CHAPTER 3

METHODOLOGY

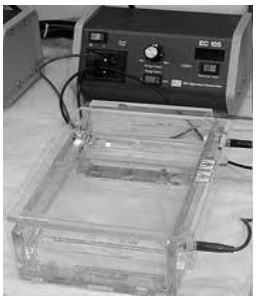
3.1 Study Design

The aim of this study was to screen for constitutional mutations in the *RB1* gene of patients with various forms of retinoblastoma. Since the incidence of Rb is relatively rare, a longer period was required to collect a reasonable number of clinical specimens for this study. Figure 3.1 illustrates the flow of techniques used to identify *RB1* mutations in the sample cohort using facilities at Paediatric Oncology Lab in University Malaya Medical Centre (UMMC) and at the Molecular Genetics Lab at the Institute of Postgraduate Studies (IPS) of University of Malaya.

This study only included patients with no family history of retinoblastoma, who were referred to University Malaya Medical Centre (as being the main local referral centre besides Hospital Kuala Lumpur and Universiti Kebangsaan Malaysia Medical Centre) from April 2011 to June 2012. The study focused on investigating germ line mutations in *RB1* gene of patients with sporadic retinoblastoma which is presumably a rare occurrence and concomitantly accounted for limited number of patients. In order to confirm the *de novo* status of an identified predisposing mutation in patient with retinoblastoma, blood specimens from respective parents were screened for *RB1* mutation. Upon identification of a proband, it was recommended to the parents of the affected patients to subject their relatives to *RB1* mutation screening. As such, this study presents report on identification of constitutional mutations in the *RB1* gene of six Malaysian patients with retinoblastoma.

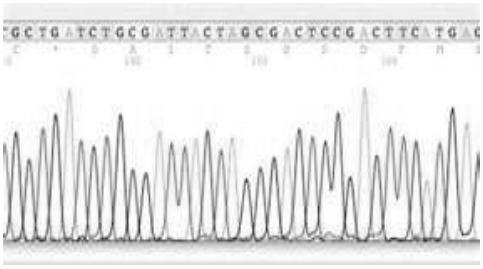
Blood sample collected from patients diagnosed with RB

DNA extraction and quantification



Agarose gel electrophoresis

PCR amplification and purification



DNA Sequencing

Identification of *RB1* gene mutation

Figure 3.1 : Schematic Representation of Experimental Design

3.2 Sampling

3.2.1 Patients

This study was carried out on a cohort of seven patients diagnosed with retinoblastoma: two unilaterally and four bilaterally affected children referred to the Paediatrics Oncology Unit of UMMC; and one adult patient who was a survivor of unilateral Rb. Peripheral blood samples were obtained from all the patients except from one affected newborn baby who was admitted to the Paediatric Oncology Ward of UMMC (main referral centre along with HKL and UKMMC) between April 2011 and June 2012. Buccal swab from the newborn baby with bilateral Rb resulted in poor DNA recovery which in turn made subsequent molecular analysis impossible. Hence, the study was conducted on only six patients. Peripheral blood samples were also successfully obtained from both parents of a child with unilateral Rb and two children with bilateral Rb to confirm the *de novo* status of identified *RB1* mutation.

The affected children were aged below five years old while the adult patient was 34 years old at study entry. Clinical features at initial presentation include proptosis, strabismus, secondary glaucoma, orbital inflammation or metastasis and leukocoria being the most distinct feature. The diagnosis of retinoblastoma was confirmed upon imaging and histopathology. All cases were sporadic as there was no known case of retinoblastoma in any of the patient's family history.

Approval from UMMC Medical Ethics Committee was sought prior to commencing this study. Written consent for blood collection and for subsequent molecular screening was obtained from all patients' parents. Details of patients and samples received prior to *RB1* mutation analysis is shown in Table 3.1.

Table 3.1 : Details of Rb Patients and Clinical Specimen Received

Patient Identification	Gender	Ethnicity	Age at Diagnosis	Sample Type	Parents' PB Samples Received?
RB-0	Female	Malay	3 months	Buccal Swab	No
RB-I	Male	Chinese	2 year +	PB	Yes
RB-II	Male	Chinese	1 year +	PB	No
RB-III	Male	Chinese	2 years +	PB	Yes
RB-IV	Male	Chinese	1 year +	PB	No
RB-V	Female	Chinese	1 year +	PB	Yes
RB-VI	Female	Chinese	2 years +	PB	No

*Highlighted patient was omitted from laboratory investigation due to poor quantity of DNA recovered from buccal swab and due to the parents' refusal of a venepuncture.

3.2.2 Clinical Specimen Collection

Peripheral venous blood as a source of DNA was obtained from all RB patients. To confirm whether the mutation was constitutional in origin, peripheral blood samples were preferably collected from both parents of the patients. Unfortunately, collection of parents' peripheral blood was not possible for three patients because they were uncontactable at the time of investigation. Upon sample collection, basic details of patients such as name, date of birth and identification number along with sample collection date and DNA extraction date was recorded.

Disinfection was performed at entry site with 70% alcohol swab prior to intravenous access for blood sampling. About 3 ml of peripheral blood was drawn from each patient and collected in an EDTA tube. Blood samples were also obtained from two healthy research colleagues of Malay and Chinese ethnicity respectively to be referred to as negative controls. Buccal swab was collected easily and painlessly from an affected newborn infant who refused to undergo invasive procedure. Before buccal swabbing, the child was abstained from milk feed for about 1 hour. Buccal swabbing was done five to six times by simply rubbing the internals of each side of the cheek using a sterile cotton swab. After collection, the buccal cell sample was not immediately processed but stored at 4°C until DNA extraction to avoid possible degradation by bacteria or nucleases at room temperature.

3.3 DNA Extraction

3.3.1 Peripheral Blood DNA Extraction

Genomic DNA was isolated from buffy coat or leucocytes present in the peripheral blood by using standard organic (phenol) extraction method. This protocol involved four stages; each stage took one day to finally liberate protein-free genomic DNA. Nucleic acids were not affected when proteins were enzymatically degraded by protease. This was followed by an extraction step selective to the physical and chemical properties that differ between nucleic acids and cellular components, shaping the basis for their isolation. Being amidst soluble contaminants formed in the extraction method, DNA was solely purified by precipitation in an ethanol-salt solution. Although this method is relatively laborious, uses

perilous chemicals and generates liquid organic waste, it yields high-quality nucleic acids (Zagone et al., 2009).

To prevent contamination and cross-contamination between samples, careful attention was given during DNA extraction. Extraction was performed in a location distant from the workstation where PCR amplification was performed.

3.3.1.1 Cell lysis

All PB samples were immediately processed upon receipt. The blood samples were kept frozen at -20°C until DNA extraction. DNA extraction began with lysis of the cells in the sample. About 3 ml of fresh EDTA-anticoagulated PB was transferred into a 15 ml falcon tube (Corning Inc, NY, USA). The content was topped to 15 ml by adding cold 1X RBC lysis buffer (Appendix I) to whole blood. The tube was then capped before gentle inversion to mix well and left to stand and incubated at room temperature for 10 minutes. The tube was centrifuged (Heraeus, Hanau, Germany) at 3,500 rpm for 10 minutes at room temperature. This centrifugation step was required to remove erythrocytes from whole blood specimen to prevent inhibition of PCR by haemoglobin prior to recovery of DNA from leucocytes.

3.3.1.2 Washing

After centrifugation, the supernatant containing lysed red blood cells was discarded carefully. Again, washing was repeated by resuspending the pellet in 13 ml 1X RBC lysis buffer. The pellet was forced to break down into smaller pieces by a repetitive series of aspiration and dispensing using a Pasteur pipette. The tube was incubated again at room temperature. The suspension was mixed gently until the pellet completely dispersed to a

homogenous mixture before a round of centrifugation at 3,500 rpm for another 10 minutes. The supernatant was discarded and excess fluid was drained off as much as possible by inversion to obtain clean white pellet which settled at the tube bottom. The pellet was resuspended with white blood cell lysis buffer formulated with 160 µl 1X SSC buffer, 40 µl Proteinase K (10mg/ml), 100 µl 10% SDS buffer and 300 µl RNase and DNase free DEPC-treated water. The mixture was then placed into an incubator (Memmert, Schwabach, Germany) overnight at 37°C for cell lysis to occur.

3.3.1.3 Protein precipitation and organic extraction of DNA

The following day, 400 µl 6M sodium chloride (NaCl) was added to the cooled 600 µl dissolved pellet (proteinase K digested cell extract). The tube was vortexed (NaCl, Tokyo, Japan) for 15 to 20 seconds to facilitate salting out. The content of the falcon tube was then transferred equally into two new 1.5 ml microcentrifuge tubes. An equal volume (1:1 ratio) of phenol-chloroform (about 500 µl) was added into each tube and subjected to vigorous vortexing to obtain a homogenous milky solution. The tubes were then spun down using a refrigerated centrifuge at 13,000 rpm, 4°C for 20 minutes. Following centrifugation, three distinct layers were observed with DNA in the clear aqueous top layer, proteins and protein-DNA complexes deposited in the whitish interface layer and phenol-chloroform being the cloudy organic layer at the bottom of tube (Zagone et al., 2009). DNA was carefully removed from the top layer by gentle aspiration using a micropipette and equal volume was transferred into two new 1.5 ml microcentrifuge tubes.

3.3.1.4 Ethanol precipitation of DNA

Ethanol-salt solution was used to concentrate diluted DNA and to remove soluble contaminants produced in the extraction method. For this, dehydration was initiated to precipitate DNA by simply adding 1 ml of ice cold absolute ethanol. The tubes were then gently inverted before overnight incubation in a freezer at -20°C. The next day, the frozen mixture was thawed in a 37°C water bath. The tube was then placed in a refrigerated microcentrifuge (Heraeus, Hanau, Germany) and spun down at 13,000 rpm for 10 minutes at 4°C. After centrifugation, the supernatant was discarded carefully. One ml ice cold 80% ethanol was added and gently mixed to wash and decompose the pellet, followed by a brief centrifugation at 13,000 rpm for 5 minutes at 4°C. Finally, the supernatant was once again discarded carefully. The resultant DNA pellet was air-dried overnight before adding sterile water or dilute salt buffer on the following day. Over-drying of DNA pellet was avoided as it would cause difficulty in resuspending DNA in water or TE buffer. In this work, water was substituted by appropriate volume of 1X TE buffer (Appendix I) to rehydrate and dissolve the DNA pellet before storage at 4°C.

3.3.2 Buccal Cell DNA Extraction

Buccal swab contained cheek cells. These cells were lysed prior to recovery of DNA. Firstly, the buccal cell sample was resuspended in a lysis mix containing 400 µl TE buffer, 20 µl 10% SDS buffer and 20 µl proteinase-K (10 mg/ml) in a 15 ml falcon tube before overnight incubation at 37°C. The following day, the lysate was pressed and removed from the tube. The solution was transferred into a labeled 1.5 ml microcentrifuge tube. Equal volume of phenol-chloroform-isoamyl alcohol solution (25:24:1) was added to the lysate in a microcentrifuge tube to isolate DNA. The contents were mixed well by vigorous vortex

for 10 minutes. The tube was then briefly centrifuged using a refrigerated centrifuge (Heraeus, Hanau, Germany) at 13,000 rpm for five minutes at 4°C. After centrifugation, distinct organic (phenol) and aqueous phases were observed. The top layer containing DNA in aqueous solution was carefully aspirated and was transferred into a new labeled 1.5 ml microcentrifuge tube using a micropipette. The DNA was precipitated by adding one-tenth volume of 4M NaCl and two volumes of ice-cold absolute ethanol. The sample was placed in freezer and incubated at -20°C for two hours before centrifugation at 13,000 rpm for five minutes at 4°C. The supernatant was carefully removed by not touching the DNA pellet which was observed after centrifugation. The DNA pellet was washed with 1 ml of 80% ice-cold ethanol and then centrifuged again at 13,000 rpm for ten minutes at 4°C. The washing of pellet was repeated again with 1 ml ice-cold absolute ethanol, followed by centrifugation under same condition. Once again, supernatant was removed and the precipitate was allowed to dry overnight. Appropriate volume of 1X TE buffer was added to the dried DNA for rehydration step. The hydrated DNA was ready to be used for subsequent manipulation.

3.4 DNA Quantification

Assessment of the yield and concentration of purified genomic DNA was done using NanoDrop 2000 ultraviolet (UV) spectrophotometer (Thermo scientific, MA, USA). Typically, the maximal absorbance for nucleotides is at 260 nm of UV light (A_{260}) while for proteins the maximal absorbance is at 280 nm (A_{280}). DNA was therefore quantified by the A_{260} measurement, while A_{260}/A_{280} ratio provided an estimation of the purity of the sample. The purity of DNA indicated by A_{260}/A_{280} ratio of 1.8 to 2.0 was required for optimal downstream applications. As contaminants absorb at 230 nm, the A_{260}/A_{230} ratio was used

as a secondary measure of nucleic acid purity and values for pure nucleic acid were expected to be higher than the respective A_{260}/A_{280} values and to be in the range of 2.0 to 2.2. Concentration of DNA was recorded in ng/µl. Water was used as the blank control for calibrating the spectrophotometer.

3.5 Storage of Genomic DNA

The DNA samples were kept in freezer at 4°C for short term storage or at -20°C for long term storage.

3.6 PCR Amplification of Exonic Regions of *RB1* Gene

The amplification process of all 27 exons of *RB1* gene was performed using PCR conditions and primer sequences as proposed by Lohmann et al. (1994). All exons were amplified in separate PCR assays except for exons 15 - 16 which were amplified in the same reaction. The lengths of exon 15 and intron sequence between exon 15 and 16 are relatively short. Thus, both exon 15 and 16 were amplified together. PCR amplifications were performed in 25 µl mixes containing 24 µl of master mix (Table 3.2) and 1 µl of DNA template. A negative control was included in each PCR run by substituting DNA template with DEPC-treated water purchased from BioBasic Inc. Table 3.2 shows the components and their concentration in the PCR master mix. PCR amplification was carried out using a thermal cycler (Eppendorf, Hamburg, Germany). The protocol consisted of initial denaturation at 95°C for 15 min, followed by 40 cycles of: denaturation at 94°C for 1 min, optimized annealing at 54°C for 40 secs and extension at 72°C for 35 secs. The whole PCR run ended with an additional extension at 72°C for 10 min. The oligonucleotide primers flanking all 27 exons of *RB1* gene are listed in Table 3.3.

Table 3.2 : PCR Master Mix Recipe

Components	Stock Concentration	Final Concentration	Volume (µl)/ reaction
H_2O (molecular biology grade)	-	-	14.55
PCR buffer (contains 1.5mM $MgCl_2$)	10x	1x	2.5
dNTP mix	10mM	200µM	0.5
DMSO	100%	5%	1.25
HotStarTaq DNA Polymerase	5U/µl	1U/µl	0.2
Forward primer	10µM	1µM	2.5
Reverse primer	10µM	1µM	2.5

Stock primers with initial concentration of 100 µM/µl were diluted with molecular biology grade DEPC-treated water to final concentration of 10 µM/µl prior usage. PCR kit (Qiagen, Hamburg, Germany) with HotStarTaq DNA polymerase and PCR buffer were used in the preparation of PCR master mix.

3.7 Selection of Healthy Control

A healthy colleague in the laboratory was selected as control for *RB1* mutational screening. In addition to the healthy control, *RB1* gene sequence found in GenBank (Accession No: L11910) was also used as the reference sequence. Before the negative control sequences were used to compare with the patient's DNA sequences, they were aligned to the reference sequence to ensure 100% concordance except for some polymorphism regions, which varied between Caucasians and Asians.

Table 3.3 : Primer Sequences

Region	Name	Sequence (5' to 3')	Temp. (°C)	Length (bp)
Exon 1	Rbi1se	GCGAATTCGTGCGCGCGCGTCGTCCTCC	62	318
	Rbi1as	GCGAATTCGGCCCCTGGCGAGGACGGGTC		
Exon 2	Rbi2se	GCGAATTCGTATGTACTGAATCAATTTG	52	263
	Rbi2as	GCGAATTCGAAGTTGGTTTTAAAATGAG		
Exon 3	Rbi3se	GCGAATTCTAACATAGTATCCAGTGTGTG	58	239
	Rbi3as	GCGAATTCGTTTCCTTTTATGGCAGAGG		
Exon 4	Rbi4se	GCGAATTCGAAATAACACAAATTTTTAAGG	55	224
	Rbi4as	GCGAATTCAGTGTAACCCTAATAAAATG		
Exon 5	Rbi5se	AGCATGAGAAAACTACTATG	55	190
	Rbi5as	CCTAACTATCAAGATGTTTG		
Exon 6	Rbi6se	GCGAATTCTTTCAGTGATACATTTTTCC	55	210
	Rbi6as	GCGAATTCAATTTAGTCCAAAGGAATGC		
Exon 7	Rbi7se	GCGAATTCTCTCATACAAAGATCTG	58	236
	Rbi7as	GCGAATTCAATAAGCAACTGCTGA		
Exon 8	Rbi8se	GCGAATTCATTGTTCTTATCTAATTTACCAC	55	241
	Rbi8as	GCGAATTCTACATCTAAATCTACTTTAACTG		
Exon 9	Rbi9se	GCGAATTCTGCATTGTTCAAGAGTCAAGAG	55	230
	Rbi9as	GCGAATTCAATTATCCTCCCTCCACAGTC		
Exon 10	Rbi10se	GCGAATTCAAAGGATAATTGTCAGTGACT	58	221
	Rbi10as	GCGAATTCTACCTATATCAGTATCAAC		
Exon 11	Rbi11se	GCGAATTCGAGACAACAGAAGCATTATAC	55	249
	Rbi11as	GCGAATTCTGAAACACTATAAAGCCA		
Exon 12	Rbi12se	GCGAATTCATTGCTTAACACATTTTC	55	185
	Rbi12as	GCGAATTCTTTGCCAAGATATTACAA		
Exon 13	Rbi13se	GCGAATTCATCCTCGACATTGATTTCTG	58	201
	Rbi13as	GCGAATTCTAGTACCACGAATTACAATG		
Exon 14	Rbi14se	GCGAATTCTGATTTTCTAAAATAGCAGGCTC	55	274
	Rbi14as	GCGAATTCTTTTAGTAGAGACAGGGTTTCAC		
Exon 15-16	Rbi15	GCGAATTCAATGCTGACACAAATAAGGTTTC	55	392
	Rbi16	GCGAATTCGATCTAAAATAAGCATTCCTTCTCC		
Exon 17	Rbi17se	GCGAATTCCAAAAAAATACCTAGCTCAAG	55	357
	Rbi17as	GCGAATTCGTTAAGAAACACCTCTCACTAAC		
Exon 18	Rbi18se	GCGAATTCAATTATGCTTACTAATGTGG	55	216
	Rbi18as	GCGAATTCAGTTTGATGGTCAACATAAC		
Exon 19	Rbi19se	GCGAATTCAACTTGAAATGAAGACTTTTCC	55	241
	Rbi19as	GCGAATTCTAGTTTCAGAGTCCATGCTC		
Exon 20	Rbi20se	GCGAATTCGACTAATTTTTCTTATTCCCAC	58	219
	Rbi20as	GCGAATTCGAGGAGAGAAGGTGAAGTGC		
Exon 21	Rbi21se	GCGAATTCCATGTAATAAAATTCTGACTAC	55	224
	Rbi21as	GCGAATTCCTATGTTATGTTATGGATATGG		
Exon 22	Rbi22se	GCGAATTCCTTTATAATATGTGCTTCTTACCAG	55	328
	Rbi22as	GCGA ATTCGTTTTGGTGGACCCATTACATTAG		
Exon 23	Rbi23se	GCGAATTCATGTAATGGGTCCACCAAAAC	58	269
	Rbi23as	GCGAATTCTTTACTACTTCCCTAAAGA		
Exon 24	Rbi24se	GTATTTATGCTCATCTCTGC	55	211
	Rbi24as	ATGAGGTGTTTGAATAACTG		
Exon 25	Rbi25se	GCGAATTCTTGAGGTTGCTAACTATGAAACAC	55	281
	Rbi25as	GCGAATTCTGGATTCCCCAGATGACCATC		
Exon 26	Rbi26se	GCGAATTCATCGAAAGCATCATAGTTAC	55	214
	Rbi26as	GCGAATTCGAAAAGACTTCTTGCAGTG		
Exon 27	Rbi27se	GCGAATTCAATGCTGTTAACAGTTCTTC	55	179
	Rbi27as	GCGAATTCTGTGAGAGACAATGAATCC		

3.8 Analysis of PCR Amplicons

3.8.1 Preparation of Agarose Gel

3% agarose gel was prepared by completely dissolving 2.1g agarose powder (Seakem, Germany) in 70 ml 1X Tris Borate EDTA (TBE) buffer (the same buffer used for electrophoresis) and boiled in a microwave oven until it became colourless. Subsequently, 1.5 µl of 10 µg/ml ethidium bromide was added to the gel solution and swirled to mix well before pouring into a horizontal casting tray. Gel comb was used to form a row of 20 wells. After cooling and polymerization, the gel was loaded in a horizontal electrophoresis apparatus and submerged in buffer in a single chamber. The solution and reagent used for agarose gel electrophoresis are shown in Appendix II.

3.8.2 Gel Electrophoresis

The PCR products or amplicons were resolved in 3% Agarose gel in 1X TBE buffer using an electrophoresis system. The wells were loaded with samples to be analysed. Each well contained 5 µl of PCR product mixed with 1 µl loading dye buffer. Samples were loaded on the 3% agarose gel and run in horizontal electrophoretic plates (BioRad, USA) at constant voltage (140V, 56W and 400mA) for 45 minutes at room temperature. The various sizes of amplicons were compared with 100 bp DNA ladder (Fermentas International Inc, Ontario, CA). Following electrophoresis, the gel was then visualized using a UV transilluminator (G-Biosciences, MO, USA).

3.9 Purification of PCR Amplicons

To ensure minimum noise level that may interfere with interpretation of the sequence result, the PCR DNA template was purified. The PCR amplified DNA was purified from unincorporated primers and dNTPs using QIAGEN's QIAquick PCR Purification kit, according to the manufacturer's protocol (Appendix III). Five volume of Buffer PB was added into one volume of the PCR sample. The content was mixed and spun. A QIAquick column was placed in a 2 ml collection tube. The mixture was then transferred into this tube and centrifuged for one minute at 9200 rpm at 4°C. The flow-through was discarded. 0.75 ml Buffer PE was added to the QIAquick column and centrifuged for one minute at 9200 rpm at 4°C. The same procedure was repeated.

Next, QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 30 µl Buffer EB or water was added to the center of the QIAquick membrane and centrifuged for one minute at 9200 rpm at 4°C. The purified PCR product was again subjected to gel electrophoresis to reveal the presence and absence of any unintended amplification products. In contrast, for electrophoresis of purified PCR products 3% glycerol is added to the sample before loading into wells.

3.10 DNA Sequencing

Purified PCR amplicons were subsequently sent to the Molecular Genetics Laboratory (Institute of Postgraduate Studies of University of Malaya) for DNA sequencing. About 15 µl of each purified PCR product were sent for sequencing with respective forward and reverse primers to aid bidirectional sequencing. Both forward and reverse primers were prepared in 3.2 µM of concentration of required volume with consideration of 1 µl needed

per reaction. The result was received in softcopy which was found to be compatible with a freeware known as *BioEdit* Sequence Alignment Editor to facilitate sequence analysis for identification of *RB1* mutation.

3.11 *In-silico* Analysis of DNA Sequence

The *RB1* gene sequence which was published by Toguchida et al. (1993) (Accession No: L11910) was used as the reference sequence in subsequent *in silico* analysis. The reference sequence was retrieved from GenBank (http://www.ncbi.nlm.nih.gov) and was used for comparison to identify mutations. First and foremost, the primer sequences were mapped to reference sequence to identify the regions at where the primers anneal using The Sequence Manipulation Suite (http://bioinformatics.org/sms/). For this, cloning adaptors which attached to most primers ($^{5'}$GCGAATTC$^{3'}$) were omitted prior to data input in The Sequence Manipulation Suite (Appendix IV). Using the same online resource, translation map was also generated with the input of *RB1* reference sequence (Appendix V). For mutational analysis, patients' DNA sequences were aligned and compared with the reference sequence and the healthy control DNA sequence by collating in BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html) and Clustal Omega (http://www.ebi.ac.uk/Tools /msa/clustalo/). Identified DNA changes and predicted amino acid changes were labelled according to standard nomenclature by Human Genome Variation Society (http://www.hgvs.org). Predicted consequences from these changes were derived from reported mutations.