Spectrum of germ-line *RB1* gene mutations in Malaysian patients with retinoblastoma

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Purpose: The availability of molecular genetic testing for retinoblastoma (RB) in Malaysia has enabled patients with a heritable predisposition to the disease to be identified, which thus improves the clinical management of these patients and their families. In this paper, we presented our strategy for performing molecular genetic testing of the *RB1* gene and the findings from our first 2 years of starting this service.

Methods: The peripheral blood of 19 RB probands, including seven bilateral and 12 unilateral cases, was obtained, and genomic DNA was extracted. Analysis of the *RB1* exons and the promoter region was conducted first using PCR and direct sequencing. Next, multiplex ligation-dependent probe amplification (MLPA) analysis was performed for patients whom the first results were negative. For patients whom either the first or second method results were positive, parental samples were analyzed to determine the origin of the mutation.

Results: Ten *RB1* mutations were identified in ten (52.6%) of the 19 probands (seven bilateral and three unilateral cases), of which 30.0% (3/10) was identified with MLPA. The detection rates in the bilateral and unilateral cases were 100.0% (7/7) and 25.0% (3/12), respectively. Three new *RB1* mutations were discovered, two in patients with bilateral RB and one in patient with unilateral RB. Interestingly, all mutations detected with the PCR-sequencing method were predicted to create a premature stop codon. Eight mutations were proven to be de novo while one mutation was inherited from the mother in a family with a positive history of RB.

Conclusions: Our results confirmed the heterogeneous nature of *RB1* mutations and the predominantly de novo origin. The high prevalence of pathogenic truncating mutations was evident among local patients with RB. The combination of PCR sequencing and MLPA is recommended for sensitive identification of heritable RB cases.

Retinoblastoma (RB; OMIM 180200) is a rapidly developing cancer of the retina. RB is the most common intraocular malignant tumor in children with 1 case reported for every 14,000 to 22,000 live births [1]. Approximately 90% of RB cases are diagnosed in children younger than 5 years old [2]. RB is responsible for 1% and 5% of the overall childhood cancer deaths and blindness, respectively [3]. In Malaysia, an average of 14.5 new RB cases are diagnosed every year [4]. The most common presenting signs are leukocoria, followed by strabismus and protopsis [5]. In developing countries such as Malaysia, late presentation is common, and consequently, high enucleation rates are observed [4-6].

Mutations that occur in the human RB susceptibility gene *RB1* (Gene ID: 5925; OMIM number: 614041) on chromosome 13q14 help to explain the clinical phenotypes of most RB cases [7]. The heritable form of RB is caused by germ-line mutations in *RB1,* and this can be transmitted to offspring as an autosomal dominant trait with 90.0% penetrance [8,9]. The proportion of bilateral cases is higher among hereditary cases than among sporadic cases [8]. Later in life, patients with heritable RB show an increased risk of developing other primary tumors such as osseous sarcoma [8]. Heritable RB can be diagnosed from any DNA-containing cells because the first *RB1* inactivating mutation occurs in the germ-line cells. The second copy of *RB1* is mutated somatically, and it can be detected only from tumor DNA [7].

More than 700 distinct *RB1* germ-line mutations has been described to date; the two most common types are deletion and nonsense mutations [10]. Sixteen hot spots of *RB1* mutations were also identified comprising 12 nonsense mutations, three splice-site mutations, and two missense mutations [10]. The majority of the recurrent mutations occur due to C to T transitions in 11 CGA codons in exons 8, 10, 11, 14, 15, 17, 18, and 23 [10]. Interestingly, more than two-thirds of the mutations in *RB1* are predicted to result in premature termination of translation yielding a truncated retinoblastoma protein (pRb) [11].

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The purpose of this study is to characterize the spectrum of germ-line mutations in local patients with RB. Such information is useful in guiding the design of the optimal genetic testing strategy for the benefit of Malaysian patients with RB and their families.

METHODS

Sample collection: Patients with retinoblastoma in Hospital Kuala Lumpur (HKL) were jointly managed by a team of pediatric ophthalmologists and oncologists. Patients were examined under general anesthesia, including indirect fundus ophthalmoscopy, fundus imaging using a wide field fundus camera (Retcam, Clarity Medical Systems, Inc., Pleasanton, CA), and ultrasonography. Tumors were characterized using the International Intraocular Retinoblastoma Classification, IIRC (ABC Classification, Group A to E). Eyes with advanced tumor with no visual potential (classified as Group E or Group D) were enucleated. Patients with bilateral disease or unilateral Group B to D were treated with systemic chemotherapy with focal consolidation using diode laser and cryotherapy. Eyes with a small tumor (Group A) received only local treatment with laser and cryotherapy. Radiotherapy was given to extraocular RB and to tumors resistant to other treatment.

The Molecular Diagnostics and Protein Unit (UMDP) of the Institute for Medical Research performs molecular genetic testing for various genetic disorders ranging from metabolic to neurodegenerative illnesses. Recently, molecular genetic testing for the *RB1* gene was offered to local patients with RB, and this enabled molecular investigation to be performed to identify the mutations that increased the patients' susceptibility to the cancer as well as to inform the family members of the affected proband of the cancer risk.

Patients were recruited at the HKL Genetic Clinic. If the probands and their families agreed to undergo the genetic test, they were guided to sign a consent form, and approximately 5 to 10 ml of peripheral blood was withdrawn and collected inside a standard EDTA tube. All blood samples, request forms, and consent forms were sent to the UMDP immediately. If a mutation was detected in the patient, parental samples were analyzed for the presence of the same mutation. The study protocol was checked and approved by the Medical Research & Ethics Committee (MREC) under project code NMRR-12–551–11436. The study protocol was in accordance with the tenets of the Declaration of Helsinki and the ARVO (Association for Research in Vision and Ophthalmology) statement on human subjects.

PCR and sequencing: PCR was used to specifically amplify the entire coding exons and the promoter region of the *RB1* gene using the HotStarTaq® *Plus* DNA Polymerase (Qiagen, Hilden, Germany). A 50 μl reaction consisted of 1X Coral-Load PCR Buffer, 2.0 mM of $MgCl_2$, 200 μM of each dNTP (Thermo Scientific, Waltham, MA), 1.5 Units of HotStarTaq *Plus* DNA Polymerase, 0.5 μM of both forward and reverse primers, 1 μl of template DNA, and double distilled water (ddH_2O). Due to the high GC content of the promoter region and exon 1, a 1X Q-solution (Qiagen) was added to the reaction mix. The PCR thermal cycling protocol began with an initial denaturation step at 95 °C for 5 min followed by a ten-cycle touchdown step of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min with the annealing temperature reduced by 0.5 °C/second every cycle. The next 25 cycles consisted of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min followed by a final extension step of 72 °C for 10 min. PCR products were run on 1.5% agarose gel under standard electrophoresis parameters to check for the presence of amplicons with the expected size.

Correct PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) per the manufacturer's instructions. Cycle sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The cycle sequencing products were purified using DyeEx 2.0 Spin Kit (Qiagen), dried on vacuum concentrators, and resuspended in 10 μl of Hi-Di Formamide. Finally, the sequencing reaction was performed on the Applied Biosystems 3500 Genetic Analyzer under standard sequencing parameters.

The primers used for PCR were designed in-house and were positioned about 100–200 bp away from the start and end of each exon. Primers were also checked for binding to regions that contain known DNA variants to ensure successful amplification of both *RB1* alleles. All PCR primers were tagged with the universal T7Promoter and T7Terminator sequence at the 5′ end, and universal T7 primers were used during cycle sequencing instead of the specific PCR primers. The presence of long stretches of the homopolymer region in particular homopolymeric A and T flanking several *RB1* exons hindered the ability to recover a good sequencing readout with a conventional sequencing strategy. Therefore, specific anchor primers were designed to overcome this problem, and these anchor primers were positioned to overlap partly on the homopolymer region and partly on the adjacent nucleotides. The combination of T7 and anchor sequencing primers enabled at least 2X coverage across regions of interest. Sequence information for all tagged PCR primers, T7 universal primers, and anchor primers is available in Appendix 1.

Mutation analysis: Sequencing results were visualized using Sequence Scanner Software (Applied Biosystems) to check for the data quality before further processing. Sequencing readouts that passed the initial QC step were assembled and compared to the *RB1* reference gene sequence (GenBank Accession: L11910) using SeqScape Software (Applied Biosystems). Visual inspection of the alignment was done to identify the DNA variants in the *RB1* gene of the patients for downstream analysis. In instances in which a possible small insertion or deletion was identified, Mutation Surveyor Software (Softgenetics, State College, PA) was used to deconvolute the overlapping signals to reveal the indel events. All variants identified as either single nucleotide variants (SNVs) or indels were reported according to the standard nomenclature for DNA sequence changes described by the Human Genome Variation Society (HVGS).

All variants identified were annotated against publicly available databases such as the Human Gene Mutation Database (HGMD) and *RB1* Locus-Specific Databases (RB1-LSDB). The pathogenicity of the DNA sequence alterations were evaluated by using MutationTaster2 software. All *RB1* mutations found were submitted to *RB1*-LSDB.

MLPA analysis: Multiplex ligation-dependent probe amplification (MLPA) analysis was performed for probands with no detectable mutation when they were screened with PCR and the sequencing method. The MLPA reaction was performed using the SALSA MLPA P047 *RB1* probemix Lot C1–0212 (MRC-Holland, Amsterdam, the Netherlands). The procedure was based on the manufacturer's protocol with a DNA starting amount of 75 ng. GeneScan™ 500 LIZ® was used as the size standard, and the MLPA products were run on an Applied Biosystems 3500 Genetic Analyzer under standard settings. The results were visualized and analyzed with Coffalyser.Net software (MRC-Holland) according to the manufacturer's instructions.

RESULTS

Throughout 2-year sample collection period beginning December 2012 to December 2014, a total of 19 blood samples from unrelated probands who requested *RB1* gene testing were received. The patients included seven (36.8%) male patients and 12 (63.2%) female patients; the majority were Malays (11 patients, 57.9%), followed by Chinese (six patients, 31.6%) and Indians (two patients, 10.5%). Twelve patients were affected unilaterally (63.2%) while seven (36.8%) were affected bilaterally. Information on clinical features, including tumor laterality, age at diagnosis, and IIRC staging, is shown in Table 1. Of all the families studied,

one was found to have history of RB in which the elder sister had been previously diagnosed with the disease.

The age at diagnosis for the patients with RB ranged from 2 to 42 months old, with an average age of 14 months for bilateral and 23.5 months for unilateral cases. Seven distinct mutations were discovered using the PCR-sequencing method. The most common mutational type was nonsense mutations (three of seven; 42.8%) followed by frameshift (two of seven; 28.6%) and splice mutations (two of seven; 28.6%). All seven mutations were predicted to create a truncated RB protein either by the direct creation of a premature stop codon or indirectly through a change in the reading frame and the loss of coding exons. Mutationtaster2 predicted all seven mutations were disease causing. An additional three mutations were found with MLPA, and they were comprised of a whole *RB1* gene deletion in two patients and a partial *RB1* gene deletion in one patient. Of the ten mutations, three (30.0%) were new mutations described in the *RB1* gene (Table 2).

Parental samples for nine of ten patients with *RB1* gene mutations were tested, and eight of nine mutations were confirmed to occur due to de novo mutations. In patient RB45, who had a positive family history of RB, the disease-causing mutation was shown to be inherited from the mother. Parental samples for patient RB05 were not available for analysis. The diagnostic yield for the PCR-sequencing method alone was 36.8% (seven mutations in 19 patients) and for MLPA was 15.8% (three mutations in 19 patients). However, when the methods were used in combination, the diagnostic yield was 52.6% (ten mutations in 19 patients). The sensitivity of the *RB1* gene mutation detection in bilateral RB was 100.0% and 25.0% for unilateral RB.

DISCUSSION

This was the first comprehensive study to reveal the spectrum of germ-line mutations in the *RB1* gene in local patients with RB. To date, only one study has been published on this subject in Malaysia, but the analysis was limited to the pocket domain B of pRb [12]. A similar demographic pattern was observed between these patients and previous reports in the distribution of unilateral and bilateral disease and the patient's ethnic background [4,5]. Late presentation was also evident; nearly half of the patients presented at stage E (Table 1).

The germ-line mutations identified in our patients were scattered along the *RB1* gene confirming the heterogeneous distribution of mutations in this gene [10]. Two mutations, R251X and R579X, were located on mutational hot-spot regions on exons 8 and 18, respectively [10]. At a frequency of 20.0% (two of ten mutations), both mutations were predicted

				TABLE 1. DEMOGRAPHIC DATA OF RB PATIENTS INCLUDED IN THIS STUDY.
Patient ID	Gender	RB laterality	IIRC staging (L/R)	Age at diagnosis (months old)
RB01	F	B	E/D	31
RB04	F	B	D/E	7
RB05	F	B	NA	NA
RB06	M	U	N/C	16
RB09	F	U	NA	41
RB12	M	B	B/E	2
RB15	M	B	C/D	27
RB18	F	U	N/E	23
RB21	F	U	NA	20
RB24	M	U	D/N	37
RB27	M	U	NA	6
RB30	F	U	NA	7
RB33	M	B	E/D	7
RB36	F	B	B/E	10
RB39	F	U	N/E	33
RB42	F	U	E/N	42
RB45	F	U	N/D	8
RB48	F	U	N/E	27
RB51	M	U	B/N	23

Abbreviation used: F, female; M, male; B, bilateral; U, unilateral; L, left eye; R, right eye; N, Normal; NA, Not Available.

to alter the CGA-arginine codons to create premature stop codons. The frequency of mutations at CGA codons we reported was similar to a previous report and thus confirmed the benefit of performing a quick survey of CGA codons in the identification of *RB1* mutations [13]. The pathogenicity of premature stop codons was attributed to the degradation of *RB1* transcripts that harbor signals for premature termination of translation via nonsense-mediated decay (NMD), and this resulted in the complete loss of pRb [14].

All sporadic RB cases in our study were caused by de novo mutations. De novo mutations were responsible for many sporadically occurring genetic diseases and represented the most extreme form of rare genetic variations due to high penetrance and damaging impact compared to inherited variations [15]. Identification of mutational origin was important especially to inform the family of the affected proband of the risk of RB to other siblings and relatives. Inherited mutations such as in patient RB45 could be traced back to the mother of the affected proband, and therefore, the siblings could be tested even before the disease begins to manifest, therefore avoiding the late presentation seen in the proband. Prenatal diagnosis could also be offered to the mother in the future.

It was also more cost-effective to perform *RB1* mutational screening in relatives at risk of RB compared to conventional ophthalmological examinations [16].

The introduction of molecular genetic testing of the *RB1* gene enabled identification of patients with heritable RB, and this has allowed for better management of patients with RB and their families. Here, our strategy resulted in the detection of disease-causing mutations in all bilateral patients and in a quarter of unilateral patients. This was possible only when the PCR-sequencing method was used alongside MLPA. Therefore, we concluded that sensitive identification of germ-line *RB1* mutations required both methods be employed. In the remaining patients in whom no germ-line *RB1* mutation was detected, analysis of the tumor sample was required to identify inactivating mutations that could also be present in the blood but in lower frequency due to germ-line mosaic mutations. Despite the high sensitivity detection of coding and promoter mutations, splice-site mutations, and large deletion and duplication mutations offered by these assays, they are not able to identify deep intronic mutations as well as promoter hypermethylation mutations. Therefore, new molecular techniques such as RNA analysis and methylation

TABLE 2. SUMMARY OF *RB1* MUTATIONS IDENTIFIED IN 10 MALAYSIAN RB PATIENTS.

Patient ID	Mutation	Mutational type	Location	Consequence (experimental evidence /putative)	Status
RB01	c.1960+1G>A	Splice	Intron 19	Confirmed to cause skipping of exon 19	Reported [17]
RB04	c.861G>C	Splice	Exon 8	Confirmed to cause skipping of exon 8	Reported [18]
RB12	c.1735C>T	Nonsense	Exon 18	Predicted to create premature stop codon, p.(Arg579*)	Reported [13]
RB15	c.751C>T	Nonsense	Exon 8	Predicted to create premature stop codon, p.(Arg251*)	Reported [13]
RB33	c.2184C>G	Nonsense	Exon 21	Predicted to create premature stop codon, p.(Tyr728*)	Novel
RB42	c.390del	Frameshift	Exon 4	Predicted to create premature stop codon, p.(Lys130Asnfs*6)	Novel
RB45[+]	c.19dup	Frameshift	Exon 1	Predicted to create premature stop codon, p.(Arg7Profs*24)	Reported [19]
RB05	-	Gross deletion	Whole *RB1* gene deletion	One copy of *RB1* gene is predicted to be lost	Reported [19]
RB36	-	Gross deletion	Deletion from Promoter to exon 3	One copy of *RB1* gene is predicted to be lost	Novel
RB48	-	Gross deletion	Whole *RB1* gene deletion	One copy of *RB1* gene is predicted to be lost	Reported [19]

Mutation nomenclature is based on Locus Reference Genomic LRG_517 for *RB1* gene. Nucleotide 1 is the A of the ATG-initiation codon.
[+] denote patient with positive family history of RB.

analysis are needed to detect the two mutation types currently missed by these assays. At present, tumor samples are not available for analysis due to logistic issues. Once this is resolved, analysis of tumor DNA will be performed especially for patients with unilateral RB. The discovery of germ-line mutations in unilateral patients is valuable because they can be segregated based on their mutational status, and this will impact the genetic counseling given to them as they age.

APPENDIX 1. SEQUENCE INFORMATION FOR ALL TAGGED PCR PRIMERS, T7 UNIVERSAL PRIMERS AND ANCHOR PRIMERS USED IN THIS STUDY.

T7Prom and T7Term represent both T7Promoter and T7Terminator universal sequencing primers that were tagged to all exon-specific PCR primer sequences. T7Prom was tagged to Forward primers while T7Term was tagged to Reverse primers. For each primer, sequences in uppercase belonged to T7Prom/Term and sequences in lowercase were exon-specific. [a] %GC primer was calculated using the GC Calculator program. [b] Predicted amplicon size (bp) was calculated using the UCSC In-Silico PCR program available at https://genome.ucsc.edu/cgi-bin/hgPcr. To account for T7 sequences

that were present on both Forward and Reverse primers, additional 39bp was added to the amplicon size predicted by the aforementioned program. To access the data, click or select the words "Appendix 1."

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