statistical package 9.0. Nearly 10% of retinoblastoma patients have cytogenetically visible 13q14 deletions (Bunin *et al*, 1989) and hence the 90^{th} percentile of 13q14 deletion frequency (log transformation value) is taken as the cutoff for considering as true deletion (Table 14). A subject with log transformation value equal to or above cutoff was taken as 13q14 deletion. The result of karyotype analysis was reported as total chromosome number, followed by the chromosomal constitution.

Table 14 : Summary of karyotype analysis (n= 59)

S No.	Sample ID.	13q14 deletion (%)	Log transformation	Result
1	N:44	3.6	0.6628	-
2	L:66	6.5	0.8751	-
3	L:73	6.6	0.8808	-
4	V:81	11.5	1.0969	-
5	L:65	11.8	1.1072	-
6	R:9	13.8	1.1703	-
7	P:54	15.6	1.2201	-
8 –10	W:49 / Q:4 / R:38	16.0	1.2304	-
11	N:68	16.7	1.2472	-
12	G1:26	18.5	1.2900	-
13	S1:78	18.5	1.2900	-
14 – 16	T:48 / R:13 / Z:61	20.0	1.3222	-
17-22	R:79/ S:26 / N:68 P:64 / Q:13 / S:45	24.0	1.3979	-
23	M:38	25.8	1.4281	-
24	N:70	26.0	1.4314	-
25	Q:33	27.0	1.4472	-
26	I1:4	27.9	1.4609	-
27-29	D1:69 / C1:66 / Q:5	28.0	1.4624	-
30	T:25	32.0	1.5185	-
31	X:35	33.3	1.5353	-
32	P:63	34.6	1.5514	-
33	Z1:71	34.8	1.5539	-
34	R:24	36.0	1.5682	+
35	N52	36.0	1.5682	+
36	Q:35	40.0	1.6128	+
37	L:39	40.9	1.6222	+
38	L:69	41.7	1.6304	+
39	N:78	56.0	1.7559	+
40	W:48	80.0	1.9085	+
Percentile / Cutoff value	50 / 1.2304	75 / 1.4054	90 / 1.5682	

NOTE: + indicate 13q14 deletion and – indicate no deletion. 19 samples had 0% 13q14 deletion.

4.4 Results

Karyotype analysis by 72-hour peripheral blood culture and GTG banding was done for one regressed retinoblastoma (1.69%), 33 bilateral (55.9%) and 25 unilateral (42.3%) patients. The 13q14 deletion frequency (in percentages) and the log transformation values for the 59 samples and the cutoff values derived by statistical analysis are shown in Table 14. The cutoff values at 5, 10 and 25th percentiles were zero. Outlier test showed normal distribution of the log transformation values (range 0.0 - 1.9085) and mean (0.93 ± 0.6745). Seven samples had log transformation values equal to/above the cutoff (1.5682) (Table 14). In this way, two unilateral (R24 and W48) and five bilateral patients (L39, L69, N52, N78 and Q35) had 13q14 deletion constituting 11.8% of the retinoblastoma patients who underwent karyotype analysis. Karyotype of a bilateral retinoblastoma patient with 13q14 deletion is shown in Figure 20.

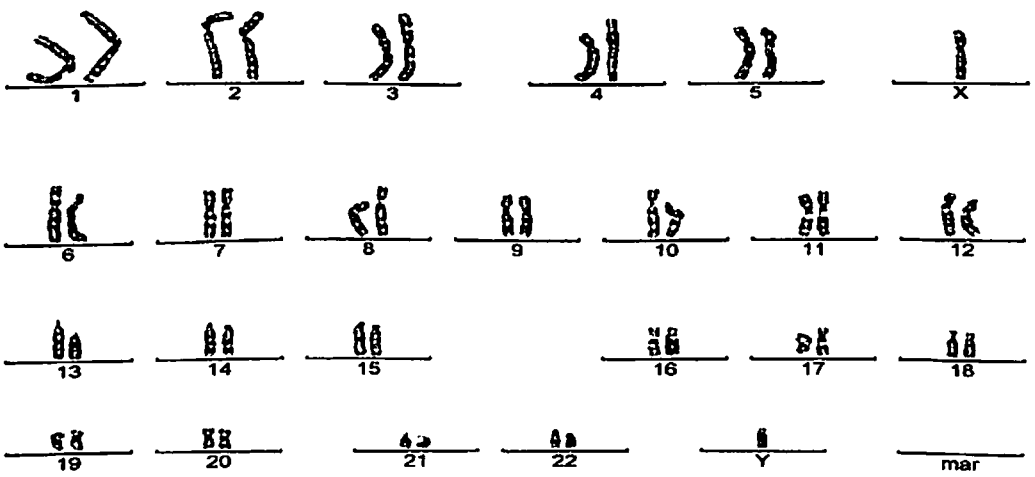

Figure 20: 13q14 deletion in a bilateral retinoblastoma patient

4.5 Discussion

In retinoblastoma genetic screening, karyotype analysis is the low-cost test providing results within few days of receipt of the blood sample. Karyotype analysis is the simple and affordable test to most of the retinoblastoma families compared to molecular genetic tests. Other advantages of karyotype analysis are, easy to master, stable characteristics of the chromosomes with clearly defined abnormal states, possibility of preservation of slides for future applications (mounted slides or in fixative), detection of multiple chromosomal abnormalities from a single specimen, interpretation of results without simultaneous parental testing (required in molecular cytogenetic deletion analysis) and quality of culture could be assessed during incubation and subsequent processing of the blood specimen by naked eye examination.

Yunis and Ramsay, (1978) concluded that sub-bands of 13q14 region should be carefully analyzed for minute deletion in retinoblastoma patients. Cells with well-banded elongated chromosomes in early metaphase are necessary for evaluation of 13q14 deletion. In a well-banded elongated metaphase, the light bands q12, q14 and dark bands q21 and q31 measure 80% of length of q arm of chromosome 13 and 13q14 deletion could be easily ascertained. Yunis and Ramsay, (1978) and Motegi, (1981) showed that, if the chromosomes are condensed, determining whether 13q14 deletion is present, is difficult. The extent of trypsinisation which in-turn affects the differential Giemsa staining of the chromosomal segments could also influence the decision whether 13q14 deletion is present. Statistical analysis was done assuming that these confounding factors might have influenced the karyotype results for the 59 blood samples.

In the present study, 13q14 deletion was apparent only in less than 50% of metaphases with frequency (0–80.0%) and mean of 17.47 for the 59 samples (Table 14). In one unilateral patient, 13q14 deletion was apparent in 80% of metaphases. Delucchi and Bostrom, (2004) reviewed the statistical methods to analyze heavily skewed psychiatric clinical study data containing zeros values and showed that proper statistical methods would result in more meaningful results and conclusions (Delucchi and Bostrom, 2004). In 19 samples in the current study, 13q14 deletion was not apparent in any of the metaphases and hence 13q14 deletion frequency was taken as 0%. The 13q14 deletion frequency (percentage of metaphases with apparent 13q14 deletion) was calculated in each case and the percentile distribution was derived using SPSS 9.0 after log transformation. Log transformation was done to convert the 13q14 deletion frequencies (did not follow normal distribution) to log transformation values (followed normal distribution). Outlier test showed normal distribution of the log transformation values. The 90^{th} percentile value i.e. 1.5682 was taken as the cutoff for 13q14 deletion. In this way 13q14 deletion was detected in two unilateral (R24 and W48) and five bilateral (L39, L69, N52, N78 and Q35), constituting 11.8% of patients. Ramprasad (2005), from the same department reported 13% of RB1 deletion by molecular deletion analysis. Deletion involving 13q region are reported in 13q deletion syndrome, breast cancer, osteosarcoma, hematological malignancies like multiple myeloma, polycythemia vera and chronic lymphocytic leukemia. The statistical approach taken in the current study might be useful in these diseases also.

4.6 Conclusions

Karyotyping is an affordable genetic test for most of the retinoblastoma families especially in a developing country like India. In the present study one regressed, 33 bilateral and 25 unilateral retinoblastoma patients were karyotyped by Giemsa

banding. Cytogenetically visible deletion of RB1 gene (13q14 deletion) by G banding was detected in seven patients (11.8%). Karyotype analysis should be interpreted by taking into consideration the technical limitations like extent of elongation of the chromosomes, variation in trypsinisation and staining intensity of the chromosomal segments and the number of metaphases with 13q14 deletion. A statistical approach could be used as an adjunct in deriving true 13q14 deletion results after karyotype analysis. The interdisciplinary approach adopted might be useful in deriving maximum benefit out of the existing techniques for reporting.

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Chapter 5

Cost analysis of screening

5.1 Aim

- To find the cost efficiency of genetic testing compared to clinical screening.

5.2 Review of Literature

Cancer genetic consultation and screening is demanded more frequently with establishment of guidelines for genetic testing and risk estimation in familial and common cancers (Hampel *et al*, 2004). Accurate, sensitive and financially viable predictive molecular genetic tests are essential to meet this increased demand. (Ganguly and Willaims 1997; Rebbeck *et al*, 1997). Disadvantages of widely used screening programmes for cancers include inter-observer variation (skin examination in cutaneous melanoma), lack of specificity or sensitivity, ability to detect the disease only at a later stage of disease (chest X ray in lung cancer), invasive nature of the procedures (sigmoidoscopy in colon cancer), arbitrary reference intervals (prostate specific antigen levels in prostate cancer) or exposure to radiation (mammography in breast cancer screening). Genetic testing might be useful as an alternative option in similar situations. In addition to reduction in mortality and morbidity the effects of the genetic tests on the quality of life and economic burden are also being increasingly ascertained. The cost effectiveness of cancer related genetic testing is of great importance for the clinicians, genetic researchers and an educated patient community apart from insurance companies (Bapat *et al*, 1999; Lawrence *et al*, 1980; Noorani *et al*, 1996; Sevilla *et al*, 2002). Collaborative research spread across different continents is gaining prominence in genetic research, enabling pooling and analyzing research outcome from different centers. In order to maximize benefits out of this, efficient and cost effective protocols that could be universally applied are needed in place before starting the research.

An earlier diagnosis could direct the treatment course and management of the retinoblastoma patient with savings of vision in the affected eye. Treatment modalities like chemo-reduction coupled with other therapies and an efficient genetic testing might contribute to better prognosis for retinoblastoma patients and families. Implementing an eye-screening programme for retinoblastoma for all children shortly after birth would be difficult and probably economically not viable due to low age restricted incidence (Shields, 1999). Currently screening for retinoblastoma is most often done on clinical examination at frequent intervals, if necessary under anesthesia, for proband and closely related young infants (Musarella and Gallie, 1987). Apart from anesthesia related risks, psychological disadvantage for the patient and parents and financial burden are additional drawbacks. Avoiding unwanted ophthalmic surveillance will save considerable amount of money, time and avoid psychological stress for these families (Gallie *et al*, 1995; Noorani *et al*, 1996; Richter *et al*, 2003).

Molecular diagnosis for retinoblastoma has been shown to have advantages over conventional clinical examination (Gallie, 1997). The knowledge of the type of RB1 mutations (egs:- low penetrance mutations, somatic mutations, splice mutations) in the family may facilitate better prognosis for an infant with mutations (Gallie, 1997; Gallie *et al*, 1999). Direct mutation identification in heritable retinoblastoma patients is important in knowing the chance of development of additional primary malignancies, osteogenic sarcoma in childhood and breast or bladder cancer later in life (Lohmann *et al*, 1997). Possibility of all types of mutations without hotspots necessitates combination of different molecular genetic techniques in developing an efficient genetic screening strategy for retinoblastoma. Multiplex PCR, heteroduplex analysis and sequencing has been used by Lohmann *et al*, (1997) as a screening method for retinoblastoma. Richter *et al*, (2003) showed significant economical benefits for the retinoblastoma families after undergoing genetic testing for RB1

mutations. Kumaramanickavel *et al*, (2003) has shown the benefits of molecular genetic analysis in retinoblastoma patient management in a study from India. In the present study cost comparison of RB1 molecular genetic screening versus conventional clinical screening for retinoblastoma patients in India was done.

5.3 Methodology

The study was done for the retinoblastoma patients who underwent clinical and genetic testing and counseling at Sankara Nethralaya, a tertiary referral center for ophthalmic diseases, in Chennai, India and consisted of a heterogeneous group scattered throughout India and also from neighbouring countries Sri Lanka and Bangladesh. On an average 60 new retinoblastoma patients are seen in a year at the Department of Vitreo-Retina. Karyotype analysis and genetic counseling for the retinoblastoma families have been ongoing in the SN ONGC Genetics and Molecular Biology department from 1993 (Harini *et al*, 2001).

Patients with retinoblastoma were enrolled in this study after being referred by the ophthalmologist or following enucleation or during genetic/social counseling. A detailed pedigree analysis was done, including family history of cancer, based on the information given by parents of the patients. The patient's parents were given non-directive counseling about RB1 genetic testing and its relevance. A detailed counseling was done after the genetic testing report became available. During enucleating surgery, blood was collected from the patients. DNA was isolated from fresh retinoblastoma tumor tissue harvested from enucleated eyeballs. Patient's blood and tumor DNA were studied with prior ethical approval by the hospital institutional review board/ethics committee.

The following criteria were used for the cost evaluation of conventional clinical examination and RB1 gene screening for the retinoblastoma patients. The proband was the first individual in the family clinically affected and all other family members remain disease free. The child is diagnosed as having retinoblastoma at the age of 24 months of age. These factors were important while calculating the number of clinical examinations proband or close relatives have to undergo by conventional screening strategy and also in the cost of genetic screening.

The conventional screening strategy followed at Department of Vitreo-retina, Medical Research Foundation consists of initial complete retinal examination under anesthesia at the age of presentation and every three months in the first year, every six months till two years of age; and yearly then, if the disease is under control. After 10 years of age the examination was stopped. The inputs valued for conventional screening were personnel time, equipment, overhead and other recurrent inputs. The cost of indirect ophthalmoscopy used normally for 5-10 minutes per procedure was calculated on an annualized basis, using 5-year working life at 5% discount per year. Examination under anesthesia occupied 1-2 hours per patient when treatment is necessary and 15-30 minutes if no treatment is necessary. Physician and laboratory examination fees were derived from the cost prevailing in the institution during 2002. The cost of examination, without anesthesia, performed at the ophthalmology clinic was calculated based on the prevailing salary of the clinician, optometrists and secretary.

The cost of identification of RB1 gene mutation in blood/tumor of bilateral and familial retinoblastoma cases was evaluated by the molecular genetic technology used in the SN ONGC Genetics and Molecular Biology laboratory in 2002. The direct costs for genetic testing included laboratory supplies, equipment, technologist's labour time, data interpretation and reporting by trained clinical or scientific professional. Supplies were valued based on replacement prices including an estimate of wastage.

The number of samples received for analysis and the current replacement costs were used as reference for cost calculation of the equipments with 5% annual discount rate and an assumed working life of five years. Twenty percent of the total testing costs were allocated for overheads, laboratory quality assurance services, general utilities and other operating inputs not identified above.

In the genetic strategy evaluated for costs, DNA from tumor/blood of bilateral/ familial cases of retinoblastoma were screened for mutations in the exons 1-27 of RB1 gene by sequencing. The specific primer sequences flanking the exonic regions were obtained from Genome Data Bank. PCR amplification was done as in chapter 1. Mutational screening was done as described in chapter 2. Any mutations detected were confirmed by sequence analysis from independent PCR and also sequencing in the reverse direction. Peripheral blood DNA of the proband was screened for the mutation detected in tumor DNA. DNA of Parents and close relatives were screened for mutations detected in peripheral blood. Karyotype analysis was done for proband and also for parents in familial cases by 72-hour peripheral leucocyte culture and Giemsa banding (Harini *et al*, 2001). Tumor DNA isolated by proteinase K digestion and phenol-chloroform extraction method was used for methylation analysis of RB1 promoter region by methylation specific PCR, poly acrylamide gel electrophoresis and silver staining (Joseph *et al*, 2004).

In the present study caution was taken in revealing the test results to the patient and families. The results were initially revealed to the consultant who referred/treated the patient. The results were explained to the parents of the patients in a genetic counseling session. In cases where the results were mailed the results were explained to the parents during their subsequent visit to the hospital.

5.4 Results

The direct costs for genetic testing and conventional clinical examination for a proband and a nuclear family with two sibs are shown in tables 15 and 16 respectively. The cost of genetic testing and clinical examination for the proband was US$ 152.27 and 535.60 respectively while for the nuclear family it was US$ 174.51 and 1071.20. The cost of sequencing of the complete coding sequence of RB1 gene, US$ 119.79, constituted 85.8% of the total cost of genetic screening for the proband. The cost of testing the sibling for the mutation detected is $1/25^{th}$ of the cost of complete RB1 gene sequencing. Enucleating surgery occupied 43.1% of the cost of the clinical strategy for the proband. In the conventional screening strategy the proband should undergo 15 each of EUA (US$ 157.20), physician checkup and blood examinations (US$ 100.65) and ophthalmologist consultations (US$ 47.10) over a period of 10 years. In a nuclear family with two sibs, the sib of a proband will have to undergo a similar clinical screening strategy if the sib is below 5 years of age.

The genetic analysis had a financial savings of 3.5 fold for the proband and 6.1 fold compared to conventional examination for a nuclear family with two sibs (Tables 15 and 16). The costs were inclusive of import duty, which was around 60% of the cost of consumables. The savings were higher if the indirect benefits due to savings in manpower and factors like transportation to/from the hospital were taken into account apart from psychological benefits due to negative test results.

Table 15: Cost of genetic screening for RB1 gene (US $)

Test	Cost/Test	Average Number of test	Cost/ Sample
Proband:			
DNA extraction	1.67	1	1.67
Cytogenetics Study	15.72	1	15.72
Methylation (PCR and PAGE)	4.19	1	4.19
Sequencing PCR 1	10.9	1	10.9
Sequencing PCR 2	119.79	1	119.79
Total			152.27
Relative:			
DNA extraction	1.67	1	1.67
Cytogenetics study	15.72	1	15.72
Sequencing PCR 1	0.41	1	0.41
Sequencing PCR 2	4.44	1	4.44
Total			22.44
Total for a nuclear family			174.51

US$ 1.00 = INR 45.575 as on 25 November 2005

Table 16: Cost of conventional screening for retinoblastoma (US $)

Input	Cost / Examination	Recommend Number of examinations	Cost / Patient
Birth to age of 5 years			
Enucleation	230.65	1	230.65
Examination under GA	10.48	9	94.32
Physician fees & Blood Test	6.71	9	60.39
Consultation	3.14	9	28.26
Age 5 yrs - 10 yrs			
Examination under GA	10.48	6	62.88
Physician fees & Blood Test	6.71	6	40.26
Consultation	3.14	6	18.84
Total cost			535.60
Cost for a nuclear family with two sibs			1071.20

US$ 1.00 = INR 45.575 as on 25 November 2005

5.5 Discussion

The possibility of predictive medicine for genetic diseases has resulted in cost benefit analysis of genetic versus conventional screening for a number of diseases. Genetic testing can be advantageous in cases where a negative result will give psychological advantages to the patients or relatives. But it has the disadvantages of insurance or employment discrimination (Lawrence *et al*, 1980). DNA diagnosis in retinoblastoma, either by direct or indirect analysis of the RB1 gene defects, will be helpful for retinoblastoma families in several ways. Some families with several severely affected patients may wish to consider prenatal diagnosis or decide not to have another child. Clinically unaffected sibs and offspring planning to start a family of their own may want to know that they really do not carry the mutation at the RB1 locus.

In the current study, the costs of genetic testing included cytogenetic screening by standard trypsin digestion Giemsa technique, DNA extraction by phenol-chloroform method, sequencing by BigDye terminator chemistry in ABI 310 automated sequencer, methylation analysis by bisulfite modification and methylation specific PCR, poly acrylamide gel electrophoresis and silver staining. Nearly six fold financial savings was obtained for a nuclear family with two sibs by genetic screening. The cost of molecular genetic analysis of RB1 gene in the proband alone was US$ 152 and for a nuclear family with two sibs was US$ 175 (Table 15). The cost of the clinical surveillance for a nuclear family with two sibs, including the proband, would be US$ 1071 over a period of 10 years (Table 16).

The savings will be even higher if the indirect costs due to low morbidity, mortality and savings in manpower, psychological benefits due to results with reduced risk for development of tumor, transportation to/from the hospital etc were taken into account. In the experience of the author, repeated examination under anesthesia and invasive

procedure of repeated blood collection before EUA examination has tremendous psychological impact on the child. Genetic testing has additional advantages of requiring to test only once in life. The additional benefits due to the shorter stay and avoiding examination under anesthesia for the close young relatives add to the direct benefits of the test. Costs for genetic counseling were not included in the calculation, as it is not charged in our hospital. Feedback from the parents of proband, obtained after genetic counseling, through a feedback form has shown the usefulness of RB1 screening and genetic counseling for the retinoblastoma families who underwent clinical management and genetic testing at Sankara Nethralaya. In India, the imported reagents and chemicals for these techniques have 60% customs duty that boosted the cost of molecular genetic screening considerably. A reduction in the customs duty will make molecular screening much cheaper than conventional examination.

In the 20 retinoblastoma families studied by Richter *et al*, (2003) 88 children avoided 313 EUA and 107 children avoided 852 clinical examinations with direct health savings equivalent to Can $6,591(US$ 4,200) per family, before counting the benefit for the children not yet born. Raizis *et al*, (2002) showed that genetic testing of the RB1 gene resulted in clinically significant benefits and cost savings to the affected individuals and their families. Noorani *et al*, (1996) showed 20-fold savings by molecular screening methodology compared to conventional screening for the retinoblastoma proband (Noorani *et al*, 1996).

The obvious advantage for the genetic screening over the conventional method for management for retinoblastoma is thought to make the former the preferred method. Genetic screening will help in directing health resources to those with high risk while eliminating the unwanted examination for close relatives without mutations (Richter *et al*, 2003).

5.6 Conclusions

Presymptomatic DNA testing offers the opportunity for disease prevention by identifying individuals with elevated risk who can benefit from improved surveillance regimens. A genetic testing service supported by counseling established at our laboratory is useful for retinoblastoma patients in this part of the world. The genetic testing of retinoblastoma is cheaper in India too like western countries and would be widely implemented in clinical practice. Further refinements in the technology will lead to still higher cost savings and clinical benefits.

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Chapter 6

Diagnostic model

6.1 Aim

❖ To establish a diagnostic model for retinoblastoma patients.

6.2 Review of Literature

Retinoblastoma is responsible for approximately 1% of all deaths caused by childhood cancer and about 5% of blindness in children (van der Wal *et al*, 2003). An ideal diagnostic model is a requisite for accurate risk prediction and effective management in retinoblastoma. Developing a diagnostic model for retinoblastoma in India, which has the highest incidence of retinoblastoma (1532 new cases/year), is worth considering and establishing (Gallie, 2004). Development of an ideal diagnostic model, in general, has been hampered due to lack of a single technique that can detect all types of mutations and non-availability of low cost laboratory techniques.

Before automated technology era, sequencing of RB1 gene was cumbersome often ending with ambiguous results. Prior to the introduction of MS-PCR, methylation analysis of RB1 gene was difficult and tedious due to the requirement of large amount of tumor DNA for Southern blotting. Even though these issues were addressed, cost effectiveness of the tests prevented implementation of an efficient diagnostic model.

An efficient diagnostic model for retinoblastoma could save considerable money, reduce the overall health care costs and also avoid unnecessary worry for the families. Richter *et al*, (2003) showed significant cost benefits for retinoblastoma patients after undergoing genetic testing. Molecular diagnostic methods for retinoblastoma could benefit over conventional clinical screening including examination under anesthesia (Joseph *et al*, 2004b). An ideal diagnostic model could eliminate the necessity for

clinical examination for not at-risk close relatives of retinoblastoma patients. Retinoblastoma molecular diagnostic methods are now used routinely in the University of Toronto, Canada and University of Essen, Germany (Lohmann *et al*, 2003). Therapeutic introduction of functionally active RB1 gene that could suppress even advanced stages of neuroendocrine tumors in RB +/- mice (Nikitin *et al*, 1999) increases the necessity of a diagnostic model and unit for retinoblastoma.

In retinoblastoma, disadvantage of clinical management of patients and relatives is the necessity of repeated eye examinations often under anesthesia. During this process several relatives who might not be at risk also have to undergo clinical examination leading to huge financial expenditure and threat for life due to anesthetic protocol. Delayed diagnosis in bilateral retinoblastoma would result in loss of the eye (Butros *et al*, 2002). Accurate and sensitive diagnostic model might avoid unnecessary anesthetic examination for at-risk relatives and also visual morbidity and mortality. Lohmann *et al*, (1996) suggested that a protocol for routine RB1 gene mutational analysis is necessary to convert scientific research into practice for benefits of at-risk individuals for retinoblastoma.

On an average 60 new retinoblastoma patients are seen in a year at Sankara Nethralaya, who come from all over India and neighbouring countries. Nearly half of these were one-child families (Harini *et al*, 2001). In Madras, retinoblastoma has been shown to be the most common eye cancer (Sunderraj, 1991). A comprehensive diagnostic model for retinoblastoma does not exist in any institution in India. A molecular diagnostic model for retinoblastoma with reliable results will be useful for the families in taking a decision to have another child. In addition it will help the ophthalmologist to decide whether a normal child can be spared unnecessary examination under anesthesia. The test results will be useful in evaluating risk of non-ocular tumors in the surviving patient. Genetic counseling and karyotype analysis has

already been established as clinical services at the SN ONGC department of Genetics and Molecular Biology. Hence it is justified to have a comprehensive diagnostic model and a unit for retinoblastoma patients. Accurate genetic testing results could be used in further management of the retinoblastoma patient and families (Kumaramanickavel *et al*, 2003) and hence is a priority in the diagnostic model.

The diagnostic model in the present study emphasize on comprehensive genetic testing - karyotype analysis, mutational screening by automated sequencing, methylation analysis of RB1 promoter region, linkage studies using intragenic markers for RB1 gene (familial cases), LOH and molecular deletion analysis, pedigree charting, counseling and clinical management. Focused CGA codon screening will help in rapid RB1 gene mutation detection since CGA arginine codons mutation accounts for 76% of single base substitutions in retinoblastoma tumors (Lohmann, 1999). LOH and Linkage analyses using RB1 intragenic and surrounding polymorphic microsatellite markers is useful in detecting allele loss and in tracking the co-segregating allele in familial cases.

Harbour (1998) proposed that a screening approach involving a series of complementary tests might allow rapid screening of RB1 germline mutations. Protein truncation test is suggested to detect nonsense and frame shift mutations together accounting for 78% of all mutations (Harbour, 1998). But protein truncation test has the disadvantages of not detecting all the types of mutations and will not give information about the exact site of mutation (Tsai *et al*, 2004). Direct sequencing was suggested as a tool to refine results from prescreening in retinoblastoma patients or to search specifically for known missense mutation sites (Harbour, 1998). Cytogenetic analysis and targeted searches for promoter or intron alterations could be reserved if mutations were not identified by other techniques (Harbour, 1998). Sen *et al*, (1999) used guided intraocular FNAB for the preoperative diagnosis of retinoblastoma.

Sutterlin *et al*, (1999) proposed the first protocol for single cell detection of retinoblastoma predisposition, applicable to preimplantation genetic diagnosis. The strategy is based on nested triplex PCR, SSCP and fragment analysis by automated fluorescence electrophoresis. DNA was isolated from single blastomere prepared by biopsy or disaggregation of spare cleavage stage embryos. In the outer triplex PCR, RB1 intragenic polymorphic locus primers and fluorescent labeled D21S1411 primers (for detection of contamination) were used. The inner nested PCRs with 35 cycles were performed on 2 μL aliquots from the outer PCR product using two inner primer pairs amplifying the mutant and the PR0.6 locus (intron 17) in two separate reactions (Sutterlin *et al*, 1999).

European Molecular Genetics Quality Network has evolved 'the best practice guidelines for molecular analysis of retinoblastoma' (Lohmann *et al*, 2002). In the direct testing strategy mutation analysis is done from peripheral blood of bilateral and from tumor of unilateral cases. In familial retinoblastoma, genotyping using co-segregating markers and mutational analysis from peripheral blood DNA are done. In isolated bilateral cases, mutation screening is done from genomic DNA and, if no mutations detected in blood, from tumor DNA. In isolated unilateral cases tumor DNA is checked for mutations. In indirect testing, polymorphic short tandem repeats loci within the RB1 gene is used for comparative genotyping of patient and parents (Lohmann *et al*, 2002).

Richter *et al*, (2003) designed a RB1 gene mutation detection strategy using a combination of quantitative multiplex PCR, double exon sequencing and promoter targeted methylation screening. Unilateral, non-familial retinoblastoma and bilateral or familial retinoblastoma probands were studied for changes in exon size and copy number by fluorescent quantitative multiplex PCR (Richter *et al*, 2003). Sequencing was done by duplex PCR using Cy5/Cy5.5 dye primer cycle sequencing kit. Allele

specific PCR was performed for four recurrent mutations. Assay ordering of QM - PCR multiplexes and allele specific PCR reduced the turnaround time for RB1 screening to 2.7 weeks (Richter *et al*, 2003).

6.3 Methodology and Discussion

The present study explains a model, which will be the ideal way for collecting and analyzing the genetic material from the retinoblastoma patients and families. The clinical strategy used for the families is also described.

6.3.1 Diagnostic strategy

Once a child is diagnosed as retinoblastoma by the ophthalmologist, the patient is referred to SN ONGC Genetics and Molecular Biology department for pre-test counseling. During this non-directive counseling session, the parents were explained about the disease, its probable progression, current treatment and basic genetic information why the disease has affected the child. In this session, the importance of genetic testing and its relevance is also specified. Extreme care is taken not to reveal the inheritance is from which parent, if one of them is affected, more particularly if it is the mother. In India where culturally the father is employed but not the mother, a divorce could be an additional disaster to both the mother and the child, which would make clinical and social managements very complicated.

a) Clinical Strategy

Complete eye examination was done including visual acuity, external and fundus examination. In uncooperative younger children clinical examination was done under general anesthesia. All first-degree children were clinically evaluated at regular intervals, if genetic testing showed risk. The at-risk children, who did not inherit the mutation in the family, are also clinically evaluated shortly after birth and as needed

for routine eye care. Careful clinical examination of an extended family is recommended if multiple members are affected. Parents of patients were clinically examined to rule out regressed retinoblastoma. The conventional screening consists of initial complete retinal examination under anesthesia at the age of presentation and every three months in the first year, every six months till two years of age; and yearly then if the disease is under control. After 10 years of age the examination was stopped. A and B scan ultrasound and CT scan of orbit and brain are done to confirm the diagnosis and also to rule out extra ocular or intracranial extension of the disease for proband. In bilateral retinoblastoma cases, eyes with large tumor mass and no visual potential were enucleated. Unilateral retinoblastoma patients underwent enucleation with follow-up screening for the other eye as described. Histopathological examination was done on the enucleated eyes, specifically looking for the type of differentiation of the tumor, the optic nerve or choroidal invasion, presence of tumor cells at the surgical cut end of the optic nerve stump and extra ocular extension. Tumors were graded according to Reese-Ellsworth classification (Ellsworth, 1977).

b) Laboratory Strategy

Peripheral blood is collected from the proband and parents of the patients and, if present, from other affected members. In surgical management the patient undergoes enucleation, subsequently tumor tissue is harvested and DNA isolated as described in appendix II. Paraffin embedded mounted sections of enucleated eyeballs of patients were obtained from pathology laboratory if fresh tumor is not available. The consumables, equipments and personnel for retinoblastoma diagnostic services are given in Tables 17 and 18 respectively.

Table 17: Important consumables in retinoblastoma screening

No.	Consumables	Company
	Karyotype analysis	
1	Methanol	Merck, India
2	Phyto haemagglutinin	Invitrogen, USA
3	RPMI medium	Himedia, India
4	Fetal bovine serum	Invitrogen, USA
5	Methotrexate	Sigma, USA
6	Thymidine	Sigma, USA
7	Colchicine	Sigma, USA
8	Giemsa stain	Sigma, USA
	PCR reagents	
9	Primers for exons 1 - 27 of RB1 gene	Bangalore Genei, India
10	*Taq* DNA Polymerase	Bangalore Genei, India
11	dNTPs	Bangalore Genei, India
12	Big Dye Terminator^{R} RR mix	PE Applied Biosystems, USA
13	POP4 polymer	PE Applied Biosystems, USA
14	Template suppression reagent	PE Applied Biosystems, USA
	Methylation analysis	
15	Methylation specific primers	Bangalore Genei, India
16	SssI (CpG methylase)	NEB Ltd, USA
	Linkage, molecular deletion and haplotyping	
17	Fluorescent microsatellite markers (RB1.20, chromosome 13 panels 17,18,19 and 67)	PE Applied Biosystems, USA
18	Genescan GS500 ROX standard; Genescan GS500 TAMRA standard	PE Applied Biosystems, USA
19	Capillary for electrophoresis, Genetic analyzer buffer	PE Applied Biosystems, USA
20	Agarose gel elution kit (PCR products)	Qiagen, USA

Table 18: List of equipments for retinoblastoma screening

No.	Equipment	Company	Used for	Number
1	Photo microscope	Nikon, USA Zeiss, Germany	Karyotyping	2
2	CO_2 incubator	Forma Scientific ASR Instruments	Incubation of culture for chromosomal studies	1
3	Varying temperature water bath	Ophthalmic instruments Co, India	Incubation of bisulfite treated DNA	1
4	PCR Thermal Cycler	PE 2700	PCR	2
5	Freezer (-80°C)	Nuaire	Storing stock DNAs	1
6	Freezer (-20°C) Refrigerator	Vest frost	Storing reagents, primers and working DNA	1
7	Micro pipettes (0.5 µL, 0.5 - 10 µL, 20 µL, 100 µL)	Pipet man, France	Setting PCRs	1 each
8	Vertical slab gel electrophoresis apparatus	Broviga instruments, India	Acrylamide electrophoresis	1
9	ABI 310 Genetic analyzer	PE Applied Biosystems, USA	Sequencing, LOH, molecular deletion and linkage analyses	1

As a first step of investigations, peripheral blood collected from proband was used for karyotyping (Harini *et al*, 2001). Cytogenetic deletions that constitute 6-8% can be identified by this method (Harbour, 1998). Automated karyoanalysis using *ikaros karyotyping system* (Zeiss-metasystems, Germany) helps to reduce cost and time for karyotyping. Translocation, though rare, could be identified and presumed to be

transmitted by one of the parents. Mosaicism, if identified, could establish the post-zygotic error and reduce the risk for the next child to nearly nil. The 13q14 deletion karyotype is confirmed by testing fluorescent microsatellite markers across the 13q14 region (D13S263, D13S153, RB1.20, D13S1320, D13S1296 and D13S156). Next loss of heterozygosity test was done to identify whether only one allele is present in the tumor (Ramprasad, 2005). Loss of heterozygosity refers to the loss of one allele at RB1 locus in tumor sample compared to normal tissue of the patient. This is the most common mechanism for the second hit in retinoblastoma (Cavenee *et al*, 1983). At this stage if there is clear autosomal dominant inheritance in the family, haplotype or linkage analysis is undertaken. This method would help to identify the risk allele that is inherited and was done taking into consideration of recombination events. Subsequently mutational screening with tumor DNA was done for the CGA codons (exons: 5, 8, 10-15, 17, 18, 23, 27). Nearly 43% of DNA mutations of retinoblastoma is caused by CGA to TGA change resulting in arginine to termination codon, hence by screening these codons the possibility of identifying the mutations in the given specimen is high (Lohmann, 1999). If no mutation is identified in the CGA codons then rest of the 15 exons are sequenced for mutation detection. Nearly 80% of a hit is due to point mutations (missense or nonsense) (Harbour, 1998). If these tests are proven to be futile then the tumor is subjected for methylation test by PCR of the promoter region (Joseph *et al*, 2004a). Methylation of retinoblastoma tumor is about 10% (Zeschnigk *et al*, 1999). If all these are negative for a hit then essential promoter of the RB1 gene is screened for nucleotide change but is extremely rare (0.48%) (Fujita *et al*, 1999). Invariably at this stage both the hits would have been identified 90% of the times. The remaining 10% chance that only one or no hits are identified; could be due to mosaicism.

In post-test counseling, caution was taken in revealing the genetic test results to the patient and families. The results were initially revealed to the consultant who referred the patient. The results, if mailed, were explained to the parents during their subsequent visit to the hospital. If any close blood relatives should undergo eye

examination, this information is conveyed giving a reassurance more than an unwarranted alarm. The results are explained and doubts are clarified depending on what socio-economic status of the families. If the parents want to test the next child for RB1 defects, the option of prenatal testing are accordingly advised.

The hierarchical structure of the diagnostic model was established based on the experience from the molecular investigations carried out in the laboratory and the inputs from literature (Figure 21). The gold standard test of DNA sequencing was used for mutational screening rather than SSCP as this is a critical test that cannot be compromised for the inherent disadvantages of the latter. Assuming the genetic testing flow chart was not exited till the last step, about 84 days are needed to complete the required tests.

DNA diagnosis in retinoblastoma is helpful for retinoblastoma families in several ways. Retinoblastoma families with several severely affected patients might consider prenatal diagnosis. Newborn infants and young children carrying RB1 germline mutation could be examined immediately after birth and then every two to four weeks. Children found not to be at-risk could be spared unnecessary ophthalmic examinations, which in very young children requires general anesthesia. Clinically unaffected sibs and offspring planning to start a family of their own might want to know that they really do not carry the RB1 mutation.

In the current study, cost and benefit analysis of genetic testing was done under research setting and currently the diagnostic model is being incorporated into regular clinical service. The usefulness of RB1 genetic testing has been proved in follow-up studies (Cohen *et al*, 2001). The feedback from the parents of probands through questionnaire has shown the advantages due to genetic testing and counseling. Educating the parents regarding genetic testing motivated them to undertake the genetic test for the proband.

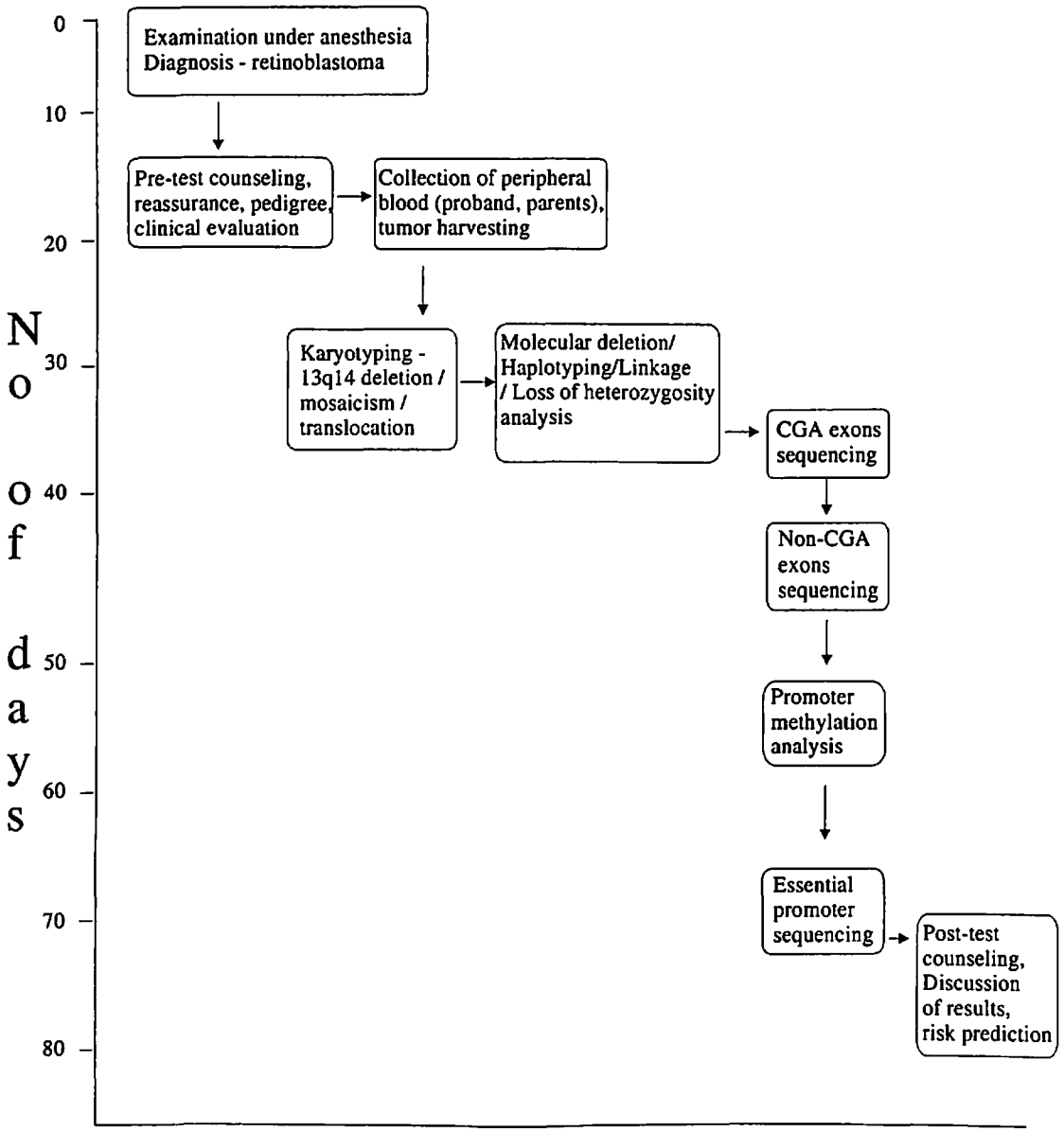

Figure 21: Diagnostic model for retinoblastoma, from day 1 it takes totally 84 days for all investigations to be completed.

6.4 Conclusions

The diagnostic model for retinoblastoma includes karyotype analysis, LOH, molecular deletion, linkage analyses (familial cases), CGA exons then non-CGA exons mutation screening, methylation analysis of RB1 promoter region and essential promoter screening. Feedback from the parents of retinoblastoma probands, after genetic counseling has shown the usefulness of RB1 screening and genetic counseling. Proper education regarding usefulness of RB1 genetic testing motivated parents to undertake the genetic test for the proband. The diagnostic model proposed in the current study offers comparatively cheaper and efficient genetic diagnosis for retinoblastoma patients from India and neighboring countries. The cost of genetic testing for the retinoblastoma families is 22 fold cheaper than the genetic analysis cost proposed by Richter *et al*, (2003).

6.5 References

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Chapter 7

Genotype-phenotype correlation analysis

7.1 Aim

- Genotype-phenotype correlation analysis in retinoblastoma patients.

7.2 Review of Literature

Genotype is defined as the genetic identity of an individual that does not show as outward characteristics. Phenotype is broadly defined as the observable trait or characteristic of an organism. But at the molecular level, phenotype starts with the translation of mRNA into a protein. Genotype-phenotype correlation is the association of a genetic variation and the resulting phenotypic abnormality. Determination of consistency of a phenotype in the presence of a particular genotype is of great importance in predicting the risk of the disease or its severity. This approach would be useful in predicting the natural history of hereditary diseases, which result in a spectrum of clinical condition ranging from subtle or severe morbidity to mortality.

Genotype-phenotype associations have been attempted with success in many diseases. In retinoblastoma patients, facial abnormalities or severe malformations have been reported due to loss of genes present on chromosome 13 (Seidman *et al*, 1987). Fukushima *et al*, (1987) proposed that chromosomal analysis should be performed to identify 13q14 deletion in retinoblastoma patients with high forehead and prominent ear lobes. Correlation between disease severity and location of truncation mutations within the epidermal keratins has been established in skin disease epidermolysis bullosa simplex (Letai *et al*, 1993). Dryja *et al*, (1997) adopted gene based approach to detect causative mutations in retinitis pigmentosa.

Retinoblastoma is a malignant tumor of the eye arising from the retinal cells that are not fully differentiated. The disease usually occurs in children below the age of five years (Brantley, Jr and Harbour, 2000). Mutations in both alleles of RB1 gene localized to chromosome 13 are required for development of the disease (Cavenee *et al*, 1985). While a *de novo* or inherited germline mutation gives the susceptibility to bilateral retinoblastoma, somatic mutations in both the alleles account for 85% of unilateral retinoblastoma without family history (Richter *et al*, 2003). It has been proposed that genotype-phenotype association might reveal the genetic factors modifying the phenotypic expression of retinoblastoma protein (Lohmann and Gallie, 2004). Development of lipoma in only one of the two retinoblastoma families with identical splice mutation resulting in skipping of exon 13 has been suggested to be due to a heritable modifier effect (Lohmann and Gallie, 2004).

Genotype-phenotype correlation studies in retinoblastoma have shown varying results in different populations. While Choy *et al*, (2002) and Ata-ur-Rasheed *et al*, (2003) did not observe any association between frameshift or nonsense mutations and phenotype in Chinese and Indian retinoblastoma patients respectively retrospective analysis of 88 germline RB1 mutation carriers from Canada and United States (Qi *et al*, 2005) broadly revealed significant association of high severe disease with truncating mutations (41/66). Seven of nine large deletions were also associated with high severity disease. However, analysis of the predicated protein length and domains disturbed with specific mutations against the severity did not show any association (Qi *et al*, 2005).

The present study aims to draw correlation between RB1 gene mutations and clinical severity of phenotype in 11 retinoblastoma patients.

7.3 Methodology

Eleven clinically and histopathologically confirmed retinoblastoma patients who reported at our tertiary eye care hospital were recruited for the study. All the cases were graded clinically as per Reese-Ellsworth classification (Table 19). In bilateral retinoblastoma, an attempt to salvage one eye was done by chemotherapy, sequential application of local therapy or radiotherapy depending upon the clinical situation. A part of the histopathological specimen without preservative and 5 ml of heparinised blood were collected for RB1 mutation screening.

Genotype

DNA was extracted by method described in appendices 1 and 2. Mutation screening was done according to Kumaramanickavel *et al*, (2003). The mutations were classified based on the type and aminoacid location in pRB. protein (Table 20).

Based on mutation type:

(i) Large deletions included mutation which resulted in loss of multiple exons.

(ii) Truncating mutations were nonsense, frameshift or splice site mutations that resulted in premature creation of stop codon and thereby a truncated protein.

(iii) Non truncating mutations, which resulted in change of aminoacids.

Phenotype

Phenotypic classification of the retinoblastoma patients was done according to criteria adopted by Qi *et al*, (2005) with modifications (Table 21).

Table 19: Reese-Ellsworth classification for retinoblastoma

(Shields and Shields, 2004)

Group	Clinical presentation
I	a. Solitary tumor, less than 4 disc diameters in size, at or behind the equator.
	b. Multiple tumors, none over 4 disc diameters in size, all at or behind the equator.
II	a. Solitary tumor, 4 to 10 disc diameters in size, at or behind the equator.
	b. Multiple tumors, 4 to 10 disc diameters in size, all at or behind the equator.
III	a. Any lesion anterior to the equator.
	b. Solitary tumor larger than 10 disc diameters in size behind the equator.
IV	a. Multiple tumors, some larger than 10 disc diameters.
	b. Any lesion extending anterior to the ora serrata.
V	a. Massive tumors involving over half the retina.
	b. Vitreous seeding.

Table 20 : Aminoacids location in pRB

No.	Domain	Aminoacids
1	N terminus	1-392
2	A/B domain	393-772
3	C pocket	773-869
4	C terminus	870-928

Predicted residual protein length and domains disrupted by the mutation were calculated for patients with truncating mutations.

Table 21: Comparison between phenotype classification criteria of Qi *et al*, (2005) and present study

Qi *et al*, (2005)	Current study
High severity (obeying any one or more criteria)	
1. Bilateral patients with age less than 1 year or unilateral disease with less than 2 years and high stage (early presentation with high stage)	1. Bilateral patients with age less than 1 year or unilateral disease with less than 2 years stage IV-V of Reese-Ellsworth classification,
2. High risk features on histopathological exam	2. High risk features on histopathological exam- Infiltration of optic nerve/choroids
3. Clinically advanced disease (neovascular glaucoma, anterior segment involvement, buphthalmos) with average age of presentation	3. and 4. Same as Qi *et al*, (2005).
4. Bilateral enucleation.	
Low severity (obeying any one or more criteria)	
1. Unilateral disease and positive family history	1. Same as Qi *et al*, (2005),
2. Bilateral patients with age greater than 1 year and low stage (late presentation with low stage).	2. Bilateral patients with age greater than 1 year and stage I- II of Reese-Ellsworth classification.
Moderate severity (obeying any one or more criteria)	
1. Absence of low or high severity criteria at diagnosis, and on review of disease course	1. Absence of low or high severity criteria at diagnosis, and on review of disease course
	2. Stage III of Reese-Ellsworth classification

Statistical analyses

1. The severity groups were analyzed against the type of mutations using 'Z' test for proportions with Statistical Package for Scientific Studies version 13.

2. Exact test based on Montecarlo assumption was used to test for differences in mean residual protein length among patients with high, moderate and low severity of disease.

3. Truncating mutations were analyzed to find association of the retinoblastoma protein domains disrupted by mutation and disease severity using exact test based on Montecarlo assumption.

7.4 Results

All the 11 patients had clinically severe form of retinoblastoma as evidenced by enucleation of one eye. Seven patients belonged to the high severity group and four were found in the moderate severity group, however, none of the patients were in the low severity class. Altogether 13 genotypes were observed in the study of which five were found in patients with moderate severity and eight in high severity of the disease (Table 22). *RB1* mutations and associated disease severities in the 11 patients are described in Table 22. Nine nonsense, one frameshift (eight bp deletion) and one splice site mutations resulting in truncation and two large deletions were detected in the study. Both the large deletions were present in high severity group, while the 11 truncating mutations were distributed as five and six in moderate and high severity groups respectively. The mean residual protein length for the moderate and high severity groups was 289 and 588 aminoacids respectively.

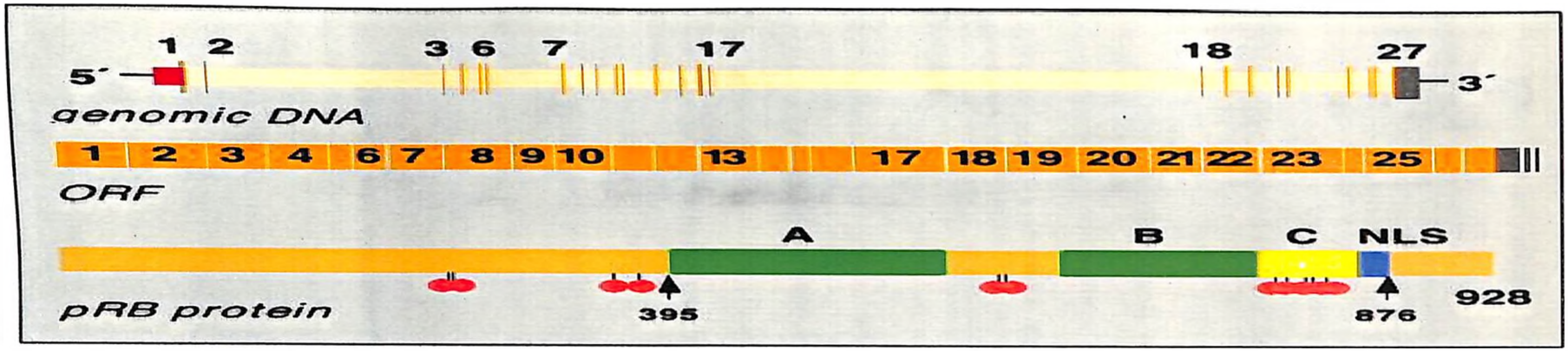
Figure 22 shows the matrix representing the genotype-phenotype (clinical and protein) correlation in retinoblastoma in this study. A total of seven truncating mutations (five nonsense, one frameshift and one large deletion) were present in the N terminus in moderate and high severity groups. A/B pocket and C pocket had two and three nonsense mutations respectively. Of these, one patient with mutation in A/B pocket (N12) and one with mutation in C pocket ($S_1$61) had another mutation in the N terminus. In the patient with intron 20 deletion, intron 2 was present; however it was not sure where the deletion started between these two introns and hence this deletion was placed between the N terminus and A/B pocket in the matrix.

Statistical testing: No statistically significant association was found between disease severity (phenotype) and mutations (genotype) ($p>0.05$) after grouping into truncating and large deletion. Distribution of truncating mutations between moderate and high severity groups were not different in four domains of the protein ($p>0.05$). However truncating mutations were found in significantly more number in the N terminus region (7/10) when compared to A/B pocket (2/10; $p=0.03$) but not for C pocket (3/10; $p=0.08$). The mean predicted protein length did not show any statistical significant difference between moderate and high severity classes.

Table 22: Phenotype and genotype data for the 11 retinoblastoma patients

S No.	ID	Clinical features	Severity	Mutation/pRB domain (exon)	Mutation type	Trunc.	Effect on protein
1	$S_1$61	Optic nerve invasion	High	Intron 2 del. /NA	Large deletion	-	Loss of protein expression
2				C>T / C(E23)	Nonsense	+	R787X
3	N15	age >1 yr, bilateral,	Moderate	8 bp del./ N (E04)	Frameshift	+	136X
4	N29	Advanced stage of disease	High	C>G /N(E07)	Nonsense	+	S215X
5	N12	Unilateral >2 yrs with no family history	Moderate	C>T /N(E08)	Nonsense	+	R251X
6				R445X/N(E14)		+	R445X
7	N19	Bilateral >2 yrs	Moderate	C>T /N (E08)	Nonsense	+	R255X
8	N34	Bilateral >2 yrs	Moderate	C>T /A(E11)	Nonsense	+	R358X
9	N8	Bilateral < 1 yr	High	Intron 11 splice /NA	Splice site	+	aa 378-405 del; aa 378-379 mutation; 380+ truncation
10	N26	Bilateral < 1 yr	High	C>T, /Spacer (E18)	Nonsense	+	R579X
11	N3	Unilateral <2 yrs Choroidal infiltration	High	Intron 20 del./ NA	Large deletion	-	Loss of protein expression
12	Q56	Unilateral, <2 yrs	High	C >A / C(E23)	Nonsense	+	S780X
13	N37	Bilateral < 1 yr	High	C>T/ C(E23)	Nonsense	+	R787X

Trunc. : Truncation



Phenotype	RB P R N Terminus (Aminoacid: 0-392) (Exon: 0-11)	O T E A/B Pocket (393-772) (12-22)	I C Pocket (773-869) (23 -	N C Terminus (870-928) 27)
High Severity	**$S_1$61 i2 Δ**; N29 S215X; N8 380X; N3 i20 Δ	N26 R579X; Q56 S780X	**$S_1$61 R787X**; N37 R787X	——
Moderate Severity	N15 136X; **N12 R251X**; N19 R255X; N34 R358X	**N12 R445X**	——	——
Low Severity	——	——	——	——

Figure 22: Matrix of genotype- phenotype (clinical and protein) correlation in retinoblastoma

(Similar hatched pattern circles denote same patient)

7.5 Discussion

Detecting RB1 mutations in retinoblastoma patients has been shown to have favorable outcome on the families in terms of quality and cost (Richter *et al*, 2003). Genotype-phenotype information is important for genetic counseling of retinoblastoma families, which would enable definite risk assessment (Lohmann and Gallie, 2004) and hence have a beneficial impact in clinical management. Varying correlations between RB1 gene mutations and disease phenotype have been reported. While no genotype-phenotype correlation could be drawn in Chinese and Indian populations, Albrecht *et al*, (2005) showed that gross deletions with one breakpoint in RB1 gene was significantly associated with bilateral retinoblastoma. In the present study, no significant association was observed between truncating mutations and disease severity, in contrast with the report of Qi *et al*, (2005). The present study had only two large deletions, both in patients with high severity, but this was too small a number for statistical significance. This result was consistent with the observation made by Qi *et al*, (2005). No correlation between mean predicted protein length and disease pattern was found for the truncating mutations. While Qi *et al*, (2005) could not draw a relationship between truncating mutations and their location, however we found that majority of the truncating mutations (7/10) were localized in the N terminus of the gene ($p=0.03$).

In general, germline nonsense or frameshift mutations are predominantly seen in patients with hereditary retinoblastoma. Those occurring in particular in the internal exons namely 2-25 have been associated with bilateral retinoblastoma (Lohmann and Gallie, 2004). Patients $S_1$61, N29, N15, N19, N34, N26 and N37, in the present study abide by this rule. However, occasionally such mutations have also been found in isolated unilateral patients (Lohmann *et al*, 1997) as in case of N12 (two nonsense mutations) and Q56 (one nonsense mutation) in the current study. Intron 11 splice

mutation in the patient, N8, resulted in bilateral retinoblastoma as mutation at invariable splice sites has complete penetrance and could have resulted in protein truncation (Lohmann and Gallie, 2004). The two large deletions intron 2 and intron 20 were found in bilateral ($S_1$61) and unilateral patient (N3) respectively. Though both are large deletions, intron 2 deletion can be expected to result in definite loss of major domains of the protein critical for most of its function. Moreover, $S_1$61 also had a nonsense mutation R787X in the C pocket, which could have disrupted interaction of A/B domain with E2F. Whereas the i20 deletion could have occurred anywhere before intron 20 and therefore some amount of protein expression and function could be expected. These collectively could possibly explain the difference in phenotype in these two patients with large deletions. The A/B pocket is attributed for the biological activities of the pRB protein such as regulation of growth and differentiation and biochemical functions like transcriptional regulation and interaction with cellular and viral proteins (DiCiommo *et al*, 2000). Seven out of 10 nonsense mutations detected in this study occurred in the N terminus region ($p=0.03$) and was associated with moderate and/or high severity form of retinoblastoma. This association is much favorable to the expected outcome of loss of protein beyond the N terminus and thereby loss of A/B pocket and its crucial function. These correlations could have been better understood had there been patients in low severity group.

7.6 Conclusion

To the best of our knowledge this is the first ever genotype-phenotype correlation study on Indian retinoblastoma patients. All the patients had moderate or high severity phenotype irrespective of the genotypes being large deletion or truncating mutations. Though significant association of the mutations and severity of disease could not be established, a positive correlation between domainwise location of the mutations and severe form of the disease was observed.

7.7 References

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Specific contributions:

The study established multiplex PCR for all the exons for RB1 gene in 16 PCRs with savings of tumor (33.3%), equipment time (46%) and reagents (30.4%). Mutation screening by automated sequencing was established for the 27 exons of RB1 gene in blood and tumor DNA of retinoblastoma patients and four novel mutations were detected. This is the first study on methylation in Indian retinoblastoma patients and demonstrated the low frequency of hypermethylation of RB1 promoter. The importance of assessing the percentage of metaphases with deletion and the usefulness of statistics in deriving meaningful 13q14 deletion results was established by the study. Cost evaluation conclusively established the economic benefits due to genetic testing of retinoblastoma in India. The diagnostic model established is the first of its kind in this part of the world catering to the needs of patients from India and neighbouring countries. To our best knowledge, this is the first ever genotype-phenotype study undertaken in India and showed an aggressive form of retinoblastoma.

Future scope of work:

Allele specific PCR for commoner stop codon mutations could be established for rapid RB1 mutation screening. Hierarchical ordering of multiplexing with sequencing at specific stages in between could be implemented to reduce the turnaround time for a sample. The quantitative multiplex PCR technology adopted by Richter *et al*, (2003) could be used to detect exonic copy number changes not detected by current methods. These improvements could result in lowered cost for genetic testing and hence affordable to most retinoblastoma families. Analysis of the CpG sites preferentially or initially methylated might lead to CpG methylation phenotype and might be useful in demethylation therapy. Lowered genetic analysis cost due to improvements in technology and optimization of diagnostic model might lead to clinical implementation of retinoblastoma screening in India. Genotype-phenotype analysis in a larger scale will give accurate data based on which molecular testing in retinoblastoma could be 'tailor made' for patients from India.

Appendix I

DNA Extraction from peripheral blood

Materials

- TE buffer pH 8.0 (1M Tris, 2 ml; 50 mM EDTA, 200 μL; and milliQ water, 7.8 ml)

- Digestion buffer (1M tris, 200 μL; Triton, 50 μL; Milli Q water, 740 μL and Proteinase K, 10 μL (15 mg/ml); Saturated NaCl; Phenol:chloroform:iso amyl alcohol (25: 24: 1); 100 % Ethanol; 70 % Ethanol

Equilibration of phenol

Phenol crystals stored at - 20^0C was allowed to come to room temperature and liquefied at 60^0C. 8-hydroxy quinolene was added to a final concentration of 0.1%. Equal volume of 0.5M Tris Chloride (pH 8.0) was added, stirred with magnetic stirrer at room temperature for 15 minutes. When the two phases separated, the upper aqueous phase was aspirated. Equal volume of 0.1M Tris Chloride (pH 8.0) was added to the organic layer and stirred for 15 minutes at room temperature. The aqueous phase was removed and the above step repeated until the pH of the phenol was greater than 7.8. After the phenol was equilibrated and the final aqueous phase removed, 0.1 volume of 0.1M Tris Chloride (pH 8.0) containing 0.2% β-Mercaptoethanol was added.

Methods

To 4.8 ml blood sample in a 15 ml tarson tube, 2 ml of digestion buffer was added and kept for complete digestion at 60°C (minimum 1 hour). Equal volume of equilibrated phenol was added, mixed well and centrifuged at 2500 rpm for 10 minutes. Then the aqueous phase was washed with equal volume of phenol: chloroform: isoamyl alcohol until the interphase was minimum. To the final aqueous layer equal volumes of chloroform: Isoamyl alcohol mixture (24:1) was added and centrifuged for 10 minutes at 2500 rpm. The process is repeated and the aqueous layer (upper layer) was transferred to a fresh tarson tube. DNA was precipitated out by the addition of 1/10 volume of saturated NaCl and two volumes of chilled absolute ethanol and kept at -20°C for 16 hours. At the end of 16 hour, the tube was brought to room temperature, centrifuged at 2500 rpm for 10 minutes. The supernatant was discarded by gently inverting the tube. Absolute ethanol was added till three -fourth of the tube, mixed well and centrifuged at 2500 rpm for 10 minutes. The supernatant was discarded and the pellet was washed twice with 70% ethanol. The tube was then blotted on a blotting paper and the mouth of the tube covered with paraffin film. Holes were made on the paraffin film and tubes were kept at 37^0C till ethanol dried. Then 500 µL of TE buffer was added to dissolve the DNA and kept at 37°C water bath overnight. The DNA samples were then kept at 65°C for 30 minutes to heat inactivate proteins.

Appendix II

Extraction of DNA from tumor and archival sections and quantification

Digestion buffer (1M tris, 200 µl; Triton, 50 µL; Milli Q water, 740 µL and Proteinase K, 10 µL (15 mg/ml)

To the tumor tissue, digestion buffer was added and kept for complete digestion at 37°C (minimum of 48 hours). The tumor from the paraffin sections after deparaffinisation were scraped with a blade; emulsified in digestion buffer, added in small amounts onto the slides; and then transferred into 0.5 ml vials by tilting. Fresh digestion buffer was added onto the slides and the process is repeated. Three hundred µL of digestion buffer is added each of the tubes and incubated at 37^0C. The specimen is then processed as for blood samples from the step of equilibrated phenol.

Quantification of DNA is done spectrophotometrically by the absorbance readings at 260 and 280 nm in a Beckmann spectrophotometer. An OD of 1 at 260 nm corresponds to approximately 50 µg/ml for the double stranded DNA. Pure preparations of DNA have OD 260/280 value of 1.8. If there is contamination with protein or phenol, the OD 260 / 280 will be significantly less than this value.

To 10 µL of stock DNA, 990 µl of milliQ water was added. The absorbance readings were taken in a Beckman DU640 spectrophotometer using Warburg-Christian concentration assay. The quantity of DNA was calculated in µgm based on the absorbance readings.

Appendix III

Details of RB1 Gene

cDNA of RB1 Gene

GI793994

BASE COUNT: 1534 a 902 c 880 g 1523 t

Gene 1..4839; ORF 138 to 2924

Exon 1	1 – 274	Exon 2	275 - 401
Exon 3	402 – 517	Exon 4	518 - 637
Exon 5	638 – 676	Exon 6	677 - 744
Exon 7	745 – 855	Exon 8	856 - 998
Exon 9	999 – 1076	Exon 10	1077 - 1186
Exon 11	1187 – 1264	Exon 12	1265 - 1352
Exon 13	1353 – 1469	Exon 14	1470 - 1526
Exon 15	1527 – 1558	Exon 16	1559 - 1635
Exon 17	1636 – 1832	Exon 18	1833 - 1951
Exon 19	1952 – 2097	Exon 20	2098 - 2243
Exon 21	2244 – 2348	Exon 22	2349 - 2462
Exon 23	2463 – 2626	Exon 24	2627 - 2657
Exon 25	2658 – 2800	Exon 26	2801 - 2850
Exon 27	2851 – 4839		

Sequence

1 tccggttttt ctcaggggac gttgaaatta tttttgtaac gggagtcggg agaggacggg

61 gcgtgccccg cgtgcgcgcg cgtcgtcctc cccggcgctc ctccacagct cgctggctcc

121 cgccgcggaa aggcgtcatg ccgcccaaaa ccccccgaaa aacggccgcc accgccgccg

181 ctgccgccgc ggaacccccg gcaccgccgc cgccgccccc tcctgaggag gacccagagc

241 aggacagcgg cccggaggac ctgcctctcg tcaggcttga gtttgaagaa acagaagaac

301 ctgattttac tgcattatgt cagaaattaa agataccaga tcatgtcaga gagagagctt

361 ggttaacttg ggagaaagtt tcatctgtgg atggagtatt gggaggttat attcaaaaga

421 aaaaggaact gtggggaatc tgtatcttta ttgcagcagt tgacctagat gagatgtcgt

481 tcacttttac tgagctacag aaaaacatag aaatcagtgt ccataaattc tttaacttac

541 taaaagaaat tgataccagt accaaagttg ataatgctat gtcaagactg ttgaagaagt

601 atgatgtatt gtttgcactc ttcagcaaat tggaaaggac atgtgaactt atatatttga

661 cacaacccag cagttcgata tctactgaaa taaattctgc attggtgcta aaagtttctt

721 ggatcacatt tttattagct aaaggggaag tattacaaat ggaagatgat ctggtgattt

781 catttcagtt aatgctatgt gtccttgact attttattaa actctcacct cccatgttgc

841 tcaaagaacc atataaaaca gctgttatac ccattaatgg ttcacctcga acacccaggc

901 gaggtcagaa caggagtgca cggatagcaa aacaactaga aaatgataca agaattattg

961 aagttctctg taaagaacat gaatgtaata tagatgaggt gaaaaatgtt tatttcaaaa

1021 attttatacc ttttatgaat tctcttggac ttgtaacatc taatggactt ccagaggttg

1081 aaaatctttc taaacgatac gaagaaattt atcttaaaaa taaagatcta gatgcaagat

1141 tatttttgga tcatgataaa actcttcaga ctgattctat agacagtttt gaaacacaga

1201 gaacaccacg aaaaagtaac cttgatgaag aggtgaatgt aattcctcca cacactccag

1261 ttaggactgt tatgaacact atccaacaat taatgatgat tttaaattca gcaagtgatc

1321 aaccttcaga aaatctgatt tcctatttta acaactgcac agtgaatcca aaagaaagta

1381 tactgaaaag agtgaaggat ataggataca tctttaaaga gaaatttgct aaagctgtgg

1441 gacagggttg tgtcgaaatt ggatcacagc gatacaaact tggagttcgc ttgtattacc

1501 gagtaatgga atccatgctt aaatcagaag aagaacgatt atccattcaa aattttagca

1561 aacttctgaa tgacaacatt tttcatatgt ctttattggc gtgcgctctt gaggttgtaa

1621 tggccacata tagcagaagt acatctcaga atcttgattc tggaacagat ttgtctttcc

1681 catggattct gaatgtgctt aatttaaaag cctttgattt ttacaaagtg atcgaaagtt

1741 ttatcaaagc agaaggcaac ttgacaagag aaatgataaa acatttagaa cgatgtgaac

1801 atcgaatcat ggaatccctt gcatggctct cagattcacc tttatttgat cttattaaac

1861 aatcaaagga ccgagaagga ccaactgatc accttgaatc tgcttgtcct cttaatcttc

1921 ctctccagaa taatcacact gcagcagata tgtatctttc tcctgtaaga tctccaaaga

1981 aaaaaggttc aactacgcgt gtaaattcta ctgcaaatgc agagacacaa gcaacctcag

2041 ccttccagac ccagaagcca ttgaaatcta cctctctttc actgttttat aaaaaagtgt

2101 atcggctagc ctatctccgg ctaaatacac tttgtgaacg ccttctgtct gagcacccag

2161 aattagaaca tatcatctgg acccttttcc agcacaccct gcagaatgag tatgaactca

2221 tgagagacag gcatttggac caaattatga tgtgttccat gtatggcata tgcaaagtga

2281 agaatataga ccttaaattc aaaatcattg taacagcata caaggatctt cctcatgctg

2341 ttcaggagac attcaaacgt gttttgatca aagaagagga gtatgattct attatagtat

2401 tctataactc ggtcttcatg cagagactga aaacaaatat tttgcagtat gcttccacca

2461 ggccccctac cttgtcacca atacctcaca ttcctcgaag cccttacaag tttcctagtt

2521 caccettacg gattcctgga gggaacatct atatttcacc cctgaagagt ccatataaaa

2581 tttcagaagg tctgccaaca ccaacaaaaa tgactccaag atcaagaatc ttagtatcaa

2641 ttggtgaatc attcgggact tctgagaagt tccagaaaat aaatcagatg gtatgtaaca

2701 gcgaccgtgt gctcaaaaga agtgctgaag gaagcaaccc tcctaaacca ctgaaaaaac

2761 tacgctttga tattgaagga tcagatgaag cagatggaag taaacatctc ccaggagagt

2821 ccaaatttca gcagaaactg gcagaaatga cttctactcg aacacgaatg caaaagcaga

2881 aaatgaatga tagcatggat acctcaaaca aggaagagaa atgaggatct caggaccttg

2941 gtggacactg tgtacacctc tggattcatt gtctctcaca gatgtgactg tataactttc

3001 ccaggttctg tttatggcca catttaatat cttcagctct ttttgtggat ataaaatgtg

3061 cagatgcaat tgtttgggtg attcctaagc cacttgaaat gttagtcatt gttatttata

3121 caagattgaa aatcttgtgt aaatcctgcc atttaaaaag ttgtagcaga ttgtttcctc

3181 ttccaaagta aaattgctgt gctttatgga tagtaagaat ggccctagag tgggagtcct

3241 gataacccag gcctgtctga ctactttgcc ttcttttgta gcatataggt gatgtttgct

3301 cttgttttta ttaatttata tgtatatttt tttaatttaa catgaacacc cttagaaaat

3361 gtgtcctatc tatcttccaa atgcaatttg attgactgcc cattcaccaa aattatcctg

3421 aactcttctg caaaaatgga tattattaga aattagaaaa aaattactaa ttttacacat

3481 tagattttat tttactattg gaatctgata tactgtgtgc ttgttttata aaattttgct

3541 tttaattaaa taaaagctgg aagcaaagta taaccatatg atactatcat actactgaaa

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3721 tataaagtac ccatctagta cttgaaaaag taaagtgttc tgccagatct taggtataga

3781 ggaccctaac acagtatatc ccaagtgcac tttctaatgt ttctgggtcc tgaagaatta

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4081 atttctagtc caaaattaca agtaatcaag ggtcattatg ggttaggcat taatgtttct

4141 atctgatttt gtgcaaaagc ttcaaattaa aacagctgca ttagaaaaag aggcgcttct

4201 cccctcccct acacctaaag gtgtatttaa actatcttgt gtgattaact tatttagaga

4321 tttgtctaac tcagaattat ttttaaaaag aaatctggtc ttgttagaaa acaaaatttt

4381 attttgtgct catttaagtt tcaaacttac tattttgaca gttattttga taacaatgac

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4621 gatattattg ctttattgct tttttgtatt ggttaaaact gtacatttaa aattgctatg

4681 ttactatttt ctacaattaa tagtttgtct attttaaaat aaattagttg ttaagagtct

4741 taatggtctg atgttgtgtt ctttgtatta agtacactaa tgttctcttt tctgtctagg

4801 agaagataga tagaagataa ctctcctagt atctcatcc //

RB1 splice junctions (Based on Genbank Accession Number: L11910)

5UTRE0001 E0001TRANS TCGCTGGCTCCCGCCGCGGAAAGGCGTC[ATG] CCGCCCAA AACCCCCCGA

E0001 I0001 CGGAGGACCTGCCTCTCGTC[AGgtgagcgagcagagccgccgtcgcctc

I0001 E0002 caattatatgattattttcatttggtagG]CTTGAGTTTGAAGAAACAGAAG

E0002 I0002 GTTTCATCTGTGGATGGAGTATTGgtaaggattttcttaaaacgttttgaa

I0002E0003 tgaaatatttgatcttta(t)6gttcccagGGAGGTTATATTCAAAAGAAAAAG

E0003 I0003 TACAGAAAAACATAGAAATC[AGgtaaagtttcttgtataaatataagcct

I0003 E0004 tgatttac(t)6ctattctttcctttgtagT]GTCCATAAATTCTTTAACTTA

E0004 I0004 CACTCTTCAGCAAATTGGAA[AGgtaaagtaaacattttattagggttaca

I0004E0005g(a)6tgttaaaaagtcataatgtttttctttcagG]ACATGTGAACTTATATATTTG>

E0005 I0005 <ACACAACCCAGCAGT[TCgtaagtagttcacagaatgttatttttcactt(a)9 g

I0005 E0006 ctg(t)8ctgctttctatttgtttaatagG]ATATCTACTGAAATAAATTCTG

E0006 I0006 GATCACATTTTTATTAGCTAAA[Ggtaagttcattatatttattaaatgcta

I0006 E0007 ctaactttctttaaaaatgtaca(t)9cagGG]GAAGTATTACAAATGGAAGAT

E0007 I0007 CATGTTGCTCAAAGAACCATAT[Agtaagtatttaatttatgccccttttac

I0007 E0008 gttcttatctaatttaccacttttacagAA]ACAGCTGTTATACCCATTAAT

E0008 I0008 GAACATGAATGTAATATAGATGAGgtaatttaacttcatgatttctttaaaac

I0008 E0009 gatcatgttgtaacttcatctttttcagGTGAAAAATGTTTATTTCAAAAAT

E0009 I0009 TAACATCTAATGGACTTCCAGAGgtaatctgaaaggaaatttaataaaatat

I0009E0010 taaaggataattgtcagtgac(t)6ctttcaagGTTGAAAATCTTTCTAAACGATA

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I0010 E0011 gtgaatgacttcacttattgttatttagT]TTTGAAACACAGAGAACACCAC

E0011 I0011 ATTCCTCCACACACTCCAGTT[AGgtatgaattttcctacttttaattatat

I0011 E0012 cacattttcctatttttatcccctctagG]ACTGTTATGAACACTATCCAAC

E0012 I0012 GAAAATCTGATTTCCTATTTTAACgtaagccatatatgaaacattatttattg

I0012 E0013 cattgatttctgtttttacctcctaaagAACTGCACAGTGAATCCAAAAGA

E0013 I0013 GTTGTGTCGAAATTGGATCACAGgtaacttgaattcattgtaattcgtggt

I0013 E0014 tcttatttttctttttgtttgtttgtagCGATACAAACTTGGAGTTCGCTTG

E0014I0014 TAATGGAATCCATGCTTAAATCAgtaagttaaaaacaatat(a)7tttcagccg

I0014 E0015 taaacaacttc(t)13aaattatctgtttcagGAAGAAGAACGATTAT>

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E0016 I0016 AGGTTGTAATGGCCACATATAGC[Agtaagttaaattttcataaataaacact

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I0018 E0019 atgaagacttttcctttaaatatatctagG]TATCTTTCTCCTGTAAGATCTC

E0019 I0019 TCTTTCACTGTTTTATAAAAAA[Ggttagtagatgattattttcaagagcatg

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E0020 I0020 TGAGAGACAGGCATTTGGACCAAgtaagaaaatcaagcacttcaccttctct

I0020 E0021 actacttttacatcaatttatttactagATTATGATGTGTTCCATGTATGGC

E0021 I0021 ATCTTCCTCATGCTGTTCAGGAGgtaggtaattttccatagtaag(t)7gataaat

I0021 E0022 taaattttac(t)24actgttcttcctcagACATTCAAACGTGTTTTGATCAAAG

E0022 I0022 ATTTTGCAGTATGCTTCCACCAGGgtaggtcaaaagtatcctttgattggaaaaat

I0022E0023 taaataaataatctac(t)6gtttttgctctagCCCCCTACCTTGTCACCAATACCT

E0023 I0023 ACAAAAAATGACTCCAAGATCA[AGgtgtgtgttttctctttagggaagtagt

I0023 E0024 taattggtatttcatcttaacttgacagA]ATCTTAGTATCAATT>

E0024 I0024 <GGTGAATCATTCGGGgtgagtattttctttctatgaaatataat

I0024 E0025 atttaaagtaaagaattctgtaatttgtagACTTCTGAGAAGTTCCAGAAAAT

E0025I0025 AGGATCAGATGAAGCAGATGGA[AGgtaggaaccagttttgaatgttttccag

I0025E0026 cacatgaaatgttttgca(t)7aatctgcagT]AAACATCTCCCAGGAGAGTCCA

E0026I0026 TCAGCAGAAACTGGCAGAAATG[Agtaagtac(t)6caccttgtgtaaacgaaat

I0026E0027 tgttaacagttcttcatccttttttccagCT]TCTACTCGAACACGAATGCAAAAG

E00273UTR
TACCTCAAACAAGGAAGAGAAA[TGA]GGATCTCAGGACCTTGGTGGA

Appendix IV

RB1 codons and aminoacids															
1	M	P	P	K	T	P	R	K	T	A	A	T	A	A	A	15
	ATG	CCG	CCC	AAA	ACC	CCC	CGA	AAA	ACG	GCC	GCC	ACC	GCC	GCC	GCT	
16	A	A	A	Z	P	P	A	P	P	P	P	P	P	P	Z	30
	GCC	GCC	GCG	GAA	CCC	CCG	GCA	CCG	CCG	CCG	CCG	CCC	CCT	CCT	GAG	
31	E	D	P	Z	Z	D	S	G	P	Z	D	L	P	L	V	45
	GAG	GAC	CCA	GAG	CAG	GAC	AGC	GGC	CCG	GAG	GAC	CTG	CCT	CTC	GTC	
46	R	L	Z	F	Z	Z	T	Z	Z	P	D	F	T	A	L	60
	AGG	CTT	GAG	TTT	GAA	GAA	ACA	GAA	GAA	CCT	GAT	TTT	ACT	GCA	TTA	
61	C	Q	K	L	K	I	P	D	H	V	R	Z	R	A	W	75
	TGT	CAG	AAA	TTA	AAG	ATA	CCA	GAT	CAT	GTC	AGA	GAG	AGA	GCT	TGG	
76	L	T	W	Z	K	V	S	S	V	D	G	V	L	G	G	90
	TTA	ACT	TGG	GAG	AAA	GTT	TCA	TCT	GTG	GAT	GGA	GTA	TTG	GGA	GGT	
91	Y	I	Q	K	K	K	E	L	W	G	I	C	I	F	I	105
	TAT	ATT	CAA	AAG	AAA	AAG	GAA	CTG	TGG	GGA	ATC	TGT	ATC	TTT	ATT	
106	A	R	V	D	L	D	F	M	S	F	T	L	L	S	Y	120
	GCA	CGA	GTT	GAC	CTA	GAT	GAG	ATG	TCG	TTC	ACT	TTA	CTG	AGC	TAC	
121	R	K	T	Y	E	I	S	V	H	K	F	F	B	L	L	135
	AGA	AAA	ACA	TAC	GAA	ATC	AGT	GTC	CAT	AAA	TTC	TTT	AAC	TTA	CTA	
136	K	E	I	D	T	S	T	K	V	D	N	A	M	S	R	150
	AAA	GAA	ATT	GAT	ACC	AGT	ACC	AAA	GTT	GAT	AAT	GCT	ATG	TCA	AGA	

RB1 codons and aminoacids (Contd.)																
151	L	L	K	K	Y	D	V	L	F	A	L	F	S	K	L	165
	CTG	TTG	AAG	AAG	TAT	GAT	GTA	TTG	TTT	GCA	CTC	TTC	AGC	AAA	TTG	
166	E	R	T	C	E	L	I	Y	L	T	Z	P	S	S	S	180
	GAA	AGG	ACA	TGT	GAA	CTT	ATA	TAT	TTG	ACA	CAA	CCC	AGC	AGT	TCG	
181	I	S	T	E	I	N	S	A	L	V	L	K	V	S	W	195
	ATA	TCT	ACT	GAA	ATA	AAT	TCT	GCA	TTG	GTG	CTA	AAA	GTT	TCT	TGG	
196	I	T	F	L	L	A	K	G	E	V	L	Z	M	E	D	210
	ATC	ACA	TTT	TTA	TTA	GCT	AAA	GGG	GAA	GTA	TTA	CAA	ATG	GAA	GAT	
211	D	L	V	I	S	F	Z	L	M	L	C	V	L	D	Y	225
	GAT	CTG	GTG	ATT	TCA	TTT	CAG	TTA	ATG	CTA	TGT	GTC	CTT	GAC	TAT	
226	F	I	K	L	S	P	P	M	L	L	K	E	P	Y	K	240
	TTT	ATT	AAA	CTC	TCA	CCT	CCC	ATG	TTG	CTC	AAA	GAA	CCA	TAT	AAA	
241	T	A	V	I	P	I	B	G	S	P	R	T	P	R	R	255
	ACA	GCT	GTT	ATA	CCC	ATT	AAT	GGT	TCA	CCT	CGA	ACA	CCC	AGG	CGA	
256	G	Q	N	R	S	A	R	I	A	K	Q	L	E	N	D	270
	GGT	CAG	AAC	AGG	AGT	GCA	CGG	ATA	GCA	AAA	CAA	CTA	GAA	AAT	GAT	
271	T	R	I	I	E	V	L	C	K	E	H	E	C	N	I	285
	ACA	AGA	ATT	ATT	GAA	GTT	CTC	TGT	AAA	GAA	CAT	GAA	TGT	AAT	ATA	
286	D	E	V	K	N	V	Y	F	K	N	F	I	P	F	M	300
	GAT	GAG	GTG	AAA	AAT	GTT	TAT	TTC	AAA	AAT	TTT	ATA	CCT	TTT	ATG	
301	N	S	L	G	L	V	T	S	N	G	L	P	E	V	E	315
	AAT	TCT	CTT	GGA	CTT	GTA	ACA	TCT	AAT	GGA	CTT	CCA	GAG	GTT	GAA	

RB1 codons and aminoacids (Contd.)																
316	N	L	S	K	R	Y	E	E	I	Y	L	K	N	K	D	330
	AAT	CTT	TCT	AAA	CGA	TAC	GAA	GAA	ATT	TAT	CTT	AAA	AAT	AAA	GAT	
331	L	D	R	R	L	F	L	D	H	D	K	T	L	Q	T	345
	CTA	GAT	CGA	AGA	TTA	TTT	TTG	GAT	CAT	GAT	AAA	ACT	CTT	CAG	ACT	
346	D	S	I	D	S	F	E	T	Q	R	T	P	R	K	S	360
	GAT	TCT	ATA	GAC	AGT	TTT	GAA	ACA	CAG	AGA	ACA	CCA	CGA	AAA	AGT	
361	N	L	D	E	E	V	N	I	I	P	P	H	I	P	V	375
	AAC	CTT	GAT	GAA	GAG	GTG	AAT	ATA	ATT	CCT	CCA	CAC	ACT	CCA	GTT	
376	R	T	V	M	N	I	I	Q	Q	L	M	M	I	L	N	390
	AGG	ACT	GTT	ATG	AAC	ACT	ATC	CAA	CAA	TTA	ATG	ATG	ATT	TTA	AAT	
391	S	A	S	D	Q	P	S	E	N	L	I	S	Y	F	N	405
	TCT	GCA	AGT	GAT	CAA	CCT	TCA	GAA	AAT	CTG	ATT	TCC	TAT	TTT	AAC	
406	N	C	T	V	N	P	K	E	S	I	L	K	R	V	K	420
	AAC	TGC	ACA	GTG	AAT	CCA	AAA	GAA	AGT	ATA	CTG	AAA	AGA	GTG	AAG	
421	D	I	G	Y	I	F	K	E	K	F	A	K	A	V	G	435
	GAT	ATA	GGA	TAC	ATC	TTT	AAA	GAG	AAA	TTT	GCT	AAA	GCT	GTG	GGA	
436	Q	G	C	V	E	I	G	S	Q	R	Y	K	L	G	V	450
	CAG	GGT	TGT	GTC	GAA	ATT	GGA	TCA	CAG	CGA	TAC	AAA	CTT	GGA	GTT	
451	R	L	Y	Y	R	V	M	E	S	M	L	K	S	E	E	465
	CGC	TTG	TAT	TAC	R	GTA	ATG	GAA	TCC	ATG	CTT	AAA	TCA	GAA	GAA	
466	E	R	L	S	I	Q	N	F	S	K	L	L	N	D	N	480
	GAA	CGA	TTA	TCC	ATT	CAA	AAT	TTT	AGC	AAA	CTT	CTG	AAT	GAC	AAC	

RB1 codons and aminoacids (Contd.)																
481	I	F	H	M	S	L	L	A	C	A	L	E	V	V	M	495
	ATT	TTT	CAT	ATG	TCT	TTA	TTG	GCG	TGC	GCT	CTT	GAG	GTT	GTA	ATG	
496	A	T	Y	S	R	S	T	S	Q	N	L	D	S	G	T	510
	GCC	ACA	TAT	AGC	AGA	AGT	ACA	TCT	CAG	AAT	CTT	GAT	TCT	GGA	ACA	
511	D	L	S	F	P	W	I	L	N	V	L	N	L	K	A	525
	GAT	TTG	TCT	TTC	CCA	TGG	ATT	CTG	AAT	GTG	CTT	AAT	TTA	AAA	GCC	
526	F	D	F	Y	K	V	I	E	S	F	I	K	A	E	G	540
	TTT	GAT	TTT	TAC	AAA	GTG	ATC	GAA	AGT	TTT	ATC	AAA	GCA	GAA	GGC	
541	N	L	T	R	E	M	I	K	H	L	E	R	C	E	H	555
	AAC	TTG	ACA	AGA	GAA	ATG	ATA	AAA	CAT	TTA	GAA	CGA	TGT	GAA	CAT	
556	R	I	M	E	S	L	A	W	L	S	D	S	P	L	F	570
	CGA	ATC	ATG	GAA	TCC	CTT	GCA	TGG	CTC	TCA	GAT	TCA	CCT	TTA	TTT	
571	D	L	I	K	Q	S	K	D	R	E	G	P	T	D	H	585
	GAT	CTT	ATT	AAA	CAA	TCA	AAG	GAC	CGA	GAA	GGA	CCA	ACT	GAT	CAC	
586	L	E	S	A	C	P	L	N	L	P	L	Q	N	N	H	600
	CTT	GAA	TCT	GCT	TGT	CCT	CTT	AAT	CTT	CCT	CTC	CAG	AAT	AAT	CAC	
601	T	A	A	D	M	Y	L	S	P	V	R	S	P	K	K	615
	ACT	GCA	GCA	GAT	ATG	TAT	CTT	TCT	CCT	GTA	AGA	TCT	CCA	AAG	AAA	
616	K	G	S	T	T	R	V	N	S	I	A	N	A	E	I	630
	AAA	GGT	TCA	ACT	ACG	CGT	GTA	AAT	TCT	ACT	GCA	AAT	GCA	GAG	ACA	
631	Q	A	T	S	A	F	Q	T	Q	K	P	L	K	S	T	645
	CAA	GCA	ACC	TCA	GCC	TTC	CAG	ACC	CAG	AAG	CCA	TTG	AAA	TCT	ACC	

RB1 codons and aminoacids (Contd.)																
646	S	L	S	L	F	Y	K	K	V	Y	R	L	A	Y	L	660
	TCT	CTT	TCA	CTG	TTT	TAT	AAA	AAA	GTG	TAT	CGG	CTA	GCC	TAT	CTC	
661	R	L	N	T	L	C	E	R	L	L	S	E	H	P	E	675
	CGG	CTA	AAT	ACA	CTT	TGT	GAA	CGC	CTT	CTG	TCT	GAG	CAC	CCA	GAA	
676	L	E	H	I	I	W	T	L	F	Q	H	I	L	Q	N	690
	TTA	GAA	CAT	ATC	ATC	TGG	ACC	CTT	TTC	CAG	CAC	ACC	CTG	CAG	AAT	
691	E	Y	E	L	M	R	D	R	H	L	D	Q	I	M	M	705
	GAG	TAT	GAA	CTC	ATG	AGA	GAC	AGG	CAT	TTG	GAC	CAA	ATT	ATG	ATG	
706	C	S	M	Y	G	I	C	K	V	K	N	I	D	L	K	720
	TGT	TCC	ATG	TAT	GGC	ATA	TGC	AAA	GTG	AAG	AAT	ATA	GAC	CTT	AAA	
721	F	K	I	I	V	T	A	Y	K	D	L	P	H	A	V	735
	TTC	AAA	ATC	ATT	GTA	ACA	GCA	TAC	AAG	GAT	CTT	CCT	CAT	GCT	GTT	
736	Q	E	T	F	K	R	V	L	I	K	E	E	E	Y	D	750
	CAG	GAG	ACA	TTC	AAA	CGT	GTT	TTG	ATC	AAA	GAA	GAG	GAG	TAT	GAT	
751	S	I	I	V	F	Y	N	S	V	F	M	Q	R	L	K	765
	TCT	ATT	ATA	GTA	TTC	TAT	AAC	TCG	GTC	TTC	ATG	CAG	AGA	CTG	AAA	
766	T	N	I	L	Q	Y	A	S	T	R	P	P	T	L	S	780
	ACA	AAT	ATT	TTG	CAG	TAT	GCT	TCC	ACC	AGG	CCC	CCT	ACC	TTG	TCA	
781	P	I	P	H	I	P	R	S	P	V	K	F	P	S	S	795
	CCA	ATA	CCT	CAC	ATT	CCT	CGA	AGC	CCT	TAC	AAG	TTT	CCT	AGT	TCA	
796	P	L	R	I	P	G	G	N	I	Y	I	S	P	L	K	810
	CCC	TTA	CGG	ATT	CCT	GGA	GGG	AAC	ATC	TAT	ATT	TCA	CCC	CTG	AAG	

RB1 codons and aminoacids																
811	S	P	Y	K	I	S	E	G	L	P	T	P	T	K	M	825
	AGT	CCA	TAT	AAA	ATT	TCA	GAA	GGT	CTG	CCA	ACA	CCA	ACA	AAA	ATG	
826	T	P	R	S	R	I	L	V	S	I	G	E	S	F	G	840
	ACT	CCA	AGA	TCA	AGA	ATC	TTA	GTA	TCA	ATT	GGT	GAA	TCA	TTC	GGG	
841	T	S	E	K	F	Q	K	I	N	Q	M	V	C	N	S	855
	ACT	TCT	GAG	AAG	TTC	CAG	AAA	ATA	AAT	CAG	ATG	GTA	TGT	AAC	AGC	
856	D	R	V	L	K	R	S	A	E	G	S	N	P	P	K	870
	GAC	CGT	GTG	CTC	AAA	AGA	AGT	GCT	GAA	GGA	AGC	AAC	CCT	CCT	AAA	
871	P	L	K	K	L	R	F	D	I	E	G	S	D	E	A	885
	CCA	CTG	AAA	AAA	CTA	CGC	TTT	GAT	ATT	GAA	GGA	TCA	GAT	GAA	GCA	
886	D	G	S	K	H	L	P	G	E	S	K	F	Q	Q	K	900
	GAT	GGA	AGT	AAA	CAT	CTC	CCA	GGA	GAG	TCC	AAA	TTT	CAG	CAG	AAA	
901	L	A	E	M	T	S	T	R	T	R	M	Q	K	Q	K	915
	CTG	GCA	GAA	ATG	ACT	TCT	ACT	CGA	ACA	CGA	ATG	CAA	AAG	CAG	AAA	
916	M	N	D	S	M	D	T	S	N	K	E	E	K	Stop		930
	ATG	AAT	GAT	AGC	ATG	GAT	ACC	TCA	AAC	AAG	GAA	GAG	AAA	TGA		

Appendix V

Cytogenetic culture

Peripheral blood culture

RPMI medium 1640 (lyophilized); Sartorius filter; laminar flow; pH strips, Millipore membrane.

Principle

Seventy-two hour peripheral blood culture was initiated using RPMI 1640, fetal bovine serum, L-Glutamine, antibiotics and phyto haemagglutinin as mitogenic agent. Methotrexate was added as synchronizing agent. The effect of methotrexate is removed by addition of thymidine; resulting in cells with good mitotic index.

❖ RPMI medium

RPMI –1640 (base)	-	10.3 gm
L- Glutamine	-	600 mg
Sodium bicarbonate	-	2.2 gm
Penicillin	-	200 mg

The ingredients were dissolved in 500 ml of autoclaved milli Q water; pH was adjusted to 6.8 and then made up to 1000 ml. The media was filtered using Sartorius filtration apparatus.

❖ MTX stock solution ($1X\ 10^{-3}$ M)

MTX [(+) Amethopterin]	-	1 mg
1N NaOH	-	0.5 ml

MTX was dissolved in 1N NaOH; made upto 2.2 ml with sterile water and stored in dark in frozen conditions.

❖ MTX working solution (1X 10^{-5} M)

MTX stock solution	-	0.1 ml
Distilled water	-	9.9 ml

Thymidine (1X 10^{-3} M)

Thymidine	-	2.5 mg
Distilled water	-	10 ml

Thymidine is dissolved in water, filtered and stored in small aliquots in frozen state. It is freshly prepared every 2-3 months.

Ethidium Bromide (0.2%): stored in amber colored bottle at 4°C.

Colchicine (0.02%); stored in amber colored bottle.

Setting of culture

Requirements: Culture vials with screw caps, 10 ml pipettes -2 Nos, Mantoux syringe - 1, CO_2 incubator, RPMI 1640 medium, fetal calf serum, rectified spirit, cotton, Bunsen burner, blood sample, laminar flow.

UV lamp in laminar airflow hood was switched on an hour before setting up culture. All the autoclaved culture vials, caps and pipette were placed in the laminar hood. Twenty ml of fetal calf serum was added to 100 ml of medium. Ten ml of medium was transferred to three culture vials.

Phyto haemagglutinin (0.2 ml) was added to each culture using mantoux syringe. After discarding the first few drops, 0.4 ml of blood (25 - 30 drops) were added slowly and mixed gently. The mouth of the culture vial was flamed and cooled before closing the vial. The culture vials were incubated in slanting position in CO_2 incubator at 37°C for 72 hours. The screw cap was kept loosened so that CO_2 can enter the vials

during incubation. Every 24 hour, the vials were shaken to remove carbon dioxide. At the end of 72 hours of incubation, 100 µL of working MTX solution (4.5×10^{-6} mg/µL, Sigma, USA) was added to the culture vial and shaken gently. The culture was incubated at 37°C for 17 hours in a CO_2 incubator.

Incubation and processing of culture

Washing of the culture was done at the end of 17 hours incubation. The culture was transferred to a conical centrifuge tube and centrifuged at 1,800 rpm for 10 minutes. The supernatant was discarded and fresh RPMI medium without serum was added. The pellet was resuspended and centrifuged at 1800 rpm for 10 minutes. The process was repeated again with fresh media and the supernatant discarded. After discarding maximum of the supernatant without disturbing the pellet, 100 µl of thymidine solution (2.5×10^{-4} mg/µL, Sigma, USA) and seven ml of FBS containing RPMI medium were added. The culture was transferred to a new culture vial and incubated further at 37°C for 5 hours.

Harvesting and metaphase preparation

Requirements: Hypotonic solution- 0.075 M KCl; Carnoy's fixative - methanol: glacial acetic acid - 3:1; glass slides.

Harvesting of cells was done after 5-hrs incubation. 100 µL of colchicine ($2x10^{-4}$ mg\µl, Sigma, USA) was added 10 minutes before harvesting. The contents of the tubes were transferred into a centrifuge tube and centrifuged at 1800 rpm for 10 minutes. The supernatant was discarded leaving as little medium as possible over the cell button. The cell button was resuspended in 5 ml of prewarmed hypotonic solution, mixed gently and incubated at 37°C for 30 minutes in a water bath. After the incubation, 3.0 ml of ice-cold freshly prepared fixative was added slowly through the

sides of the tube and mixed gently. The contents were centrifuged and the supernatant discarded. The fixation step was repeated twice with 5.0 ml of ice-cold fixative. The cells were resuspended in a small volume of fixative (0.5 - 1.0 ml).

Metaphase slides were prepared by standard methods. New slides were treated with dichromate solution and then washed in several changes of distilled water. The slides were then washed successively in 0.1% HCl and milli Q water. Before use, the slides were washed in running tap water and stored in milli Q water inside a freezer to chill the slides. Metaphases were prepared from the cell suspension by dropping 3 - 4 drops onto each chilled slide from a height suitable for spreading. The slides were kept in slanting position to drain off excess fluid, allowed to air dry and then kept on a 37^0C hot plate for 5 minutes. The slides were labeled with the reference number and year of the patient. The metaphases are examined under low and high magnification to check cell density, spread and quality of chromosomes. If the cell density is high, the suspension was diluted further by adding few more drops of ice-cold fixative. If the cell density is low, the contents of the tube are centrifuged; the pellet is resuspended in a smaller volume of fixative. The slides were then incubated at 65°C for 16 hours and stored in slide boxes till Giemsa banding is done.

Giemsa banding

Principle: Differential extraction of proteins from regions of chromosomes during fixation and trypsinisation may be the mechanism by which G-bands are obtained.

Reagents

Trypsin–EDTA solution: The reagent is freshly prepared just before use and mixed using a magnetic stirrer for 30 minutes.

Trypsin	–	20 mg
EDTA	–	10 mg
Sorrensen's buffer	–	50 ml

Sorensen's buffer pH 7.0: Mixed Disodium hydrogen phosphate (5.112 gm / 600 ml) and Potassium dihydrogen phosphate (3.264 gm / 400 ml) and stored at 4°C.

Working Giemsa stain: prepared by mixing 48 ml Giemsa buffer and 2 ml Giemsa stain; filtered and froth is removed using blotting paper.

Giemsa buffer: Sodium dihydrogen phosphate (0.2 M-6.2 gm/200 ml) and Disodium hydrogen phosphate (0.2 M -11.36 gm/400 ml) were mixed and stored at 4°C.

Procedure

Heated metaphase slides were treated with trypsin-EDTA solution (1 dip). The slide was rinsed in two changes of water and then dipped in Giemsa stain for 2-5 minutes. The trypsinisation and staining time were varied to get optimal staining intensity. After staining, the slides were rinsed in water and examined under the microscope for adequacy of staining. Suitable stained slides were overlaid with DPX mountant, covered with clean cover slip without air bubbles and kept in horizontal position overnight. The excess amount of DPX was removed by cleaning with xylene.

Appendix VI

Homology modeling for RB1 mutants

Modeling of mutants has been done with the help of swissmodel at ExPaSy server to understand the effect of mutations at the three-dimensional level for the A/B domain, Spacer and C terminus mutants.

Principle: PDB, or Protein Data Bank, is an international repository of 3-D protein structures primarily determined by X-ray crystallography and solution nuclear magnetic resonance. Theoretical homology models are built by threading a sequence onto an x-ray or NMR model of one or more homologous proteins.

Once the server starts working on the submission, a welcome e-mail from Swiss-Model with Process Identification code corresponding to the request is received. The programs at the expert Protein Analysis System compares the submitted sequences to find all similarities with sequences of known structures and templates with above 25% sequence identities and projected model size larger than 20 residues. The molecular graphics program Deepview aligns the given sequences and creates a preliminary three-dimensional model, which is submitted to Swiss model server. After receiving the acknowledgement of submission, modeling results are received within four hours via e-mail as an attached PDB file.

In the first step R445X, R579X, S780X, R787X and wild type pRB with N terminal aminoacids included were submitted in FastA file format to the ExPaSy server. In the next step mutants R445 (A domain), R579 (spacer) and wild type pRB large pocket were submitted with omission of N terminus aminoacids. In the last step the wild type N terminus aminoacids alone were submitted for modeling. The generated structures were viewed using deepview downloaded from the server.

Appendix VII

Genbank accession number for mutations

No.	Patient ID	Mutation	Detected in	Accession Number
1.	N15	Exon 4 eight -bp deletion (g.41945 –41952)	Tumor	AY740680
2.	N29	S215X	Blood	AY643840
3.	Q56	S780X	Tumor	AY642690
4.	S_1 61	R787X	Tumor	AY642692
5.	N37	R787X	Blood	AY642691

Publications

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Joseph B, Sivanarayana K, Vidhya A, Shanmugam MP, Kumaramanickavel G. Mutational screening of retinoblastoma - A novel mutation of RB1 gene. 26th annual session of Indian Society of Human Genetics conference, Rajiv Gandhi Centre for Biotechnology, Trivandrum, India, February 2002.

Brief C.V. of the candidate

Mr. Biju Joseph obtained his B.Sc (Medical Laboratory Technology) degree from Kerala University in 1994. After working as Medical Laboratory Technologist for a short duration, he obtained his M.S. (Medical Laboratory Technology) degree of Birla Institute of Technology and Science, Pilani in 1998 with course work at Medical Research Foundation, Chennai. He was vocational lecturer in Medical Laboratory Technology at VHSS Ettumanoor, Kerala before joining the SN ONGC department of Genetics and Molecular Biology, Sankara Nethralaya as Junior Scientist. He established automated sequencing on ABI 310 sequencer for RPE65 gene in the department. As part of thesis work, patient recruitment, blood and tumor collection, and experiments for DNA diagnostic methods for retinoblastoma was done with Dr. G. Kumaramanickavel as supervisor and with a senior research fellowship from Department of Biotechnology, Govt. of India. He was awarded the Indian Eye research Group travel fellowship, 2000. He also cleared the all India joint Lectureship examination 2002, conducted by Council for scientific & Industrial research-University Grants Commission, Govt. of India. As an off-campus faculty of Birla Institute of Technology and Science, Pilani, at Medical Research Foundation, Chennai, he taught molecular biology and human genetics for undergraduates and post-graduates. His achievements include improving quality of DNA by chloroform-isoamyl alcohol purification step, implementing sample and cost saving measures - multiplex PCR and avoiding gel elution in RB1 mutation screening, and foreign currency savings by in-house preparation of hybridization buffer for RB1 gene FISH. He has five publications and presentations each as first author. His research interests are in genomics of cancer.

Brief CV of the supervisor

Dr. G Kumaramanickavel is the Reader & Head of SN ONGC Department of Genetics & Molecular Biology, Vision Research Foundation, Sankara Nethralaya, Chennai, India. He did MBBS (1980) and MD - Physiology (1986) from Madras Medical College, University of Madras, India. He is the President of the Asian Society for Eye Genetics and Mr. YT Cheng Visiting Scholar, Dept of Ophthalmology & Visual Sciences, Chinese University of Hong Kong, China. As a Fogarty Visiting Associate, he worked with Dr. Fielding Hejtmancik in Ophthalmic Genetics & Clinical Services branch, National Eye Institute, National Institute of Health, United States of America. Between 1992 and 96 he was Research Fellow, Ocular Gene Mapping Laboratory, Department of Biochemistry, University of Otago, Dunedin, New Zealand and worked with Dr. Michael Denton. He received the Swarnalatha Punshi Award (Best Research worker) 2001, Sankara Nethralaya, ISCA Young Scientist Award, Platinum Jubilee Session of the Indian Science Congress Association (1988) and Indira Vasudevan Award, Indian Association of Bio-medical Scientists (1986), India. He works on ocular genomics and is involved in gene mapping, mutational screening and genetic association studies in ophthalmic diseases like age-related cataract, open angle and angle closure glaucomas, diabetic retinopathy, retinitis pigmentosa, retinoblastoma and molecular diagnostics. He receives funding approximately for US $ 100, 000 per annum for these projects from Indian Federal (Centre for Scientific Industrial Research, Indian Council of Medical Research, Department of Science & Technology, Department of Biotechnology, Oil and Natural Gas Corporation Limited) and private (R.D. Tata Trust, Chennai Willingdon Corporate Foundation) agencies. He is involved in two major epidemiological projects on glaucoma and diabetic retinopathy. He has published more than 50 articles in *Nature Genetics, Human Molecular Genetics, Investigative*

Ophthalmology and Visual Sciences, Community Genetics, Ophthalmic Epidemiology, American Journal of Medical Genetics and Genomics. He has 5 graduate students working with him and is also a PhD examiner. He along with his colleagues has trained 15 masters' students who worked on specific genetics dissertation topic. He runs a genetic counseling clinic at the hospital, where in eight years nearly 6000 new patients with ocular genetic diseases have been counseled and teaches genetics for ophthalmologists and para-ophthalmic courses. His department collaborates with Dr. Michael J Denton (University of Otago, New Zealand), Dr. Fielding J Hejtmancik (NEI, NIH, USA) Dr. Cathy McCarty (Marshfield Clinic, USA), Dr. Brenda Gallie (University of Toronto, Canada), Prof. Calvin Pang (Chinese University of Hong Kong) and Dr. Eric Yap (National University of Singapore). He is a reviewer for journals *Indian Journal of Ophthalmology, Clinical Genetics and Neoplasia*. Areas of Interest: Ocular genomics and is involved in gene mapping, mutational screening and genetic association studies in ophthalmic diseases like age-related cataract, open angle and angle closure glaucomas, diabetic retinopathy, retinitis pigmentosa, retinoblastoma and molecular diagnostics.