CHAPTER 4

RESULTS

4.1 Patients and Control

Between November 2009 and June 2012, a total of seven patients (11 eyes), diagnosed with retinoblastoma were seen in University Malaya Medical Centre. These were confirmed cases of unilateral and bilateral retinoblastoma by clinical presentation, imaging and histopathological analysis upon enucleation, as removal of an eye was performed in all patients. All the patients subjected to *RB1* molecular screening were Chinese (100%), after excluding a Malay infant who was subsequently eliminated from the study due to insufficient genomic DNA recovered from the buccal swab. There were no Indian patients observed during this study course. Table 4.1 and 4.2 summarize the clinical history and outcome of patients enrolled in this study. The study group consisted of four male patients and two female patients. Notably, the patients had neither family history of retinoblastoma nor found to have other non-ocular malignancies. Leukocoria was the main clinical feature at initial presentation. All the patients were under the age of five years old except an adult who was 34 years old at study entry. The age at diagnosis ranged from 17 months to 33 months. DNA from two normal samples were used as negative controls. The negative or healthy controls were of Chinese and Malay ethnicity respectively.

Table 4.1 : Description of Retinoblastoma Patients Enrolled in *RB1* Mutation Screening Study

Patient	Gender	Laterality	Age at diagnosis	Age at study entry
RB-I	Male	Unilateral	28 months	28 months
RB-II	Male	Unilateral	24 months	24 months
RB-III	Male	Bilateral	33 months	57 months
RB-IV	Male	Unilateral	16 months	418 months
RB-V	Female	Bilateral	17 months	17 months
RB-VI	Female	Bilateral	32 months	32 months

Table 4.2 : Treatment Received by Patients with Retinoblastoma in reference to the Laterality and Severity of Their Disease

Patient	Laterality	Enucleation / Therapy
RB-I	Unilateral	Right eye enucleated
RB-II	Unilateral	Left eye enucleated
RB-III	Bilateral	Right eye: chemotherapy + external beam radiotherapy Left eye enucleated
RB-IV	Unilateral	Left eye enucleated
RB-V	Bilateral	Right eye: enucleated Left eye: chemotherapy
RB-VI	Bilateral	Right eye: chemotherapy Left eye enucleated

4.2 DNA Extraction

Peripheral blood samples were collected from six patients along with a buccal swab from an infant with bilateral retinoblastoma. Genomic DNA was isolated from peripheral blood leukocytes in six cases according to standard protocol. The complete extraction and isolation of genomic DNA took approximately four days to yield proportionately higher concentration of pure DNA. Approximately 3 ml of blood was obtained from each patient with retinoblastoma and healthy controls yielded adequate DNAs to facilitate the entire study of *RB1* mutation screening. The quantity and purity of DNA obtained upon extraction is shown in Table 4.3.

However, the buccal cell sample yielded low quantity of DNA and thus found to be insufficient for subsequent molecular analysis. DNA isolated from oral buccal cells was subjected to sample spectra measurement and subsequently showed a high A260/230 ratio and a low A260/280 ratio (Table 4.3). Although blank measurement was performed on a clean pedestal, the DNA sample consistently showed high A260/230 ratio. Presumably, this was due to use of inappropriate solution for the blank measurement as the blank solution should be the same pH and of a similar ionic strength as the sample solution. In this study, water was used as the blank. Although using water for the blank measurement for samples dissolved in TE may result in low A260/230 ratio, the patient's DNA sample conversely exhibited high A260/230 ratio. Furthermore, when sample purity was assessed, a low A260/280 ratio indicated possible presence of contaminant(s) absorbing at 280 nm or less. Thus, mutational screening was not possible for this new-born baby with bilateral RB and hence was eliminated from the study.

Table 4.3 : Quantity and Measurement of Purity of Genomic DNA Extracted from Clinical Specimens of Patients and Parents

Sample ID	Sample Type	Nucleic Acid Concentration (ng/µl)	Absorbance (A)	
			260/280	260/230
RB-0	Buccal swab	25.7	1.71	42.14
RB-I	Peripheral blood	3857.5	1.82	2.24
RB-I-F	Peripheral blood	4019.7	1.85	2.28
RB-I-M	Peripheral blood	1300.4	1.80	2.12
RB-II	Peripheral blood	1866.6	1.81	1.96
RB-III	Peripheral blood	540.9	1.80	1.94
RB-III-F	Peripheral blood	1167.8	1.83	2.19
RB-III-M	Peripheral blood	1225.7	1.81	2.17
RB-IV	Peripheral blood	1257.7	1.88	2.55
RB-V	Peripheral blood	980.0	1.80	2.12
RB-V-F	Peripheral blood	840.9	1.85	1.99
RB-V-M	Peripheral blood	619.1	1.80	1.84
RB-VI	Peripheral blood	3356.1	1.95	2.58

F: Father; M: Mother.

4.3 Primer Map and PCR Amplification of *RB1* Gene

The Sequence Manipulation Suite which is a web-based computer program was used to examine the specificity of the *RB1* primers. When *RB1* DNA sequence along with a set of primer sequences were uploaded onto the analyser, Primer Show returned a textual map displaying the exact annealing positions of the primers and the corresponding exonic regions of *RB1*. The output (Primer Map) was used to determine the specificity of each primer pair for amplification of relative *RB1* exons. Appendix IV presents Primer Map which highlights the exons of *RB1* and annealing positions of corresponding forward and reverse primers.

For PCR amplification of *RB1*, primer sequences were adapted from Lohmann et al. (1994). The exon-specific forward and reverse primers were tagged with a common 5' tag sequence of eight nucleotides (GCGAATTC). Primer pairs specific for exon 5 and 24 respectively did not contain any universal tag. When primer mapping was attempted, it was not feasible for the program to anneal primer sequences with the tag. Hence, forward and reverse primers were uploaded onto Primer Show without tag sequence. Table 4.4 shows the list of *RB1* primers without common 5' tag sequence. As specified by Primer Map, minor modifications were made to primer oligonucleotides prior to PCR amplification, i.e., change or addition of a single nucleotide in the primer sequence. The highlighted nucleotides in the reverse primer sequences specific for exons 2, 3 and 18 respectively depict the modifications made as shown in Table 4.4.

The *RB1* DNA sequence consisted of 180,388 bases. The gene comprised 27 exons. When the integrity of primers was examined using Sequence Manipulation Suite, the reverse primer for exon 14 showed to be non-specific. The oligonucleotide found to anneal at two different sites in between exon 2 and exon 3 of *RB1* gene. Forward primer of exon 23 was found to be complementary with reverse primer of exon 22. However,

formation of self-dimer was not feasible in this study since each amplication reaction of exon 22 and exon 23 was conducted separately. All *RB1* exons were individually amplified using primers complementary to flanking intron sequences. The PCR-amplified products ranged in size from 179 to 586 bp and each included a flanking intron sequence.

Table 4.4 : *RB1* Primer Sequences

Exon	Forward/ Reverse Primer	Primer Sequence
Exon 1	1-F	5'GTGCGCGCGCGTCGTCCTCC3'
	1-R	5'GGCCCCTGGCGAGGACGGGTC3'
Exon 2	2-F	5'GTATGTACTGAATCAATTTG3'
	2-R	5'GAAGTTGTTTTTAAAATGAG3'
Exon 3	3-F	5'TAACATAGTATCCAGTGTGTG3'
	3-R	5'ATTTCCTTTTATGGCAGAGG3'
Exon 4	4-F	5'GAAATAACACAAATTTTTAAGG3'
	4-R	5'AGTGTAACCCTAATAAAATG3'
Exon 5	5-F	5'AGCATGAGAAAACTACTATG3'
	5-R	5'CCTAACTATCAAGATGTTTG3'
Exon 6	6-F	5'TTTCAGTGATACATTTTTCC3'
	6-R	5'AATTTAGTCCAAAGGAATGC3'
Exon 7	7-F	5'TCTCATACAAAGATCTG3'
	7-R	5'AATAAGCAACTGCTGA3'
Exon 8	8-F	5'ATTGTTCTTATCTAATTTACCAC3'
	8-R	5'TACATCTAAATCTACTTTAACTG3'
Exon 9	9-F	5'TGCATTGTTCAAGAGTCAAGAG3'
	9-R	5'AATTATCCTCCCTCCACAGTC3'
Exon 10	10-F	5'AAAGGATAATTGTCAGTGACT3'
	10-R	5'TACCTATATCAGTATCAAC3'
Exon 11	11-F	5'GAGACAACAGAAGCATTATAC3'
	11-R	5'TGAAACACTATAAAGCCA3'
Exon 12	12-F	5'ATTGCTTAACACATTTTC3'
	12-R	5'TTTGCCAAGATATTACAA3'
Exon 13	13-F	5'ATCCTCGACATTGATTTCTG3'
	13-R	5'TAGTACCACGAATTACAATG3'
Exon 14	14-F	5'TGATTTTCTAAAATAGCAGGCTC3'
	14-R	5'TTTTAGTAGAGACAGGGTTTCAC3'
Exon 15-16	15-F	5'AATGCTGACACAAATAAGGTTTC3'
	16-R	5'GATCTAAAATAAGCATTCCTTCTCC3'
Exon 17	17-F	5'CAAAAAAATACCTAGCTCAAG3'
	17-R	5'GTTAAGAAACACCTCTCACTAAC3'
Exon 18	18-F	5'AATTATGCTTACTAATGTGG3'
	18-R	5'AGTTTGAATGGTCAACATAAC3'
Exon 19	19-F	5'AACTTGAAATGAAGACTTTTCC3'
	19-R	5'TAGTTTCAGAGTCCATGCTC3'
Exon 20	20-F	5'GACTAATTTTTCTTATTCCCAC3'
	20-R	5'GAGGAGAGAAGGTGAAGTGC3'
Exon 21	21-F	5'CATGTAATAAAATTCTGACTAC3'
	21-R	5'CTATGTTATGTTATGGATATGG3'
Exon 22	22-F	5'CTTTATAATATGTGCTTCTTACCAG3'
	22-R	5'GTTTTGGTGGACCCATTACATTAG3'
Exon 23	23-F	5'ATGTAATGGGTCCACCAAAAC3'
	23-R	5'TTTACTACTTCCCTAAAGA3'
Exon 24	24-F	5'GTATTTATGCTCATCTCTGC3'
	24-R	5'ATGAGGTGTTTGAATAACTG3'
Exon 25	25-F	5'TTGAGGTTGCTAACTATGAAACAC3'
	25-R	5'TGGATTCCCCAGATGACCATC3'
Exon 26	26-F	5'ATCGAAAGCATCATAGTTAC3'
	26-R	5'GAAAAGACTTCTTGCAGTG3'
Exon 27	27-F	5'AATGCTGTTAACAGTTCTTC3'
	27-R	5'TGTGAGAGACAATGAATCC3'

4.4 Mutation Analysis by DNA Sequencing

Comprehensive investigation of whole coding region (27 exons) of the *RB1* gene by amplification and bidirectional sequencing in search for mutations in six patients with retinoblastoma revealed a total of two heterozygous mutations, corresponding to one mutation for each patient. The mutations were carried by a male and a female patient with bilateral retinoblastoma: RB-III and RB-V respectively. Nevertheless, these two mutations were an identical aberration detected in exon 23 of *RB1*. The summary of mutation screening and the details of mutations identified in patients RB-III and RB-V are listed in Table 4.5.

Table 4.5 : Summary of Mutation Analysis in Patients with Retinoblastoma

Patient	**Age/ Sex/ Laterality**	**Mutation**	**Occurrence**	**Location in *RB1***	**Consequence**
RB-I	2 yrs/ M/ Unilateral	Nil	-	-	-
RB-II	2 yrs/ M/ Unilateral	Nil	-	-	-
RB-III	2 yrs/ M/ Bilateral	g.162237C>T (R787X)	Constitutional	Exon 23	Termination
RB-IV	1 yr/ M/ Unilateral	Nil	-	-	-
RB-V	1 yr/ F/ Bilateral	g.162237C>T (R787X)	Constitutional	Exon 23	Termination
RB-VI	2 yrs/ F/ Bilateral	Nil	-	-	-

Age represents age at diagnosis. Occurrence in constitutional (blood leukocytes) cells is indicated. M: male patient and F: female patient.

The causative point mutation was a single base substitution in CGA codon, located in exon 23 of *RB1* gene. When *RB1* exon 23 forward sequence of patients, healthy control and reference (GenBank Accession No. L11910) were aligned, a C to T transition was observed, and thus changed CGA codon to TGA (Figure 4.1a). The mutation was confirmed when single base change was observed at the same corresponding location in the complementary reverse sequence (Figure 4.1b). Figure 4.2 shows electropherograms which shows the mutation spot in the *RB1* exon 23 of RB-III and RB-V with respect to wild-type sequence. The wild-type codon (CGA) encodes for arginine. In comparison with wild-type sequence, the mutant allele was found to show a single base substitution (CGA→TGA) which caused an alteration of the reading frame and gave rise to a premature stop codon (L11910: g.162237C > T; R787X). The alteration was a nonsense mutation, located 34 bp from the 5' end of exon 23 and changed codon 787 encoding arginine (CGA) to a stop codon (TGA). Hence, this mutation resulted in a premature termination at amino acid 786.

Mutation was identified in two unrelated children with bilateral retinoblastoma: RB-III and RB-V. RB-I, RB-II, RB-IV and RB-VI did not show any mutation in exonic region of *RB1*. The first three patients had unilateral retinoblastoma. Although RB-VI had bilateral retinoblastoma, DNA sequence analysis ruled out the possibility of a mutation in coding region of *RB1*. Mutation analysis which scanned the coding parts of exons but not the promoter region and 3' UTR provided no evidence for a second mutation.

When translation of exon 23 of reference, control and patients were aligned in Clustal Omega, the arginine residue was observed to be substituted by a stop codon, suggesting premature termination of translation (Figure 4.3).

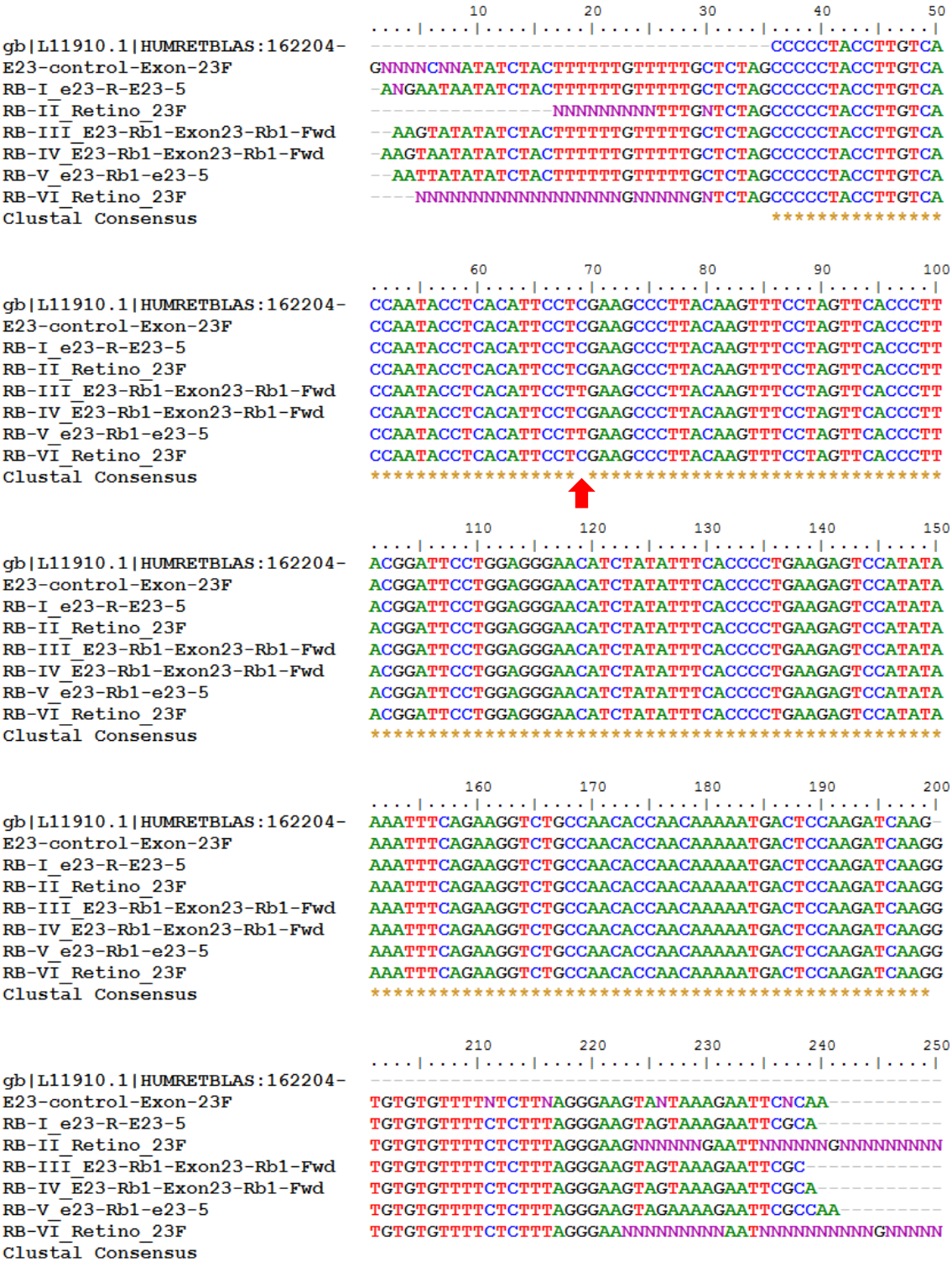

Figure 4.1a : Clustal Omega Alignment of *RB1* Exon 23 Forward Sequences of Normal Control and Patients. The Arrow Denotes a C to T Transition at Base 34 from 5' end of the Exon Sequence, Altering a CGA Codon (Arginine) to TGA (Stop Codon) in RB-III and RB-V.

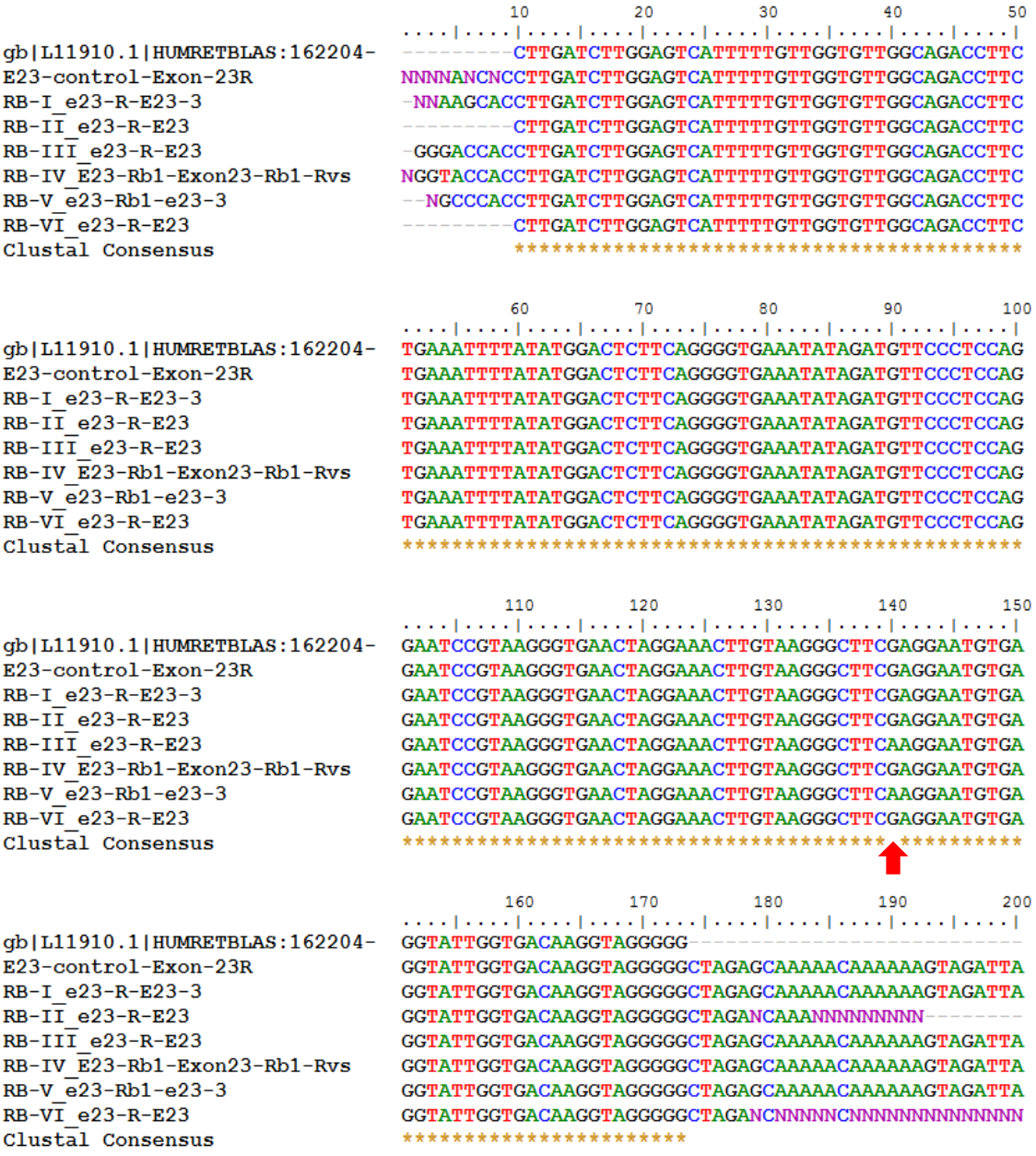

Figure 4.1b : Clustal Omega Alignment of *RB1* exon 23 Reverse Sequences (in 5' to 3' Order) of Normal control and Patients. The Arrow Denotes a Complementary Nucleotide Change Corresponding to Transition Mutation in RB-III and RB-V.

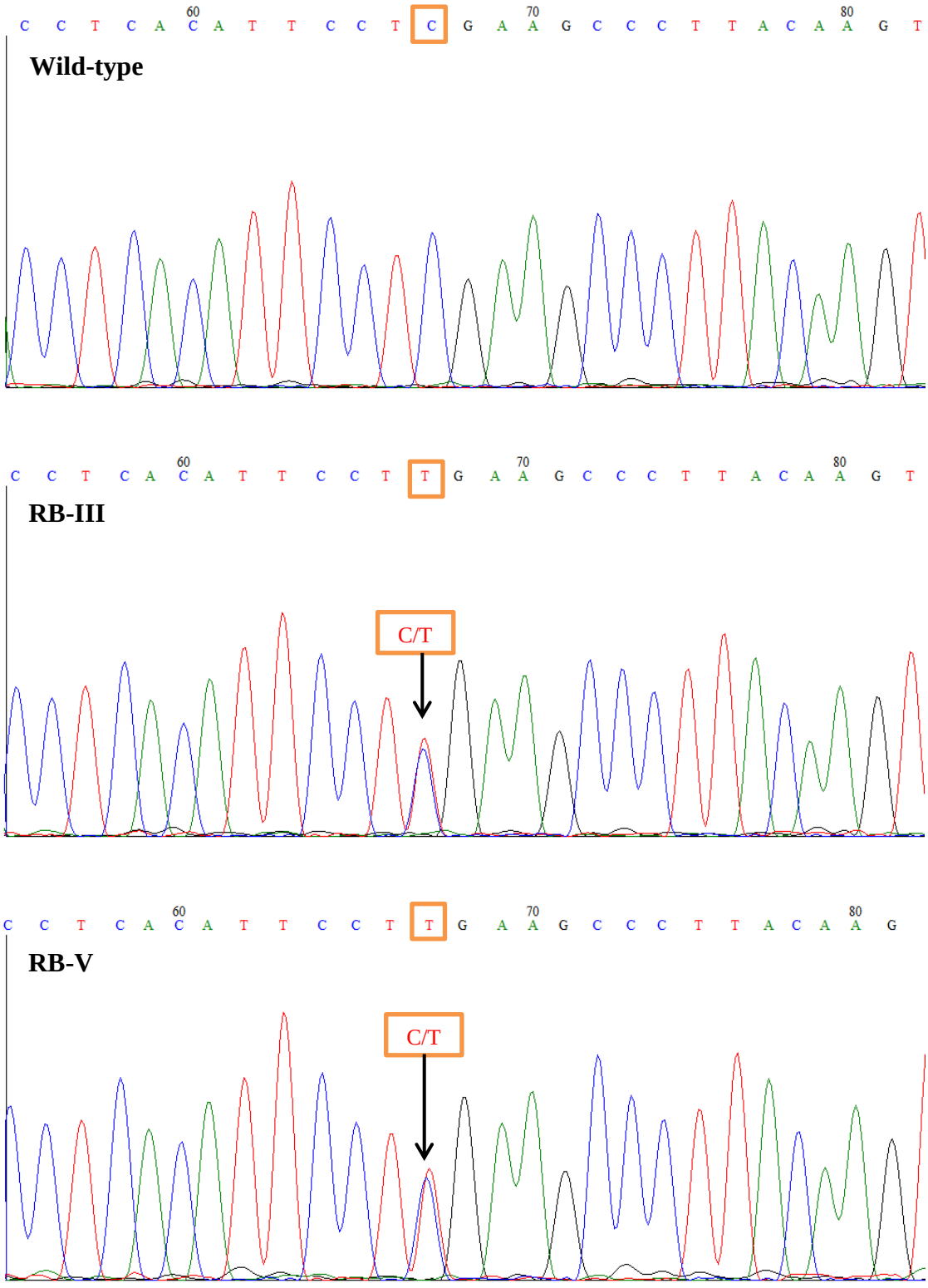

Figure 4.2 : Nucleotide Sequence of a Fragment of *RB1* exon 23. Nonsense Mutation g.162237C>T Changes the Wild-Type Codon CGA for Mutant TGA (Stop Codon) in RB-III and RB-V.

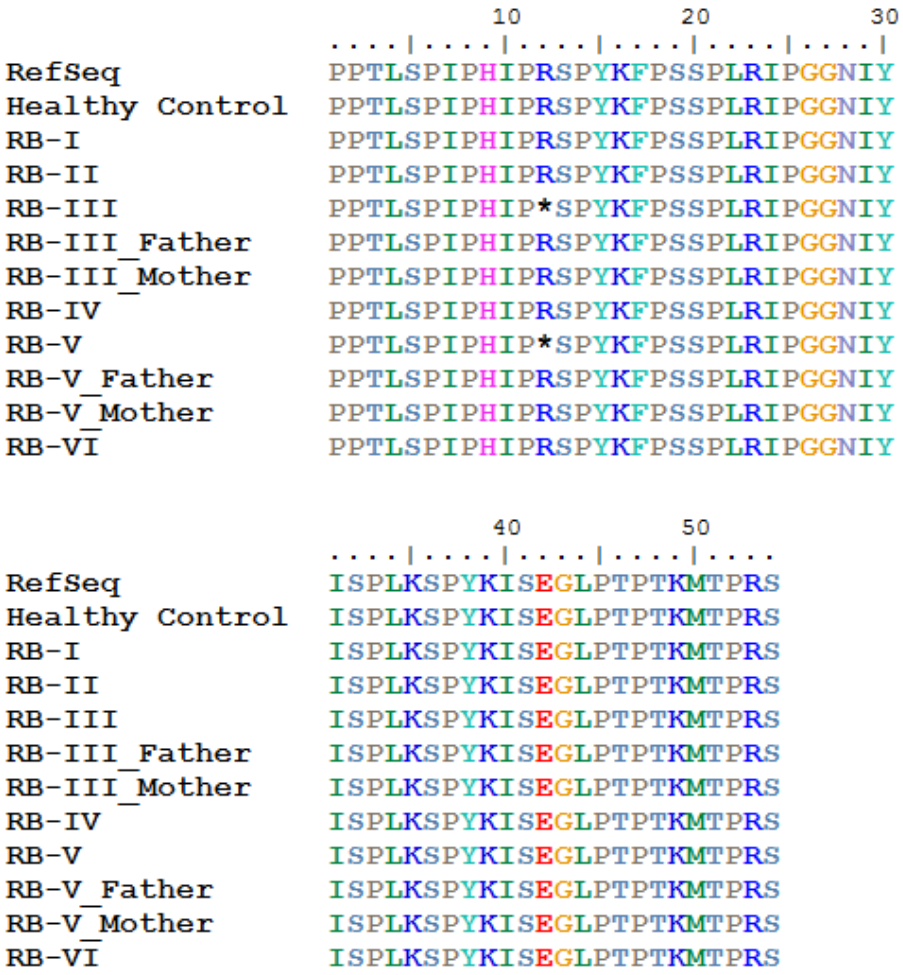

Figure 4.3 : Clustal Omega Protein Sequence Alignment. (*) Denotes a Stop Codon Replacing Arginine (R), Suggestive of Premature Termination of Translation as a result of g.162237C>T Point Mutation.

Although the germinal mutations in *RB1* were identical, both RB-III and RB-V were unrelated individuals. The patients shared common characteristics such as ethnicity and laterality of Rb. Furthermore, both patients did not possess family history of retinoblastoma. The patients were the first in the family to be diagnosed with retinoblastoma. The aforementioned findings proved that the predisposing mutation was constitutional in RB-III and RB-V.

Figure 4.4 and 4.5 show the vertical arrangements of electropherograms, demonstrating absence of mutation g.162237C>T in *RB1* gene of parents. Based on the sequence analysis, the mutation was not observed in either parents of the patient. Therefore, it was deduced to be *de novo* germ-line mutation.

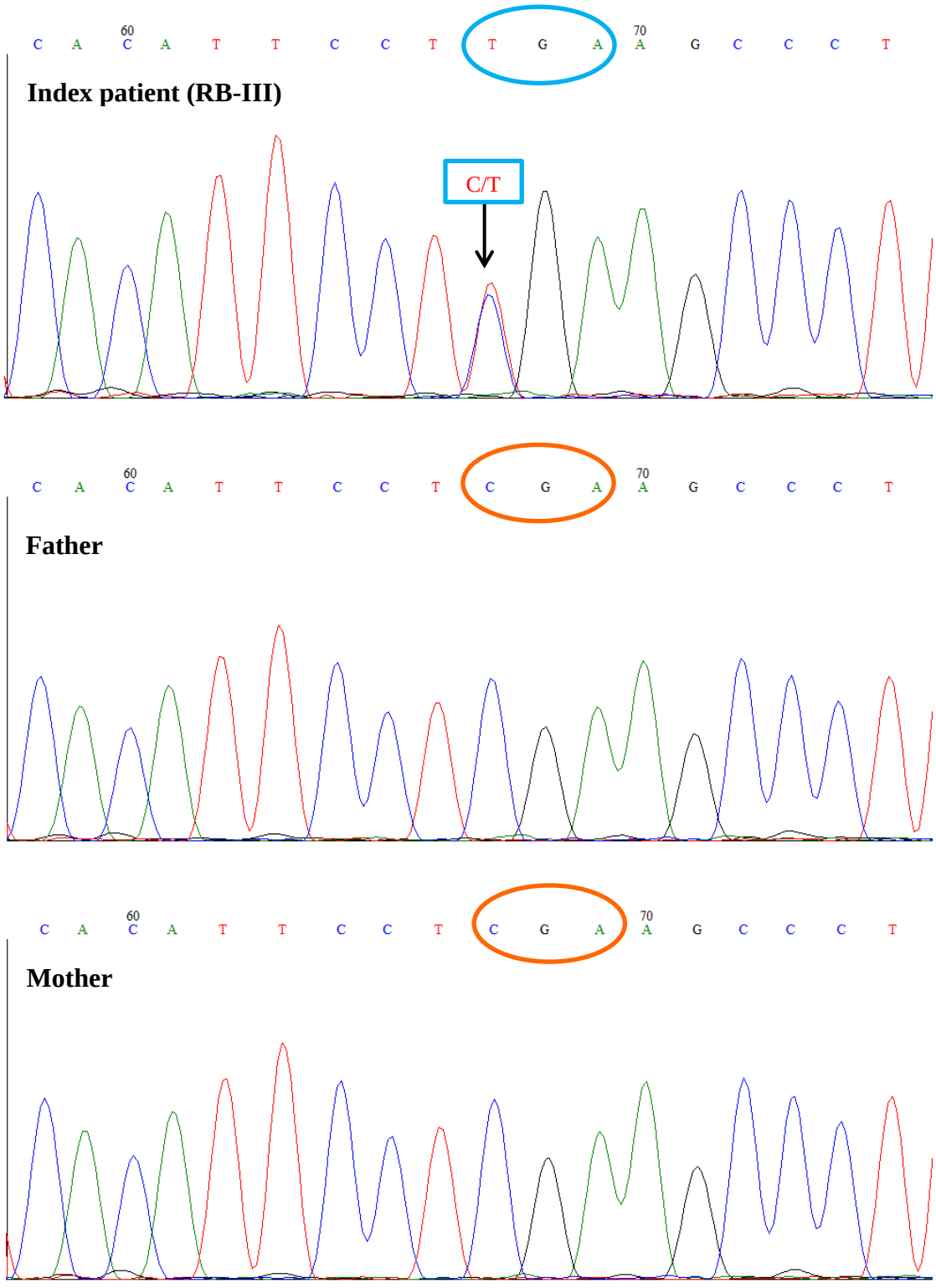

Figure 4.4 : *RB1* Exon 23 Transition Mutation in Blood DNA of Bilateral Retinoblastoma Patient, RB-III. *Top*, *RB1* Exon 23 Sequence of Index Patient Using Forward Primer, Indicating a C > T Transition. *Centre & Bottom*, *RB1* Exon 23 Sequence of Wild-Type Father and Wild-Type Mother Using Forward Primer.

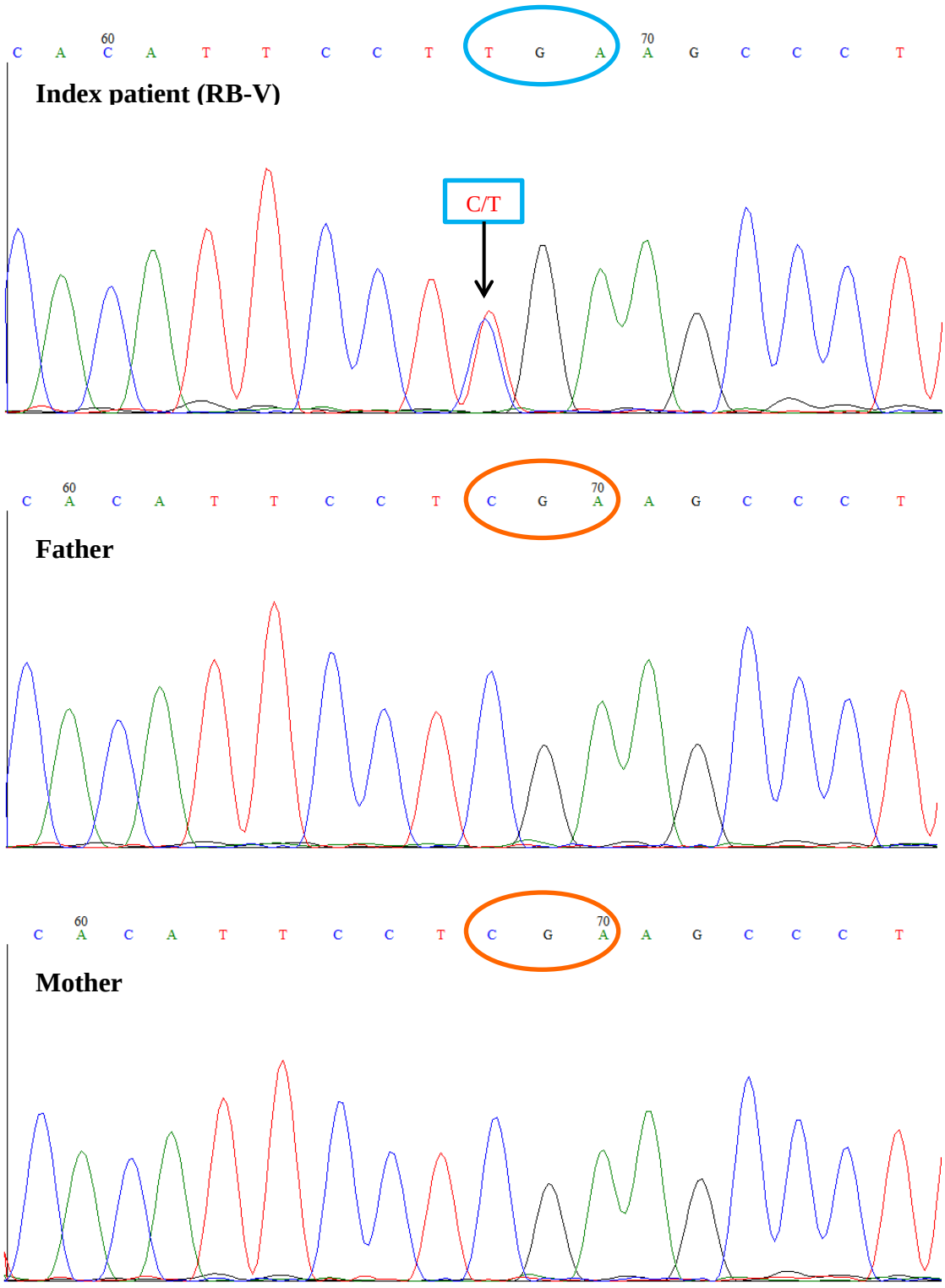

Figure 4.5 : *RB1* Exon 23 Transition Mutation in Blood DNA of Bilateral Retinoblastoma Patient, RB-V. *Top*, *RB1* exon 23 Sequence of Index Patient Using Forward Primer, Indicating a C > T Transition. *Centre & Bottom*, *RB1* Exon 23 Sequence of Wild-Type Father and Wild-Type Mother Using Forward Primer.

CHAPTER 5

DISCUSSION

RB is known as the retinoblastoma protein and has been identified as a vital tumour suppressor. In almost all human cancers, RB is believed to be directly or indirectly inactivated. The loss of RB function is the initiating event in the formation of familial retinal tumours as well as sporadic cases (Knudson, 1971). Although five to eight percent of deletion mutations in the *RB1* gene can be detected cytogenetically, nearly all submicroscopic mutations are identified through molecular approaches. However, the complexity of *RB1* gene along with the absence of mutation hotspots makes mutation screening a difficult task despite the availability of various modern molecular tools (Mamatha et al., 2006). Therefore, a comprehensive screening of 27 exons of *RB1* for mutations was performed to address the non-preferential localisation of mutation in the gene (Szijan et al., 1995).

RB1 gene exhibits a high degree of mutational heterogeneity as evidenced by the existence of over 900 reported mutations till 2009 (Parsam et al., 2009). Besides polymorphisms, any alteration discovered in the *RB1* coding region is predicted to be a predisposing mutation (Schubert et al., 1997). In response to this, many genetic studies around the world have reported a wide spectrum of *RB1* mutations in patients with retinoblastoma that of prognostic value. However, there are no studies to date that have identified nor described *RB1* mutations in a Malaysian cohort of Rb patients. As a first step toward detection of mutations, blood specimens from patients diagnosed with retinoblastoma at UMMC were screened to elucidate the predisposing mutations. The

results of the peripheral blood screen and *in silico* analysis had corroborated the presence of constitutional mutations in the *RB1* gene of two patients with bilateral retinoblastoma.

5.1 Detection of an Identical Germ-line Mutation in the *RB1* Gene of Two Patients with Bilateral Retinoblastoma

Of the 46 arginine codons in the retinoblastoma gene, 14 are encoded by CGA or CGG and 12 are targets for recurrent mutations within the open reading frame (Mamatha et al., 2006; Richter et al., 2003). The inclination of cytosine residue for deamination accounts for recurrent transitions at CpG sites (Kumaramanickavel et al., 2003). More than 50% of mutations reported in CGA codons are located in exon 5, 8, 10 to 15, 17, 18, 23 and 27 (Mamatha et al., 2006). In this study, a C > T base change which converted CGA (arginine) to TGA (stop codon) was observed in exon 23 of the *RB1* gene. Furthermore, Cowell et al. (1994) and Parsam et al. (2009) have described similar mutations at CpG dinucleotides. Representing a large proportion of described mutations in an *RB1* mutation database, C→T common transition is likely to result from 5-methyl-cytosine deamination in dinucleotide CG (Babenko et al., 2002). The data of this experiment was in consistence with various reports throughout the world as no single mutation was spotted within terminal exons of *RB1* though exons 26 and 27 have two CGA codons. Mutations present within open reading frame of *RB1* except the terminal exons are referred as oncogenic mutations. (Alvarez, 2008; Lohmann et al., 1996). Thus, being coherent to exon 23, g.162237C>T is a possible oncogenic mutation in the *RB1* gene of RB-III and RB-V. The mutation has been previously reported as a recurrent mutation in children with retinoblastoma (Parsam et al., 2009).

The causative point mutation was identified in the proband and not in the parents, explaining the formation of sporadic form of the disease in these two cases. Particularly, the absence of family history and the absence of mutations in the parents blood DNA strongly suggested that the mutations should have occurred *de novo* in the patients. Furthermore, the results obtained in this study were in agreement with Parsam et al.'s (2009) report that a large number of patients with bilateral Rb have sporadic form of the disease as a result of a *de novo* mutation that arises in the germ-line or embryo.

Predisposition to retinoblastoma is characterised by germ-line mutation of one allele of the retinoblastoma gene. In retrospect, hereditary Rb is caused by a heterozygous mutation in the *RB1* gene (Knudson, 1971, Vogel, 1979). This notion was substantiated by the results of this experiment which manifested the mutations in heterozygous state. The findings agreed with statement that most patients with hereditary retinoblastoma or heterozygous for mutations that result in a premature termination codon develop bilateral disease (Dundar et al., 2001; Lohmann, 1999). Though all three children with bilateral Rb did not demonstrate familial transmission of the disease, *de novo* germ-line mutations were only discovered in two patients, RB-III and RB-V. Based on the observation, the germ-line mutation was not inherited from their parents. Thus, RB-III and RB-V should have acquired the mutation during gametogenesis or gestation. In view of this, the germ-line mutation was deduced as a hereditary *de novo* mutation (Dehainault et al., 2007).

Findings of this mutation screen revealed a total of two small mutations, both being identical single base substitution present in two patients of Chinese ethnicity and with bilateral Rb. Notably, this finding was in consonance with the report by Zhang and colleagues (2011) in which the nature of *RB1* germ-line mutations in Chinese was disclosed. Identical C→T transition affecting codon 787 was detected as a heterozygous germ-line mutation in the leukocyte DNA of a patient in a study conducted on a cohort

of eight Chinese patients. Their findings summarized that alterations involving shorter base pairs are common in the retinoblastoma gene of Chinese patients. However, frequency and the commonness of the identified mutation in Chinese ethnicity were unknown and incoherent with small sample size for further discussion. Besides, association of the mutation with differences that exist in genetic background between various ethnic groups could not be ruled out as all patients represented single ethnicity.

5.2 Parental Origin of Mutations

The genotypic data indicated that the father and mother of RB-III were C/C homozygotes at g.162237. Similarly, both father and mother of RB-V had also exhibited homozygous state at *RB1*'s genomic position 162237. Thus, g.162237C>T was not constitutional in the proband's progenitors. The mutation could have occurred either in the maternal or the paternal RB1 allele, as a *de novo* event in germ-lines or in the zygote.

5.3 Association of Identified Germ-line Mutation with Laterality of the Disease

Interestingly, the point mutation was found as the only alteration in two patients with retinoblastoma. Both patients had bilateral retinal tumours. This finding was consistent with Dommering et al. (2012), Lohmann et al. (1996), Orsouw et al. (1996) and Parsam et al. (2009), in which the same alteration was identified as a recurrent mutation in patients with bilateral retinoblastoma. Conversely, another finding reported the same mutation which caused C2359T change in the nucleotide sequence of a patient with sporadic unilateral RB (Babenko et al., 2002). In view of previous reports and observation of this study, g.162237C>T is associated with the development of retinoblastoma (Babenko et al., 2002; Cowell et al., 1994). Being a constitutional mutation, it is the only alteration that can be linked to bilateral retinoblastoma in RB-III

and RB-V. As deduced by Kumaramanickavel et al. (2003) and in consistence with this observation, bilateral retinoblastoma was speculated to be the consequence of allele with premature termination of coding sequence.

5.4 Consequence of Mutation g.162237C>T Found in *RB1* Gene

The predisposing mutation identified as g.162237C>T in two children with bilateral retinoblastoma was a previously reported and recurrent mutation. Cs in this position was observed to be highly conserved, being part of the consensus reference. This was confirmed by analysis of Genbank reference data and healthy control sequences. The loss-of-function mutation had consequently resulted in a stop codon instead of an amino acid (Arginine) by replacing CGA with TGA. Thus, the mutation had been found to negatively affect translation by bringing to a halt with premature termination. With respect to this, the mutation is speculated to have pathogenic clinical significance (Lohmann et al., 1999).

The identical mutation in the *RB1* gene is assumed to have led to similar phenotype in both unrelated patients. Cowell and Bia (1998) have indicated that a large number of mutations identified in patients with severe phenotype subsequently lead to premature termination. The stop codon arises either as a result of nonsense mutation or frameshift mutation. Parsam et al. (2009) had identified the consequence of the single base pair mutation, g.162237C>T, as being a nonsense mutation. The termination codon leads to a truncated non-functional retinoblastoma protein (Crosby et al., 2009). This protein is unable to regulate the cell cycle (Mamatha et al., 2006).

According to Henley et al. (2010), a cancer derived mutation in exon 23 of *RB1* gene may specifically disrupt LXCXE dependent interactions in cell cycle regulation. LXCXE is a peptide motif used by a number of cellular proteins, particularly, chromatin regulators such as histone deacetylases and methyltransferases to interact with pRb. The C-terminal region of pRb which regulate access to the LXCXE binding cleft is encoded by exon 23. Henley and colleagues (2010) published evidence which explained that mutation in this region compromises pRb's ability in cell cycle regulation even when it is heterozygous with a wild type copy of the *RB1* gene.

Sufficient synthesis of functional proteins is important in preventing tumorigenicity in the developing retinal cells (Cowell & Bia, 1998). A full length retinoblastoma protein (pRb) has several functional domains, with pocket domain extending from residue 379 to 792 and larger domain extending from residue 379 to 928. The larger domain is responsible for growth suppression function. In short, the functional domains are observed to be disrupted in truncated proteins that are synthesized as a result of mutation in the *RB1* gene. Hence, an oncogenic mutation may therefore cause initiation of tumour development (Szijan et al., 1995).

5.5 Possible Causes for Absence of Germ-line Mutations in RB-I, RB-II, RB-IV and RB-VI

Many children with heritable form of retinoblastoma do not show distinct clinical signs or family history suggestive of the disease (Chen et al. 2003). This is primarily because unilateral phenotype is observed in both sporadic retinoblastoma and in one-third of patients with heritable retinoblastoma. Thus, this study concomitantly screened both unilateral and bilateral patients for germ-line *RB1* mutations indicative of heritable disease. However, none of the patients with unilateral retinoblastoma showed constitutional mutation in the retinoblastoma gene. This observation supported the idea that only 15 to 17% of unilateral retinoblastomas have a germ-line origin and more than 85% of unilaterally affected are patients with non-hereditary Rb (Leone et al., 2003). Hence, this finding was consistent with the hypothesis that non-heritable and sporadic cases of retinoblastoma are always unilateral. Owing to post-conceptional accumulation of somatic mutations in both alleles of the retinoblastoma gene, these patients were assumed to have developed sporadic non-heritable retinoblastoma (Chen et al., 2003). In this case, the first and second *RB1* mutations can be detected in the tumour only. Nonetheless, analysis of DNA from tumour tissue was not possible and therefore presence of somatic (non-heritable) mutations in the *RB1* could not be proved, irrespective of lack of family history, absence of mutation and unilateral phenotype.

A mutation might not be detectable using conventional mutation analysis technique owing to its location in other than coding region of the gene. Concluding from past findings, intronic alterations are observed in 12% of patients while nucleotide changes in the promoter region accounts for 2% of patients (Mamatha et al., 2006). It is believed that deep intronic changes could render negative impact on normal splicing and thus be responsible for patient's disease (Dehainault et al., 2007). In patient RB-V, mutation might be located deep within the intronic region or was not spotted in blood DNA due

to mosaicism. According to Quah (2005), mosaicism is possible when a mutation arise in the retinoblastoma at some point during embryogenesis or post zygotic event. As an implication of mosaicism, the bilaterally affected individual may not be a germ-line carrier. Since the retinoblastoma in an individual with genetic mosaicism may be unilateral, unifocal, multifocal or bilateral, the retrospective screening of parents of unilaterally affected patients is warranted.

5.6 Molecular Testing by PCR-Sequencing

Close to 50% of the offspring of an affected individual are expected to develop retinoblastoma (Schubert et al., 1997). This is because retinoblastoma is generally transmitted to successive generations in an autosomal dominant fashion. This study had confirmed the presence of predisposing germ-line mutations in the *RB1* gene and thus heritable form of the disease in RB-III and RB-V by employing molecular approaches. Of six patients, the presence of germ-line mutations in two patients with bilateral retinoblastoma was corroborated by uniplex PCR and bidirectional sequencing.

RB1 gene is a relatively long gene that comprises 27 exons. Thus, the amplification of target sequences of *RB1* gene in this study incorporated a total of 189 individual uniplex PCR reactions [(six patients + one control) x 27]. The figure does not include the number of PCR reactions that were repeated when gel analysis showed PCR contamination or multiple bands. Apart from being time-consuming, the approach was also found to be limited by sample quantity and not economical as expected. More reagents such as PCR buffer, primers, dNTPs and *Taq* DNA polymerase enzyme were consumed when target sequences were amplified separately at different annealing temperatures. In response to similar finding, Mamatha et al. (2006) recommended uPCR strategy for mutational screening of small genes or genes with mutation hotspots. At UMMC's Paediatric Oncology Department, on an average two patients with

retinoblastoma are seen in a year. Rapid DNA based diagnostic services are thus not necessarily required unless there's a need for sensitivity of detection and considerable saving of time, effort and reagents.

Lohmann et al. (1996) and Parsam et al. (2009) reported point mutation detection rate of 80% to 90% when a similar approach by using a combination of PCR and sequencing was performed. However, in this molecular study the mutation detection rate achieved in patients with unilateral Rb was 0/3 (0%) and in patients with bilateral Rb was 67%. Overall, the mutation detection rates in sporadic cases of retinoblastoma were found to be relatively low, similar to finding by Lohmann et al. (1996). The low mutation detection rate in patients with bilateral retinoblastoma reflected the difficulty of screening a large gene with extensive mutational heterogeneity (Lohmann et al., 1996).

5.7 Significance of Mutation Screening for Genetic Counselling and Patients Management

Friend et al. (1986) stated that the risk of osteosarcoma development increases when loss of *RB* occurs in children and teenagers. Additionally, in more than 90% cases of human small-cell lung carcinoma (a lung cancer subtype) *RB* is found to be inactivated. Analysis of blood DNA of patients with retinoblastoma aided in the discovery of germinal mutation, which confirmed the loss of heterozygosity of *RB1*. The defective *RB1* is inherent in all somatic cells which consequently increases the risk of tumorigenesis in other organs and tissues. In considering this, the identification of germinal mutation in patients RB-III and RB-V also necessitates molecular screening of *RB1* in probands' relatives.

Studies have correlated the incidences of second malignancies in survivors with predisposing mutations in *RB1* with earlier treatment of retinoblastoma (Cowell, 1994). In particular, radiation therapy enhances the risk of second cancer in the irradiated field (Dryja et al., 1986). RB-III had received radiation therapy as part of his treatment for right eye retinoblastoma. Having a predisposing mutation in the *RB1* gene, RB-III possesses high chances for development of secondary tumours in adult life. Conversely, RB-V presumed to have lesser risks as many reports mention that there's no increased risk for second cancers from chemotherapy (Nale, 2009). Furthermore, the validation of constitutional origin of mutations in patients RB-III and RB-V allowed their inclusion in a high risk group, in whom close monitoring is recommended because they are particularly more prone to develop secondary tumours in adult life when compared to non-carriers (Draper et al., 1986).

Mutation analysis of both parents of RB-III and RB-V showed only one person was affected in their respective family, indicative of simplex retinoblastoma case. Index patient were predominantly presented with multifocal retinoblastoma. Presence of germ-line mutation in the *RB1* of these individuals indicated the risk to each offspring as 50% for being a carrier and 45% for developing the disease. Furthermore, in the event of two unaffected parents having one child with retinoblastoma, i.e., RB-III and RB-V, the risk of second child in the family developing retinal tumour is about 5%. Nonetheless, the risks were slightly higher for the patient's siblings since RB-III and RB-V had multifocal disease (Field et al., 2007; Quah, 2005).

CHAPTER 6

Conclusion

Realising the significance of identification of a mutation in the *RB1* gene in sporadic cases of retinoblastoma for accurate genetic counselling (Shimizu et al., 1994), a simple molecular approach primarily consisting of PCR and sequencing was carried out to screen for *RB1* mutations in six patients with sporadic retinoblastoma – three bilateral patients and three unilateral patients. The entire coding region of *RB1* gene was screened for mutations by comparison with GenBank reference data (L11910) and normal healthy control. An identical oncogenic mutation, g.162237C>T (R787X) was discovered in two bilaterally affected patients. The sporadic development of heritable retinoblastoma in these children is caused by a *de novo* germ-line mutation in the *RB1* gene. The nonsense mutation found in CGA codon located within exon 23 of *RB1* gene reportedly truncates the normal retinoblastoma protein. However, predisposing germ-line mutation was not identified in a bilaterally and three unilaterally affected patients.

A small proportion of patients may have a heritable mutation despite the lack of family members with retinoblastoma (Chen et al., 2003). Heritable retinoblastoma is associated with a predisposition to develop other malignancies in later life. Moreover, prior treatment and/or genetic susceptibility of *RB1* enhance the risk for the development of second malignancy in patients with hereditary Rb (Pauser & Grimm, 2008). Thus, identification of *RB1* germ-line mutation signifying heritable form of retinoblastoma was of prognostic value for providing precise risk prediction and valuable genetic counselling to affected individuals and their families. The findings of this experiment suggest that all sporadic and bilaterally affected cases must be considered as carriers of

germ-line mutations and thus subject them to mutational analysis. Testing unilaterally affected patients allows detection of possible germ-line mutation. The finding of this molecular analysis emphasized the importance of mutation screening for predictive diagnosis in families with sporadic bilateral retinoblastoma. Identification of distinct germ-line mutations clarified the risk of retinoblastoma for all family members of bilaterally affected RB-III and RB-V.

6.1 Suggestions for Future Work

Though Malays represent the largest ethnic group in Malaysia, all the subjects involved in this study were Chinese patients. Amongst, a reported and recurrent mutation was spotted in two patients of opposite genders. However, it is too soon to conclude that the identical oncogenic mutation detected in two patients is a common recurrent mutation in Chinese population. Based on the observation, it was impossible to examine adequate number of Rb patients due to rare occurrence of the disease and incomplete local survey system (Yusof et al., 2010). However, this could be rectified by conducting a multicentre study, by prolonging the duration of the study and recalling previously treated patients. A larger sample size would be preferred to obtain reliable estimate of mutation detection rate and to describe the wide spectrum of *RB1* mutations in Malaysian population.

The large size of the gene and the lack of mutation hotspots necessitate the development of rapid DNA based diagnostic procedures such as multiplex PCR (Orsouw et al., 1996). Unlike uPCR, multiple target sequences are simultaneously amplified under identical conditions in a single reaction in mPCR. Therefore, this strategy is favoured over uPCR because it is believed to be time and cost effective when employed in mutation screen of *RB1* gene (Mamatha et al., 2006). Furthermore, multiplexing would

be an effective and economical approach as being more robust and less time-consuming in molecular testing (Parsam et al., 2003).

Of particular interest, a DNA genotyping technology known as next-generation sequencing would offer an alternative to expensive and traditional Sanger sequencing method. This new technology enables sequencing in parallel, and hence a large number of DNA fragments can be simultaneously sequenced. Since hundreds of thousands or millions of fragments are sequenced concurrently, most next-generation sequencing techniques such as pyrosequencing prove to be rapid technologies than traditional Sanger sequencing method (Pierce, 2012). Hence, time-saving and cost-effective next-generation sequencing method is favoured over Sanger sequencing for mutation screening when large gene such as *RB1* with presumably many target sequences is involved.

The main obstacles to achieve competent detection rate of mutations in the *RB1* were the large size of the gene, the presence of mosaicism and possibility of mutations within non-coding regions that were not screened. Molecular analysis at cDNA level may function as an adjunct when a mutation is not detected by routine DNA analysis (Parsam et al., 2011). Furthermore, deep intronic mutation in the retinoblastoma gene shall be investigated at the cDNA level, especially in cases of hereditary retinoblastoma or when an expected mutation is not detected by classical approaches (Dehainault et al., 2007). On the other hand, identification of *RB1* germ-line mutations can be initiated by screening CGA codons first as almost 50% of the reported mutations predominantly found in these regions. The remaining exons of *RB1* gene can be screened for alterations if no mutation detected in the 12 CGA codons.

The experiment was solely designed to investigate peripheral blood DNA to identify germinal mutation in the *RB1* gene of patients with different laterality of retinoblastoma. Furthermore, samples were not selected based on their presumed heritability prior screening, and hence both unilateral and bilateral Rb patients were included in the search for constitutional mutations. However, the observation indicated that it was not feasible to detect mutations with the analysis of peripheral blood DNA alone in patients with unilateral retinoblastoma. Hence, it is highly recommended that tumour material is first tested for *RB1* mutations in unilateral retinoblastoma cases, and if there any, peripheral blood DNAs of patient and patient's parents are considered for retrospective study in order to confirm hereditary retinoblastoma (Braggio et al., 2012; Field et al., 2007). The finding of this molecular analysis supports the idea that patient's leukocyte DNA may be analysed in cases of bilateral retinoblastoma when surgery material is not available (Babenko et al., 2002).

The peripheral blood DNA extraction method employed in this work showed to be time-consuming and laborious. The whole process of DNA extraction took approximately four days to yield protein-free genomic DNA. In addition, this procedure usually incorporates hazardous organic solvents (phenol-chloroform) and also involves multiple wash steps necessary for concentrating contaminant-free DNA. A large number of wash steps leads to low DNA recovery from blood sample. As described by some principal molecular biologists, traditional method such as phenol chloroform extraction can also be replaced by simpler, nontoxic and inexpensive method for rapid isolation of DNA from whole blood (Ciulla *et al*., 1988; Salazar *et al*., 1998). It is learnt that similar DNA yields could be obtained through different methods of extraction which are simple and reliable. Therefore, a better method of DNA extraction should be employed to facilitate fast and efficient processing of blood samples from vast number of patients.