Molecular Genetic Profiling of Filipino Patients with Retinoblastoma: A Preliminary Study

Pia Magdalena R. Mendoza, MD,[1] Prospero Ma. C. Tuano, MD,[1] Lee G. Verzosa, MD,[1] Remedios Felisa S. Huerto, MD,[1] Ma. Luisa D. Enriquez, PhD,[2] and Emilio L. Macias, MD[1,3]

[1]International Eye Institute
St. Luke's Medical Center,
Quezon City, Philippines

[2]Research and Biotechnology Division
St. Luke's Medical Center
Quezon City, Philippines

[3]Department of Ophthalmology
Jose R. Reyes Memorial Medical Center
Manila, Philippines

Correspondence: Pia Magdalena R. Mendoza, MD
St. Luke's Medical Center
Quezon City, Philippines
Email: piam4@yahoo.com

Disclosure: Financial support for this study was provided by the Research and Biotechnology Division of St. Luke's Medical Center-Quezon City. The authors have no proprietary or financial interest in any of the products or instruments used in this study.

ABSTRACT

Objective: To detect and characterize retinoblastoma susceptibility gene (RB1) mutations in tumor samples collected from Filipino patients with retinoblastoma.

Methods: Six tumor samples were obtained from Filipino patients diagnosed with retinoblastoma. DNA was extracted from the tumor samples and exons 13-21 of the RB1 gene were amplified by polymerase chain reaction (PCR). PCR amplification products were subsequently purified and sequenced. Mutation detection and characterization were done by alignment of obtained sequences to the RB1 reference sequence from NCBI GenBank using Bioedit® software. The identified mutations were correlated with clinical presentation and family history. These mutations were also compared to known mutations reported in the RB1 Gene Mutation Leiden Open Variation Database (LOVD).

Results: Mutations were detected in two out of the six samples. In a patient with unilateral disease and no family history, two mutations were identified: a novel CGT>AGT (Arginine → Serine) missense mutation in position c.1861 of exon 19 and a previously reported CGA>TGA (Arginine → STOP) nonsense mutation in position c. 1735 of exon 18. A possible large exonic deletion was identified in a case of unilateral disease with no family history.

Conclusion: We were able to identify both novel and known mutations in the RB1 gene of Filipino retinoblastoma cases using DNA sequencing techniques. These techniques may be applied to further characterize the genetic mutations of Filipino retinoblastoma cases and their families in developing a rational method of genetic testing for early diagnosis and counseling.

Keywords: Retinoblastoma, Gene mutation, RB gene, Retinoblastoma susceptibility gene

Philipp J Ophthalmol 2012; 37:39-44

Retinoblastoma (RB) is the most common primary intraocular malignancy of childhood, with an incidence rate of 1 in 15,000 to 20,000 livebirths in Western countries and no gender predilection.[1] In the Philippines, studies show that the incidence through the years has increased from 79.6 to 237 per 100,00 eye cases.[2] and is higher compared to the incidence in other countries.[3] The relatively high incidence of RB in the Philippines, coupled with socio-economic conditions resulting in delayed detection and inadequate treatment, makes the disease a cause of significant morbidity and mortality.[4,5]

Tumorigenesis in retinoblastoma is due to inactivation of both alleles of the RB susceptibility gene RB1.[6,7] RB1 is a tumor suppressor gene whose deletion or mutation causes unregulated proliferation of embryonal retinal cells. It is located on chromosome 13q14, spans 180 kbp, and has 27 exons. All 27 exons contribute to the 2.7 kb open reading frame which codes for a 110 KDa nuclear phosphoprotein known as pRB.[8] pRB functions as a regulator of cellular replication.[9] Any mutation in exons 13-21 of the RB1 gene, which code for the functional domain of the protein, will result in reduced function of pRB.[10-11]

In hereditary cases (40%), there is a germline RB1 mutation present in constitutional cells and predisposition to RB is transmitted as an autosomal dominant trait. The disease then typically presents at an earlier age, is bilateral, and tumors are multifocal. In non-hereditary cases (60%), there are two distinct RB1 mutations acquired in both RB1 alleles that occur in somatic cells. The disease usually presents at a later age, is unilateral, and tumors are unifocal. However, not all cases follow these classical hereditary and non-hereditary forms. Some patients with RB exhibit reduced expressivity, such as in 15% of unilateral and non-familial cases that actually have germline mutations. There are also reports of incomplete penetrance (not all carriers develop clinically evident tumors), as well as mutational mosaicism.[12-14.] Clinical presentation and family history, combined with molecular genetic testing of tumor and/or peripheral blood, are used to determine if a patient has a heritable or non-heritable mutation. This information can then be used to determine the genetic status of the parents of a proband with RB, as well as the risk of inheriting the cancer-predisposing mutation in the proband's siblings and offspring.

Genetic testing is, therefore, a valuable adjunct to the clinical management of RB patients and their family members, since it gives diagnostic certainty, facilitates timely treatment, reduces unnecessary examinations, aids genetic counseling for reproductive planning, and allows early tumor detection in at-risk individuals. Because of these benefits, there is a significant reduction in hospital expenditure and psychological stress for patients and their families who undergo genetic testing for RB.[15]

The varied genotype-phenotype relationships in RB are due to the wide array of genetic variations linked to RB1, which include chromosomal rearrangements, large exonic deletions, hypermethylation of the gene promoter region, small length mutations, and single nucleotide substitutions. The expressivity and penetrance of RB depend on the functional consequence of the predisposing RB1 mutation. Whether the mutation translates into a pRB protein product that is either totally absent, present but with total loss of function, or abnormal but with some residual function, determine the severity of the disease. The majority of germline mutations that have been identified in familial RB are nonsense or frameshift mutations within exons 2-25 causing multifocal and bilateral tumors due to absent RB protein (pRB).[16-17] Sequence analysis was used to identify small length mutations and single nucleotide substitutions in exons and splice site consensus regions which accounted for about 70% of oncogenic RB1 mutations.[18] A single nucleotide polymorphism in intron 18 of RB1 has been shown to be prevalent in Southeast Asian populations, including Filipinos.[19-20]

We initiated this study to detect and characterize the RB1 gene mutations in tumor samples collected from Filipino patients with retinoblastoma. We identified and characterized the type of mutations in exons 13-21 of the RB1 gene by polymerase chain reaction (PCR) amplification and sequencing and correlated the mutations with patients' clinical presentation and family history. We also compared the mutations to known mutations reported in the international RB1 gene mutation database.

To the best of our knowledge, this is the first extensive molecular study on the RB1 gene in the Filipino population. The results derived from this study will serve as baseline molecular-genetic data on retinoblastoma patients in the Philippines. The information may provide new insights into this malignancy, which will contribute to the improvement in the diagnosis and management of the disease. This study is also an initial effort towards establishing a more comprehensive genetic screening protocol for retinoblastoma patients in the country.

METHODOLOGY

Six Filipino patients with retinoblastoma, consulting at St. Luke's Medical Center (SLMC) and Jose R. Reyes Memorial Medical Center (JRRMMC), underwent complete physical and ophthalmologic examinations. B scan ultrasound and/or CT scan of the orbit and brain were performed to assess the extraocular or intracranial extension of the disease. Tumors were graded according to the Tumor, Node, Metastasis (TNM) classification scheme. Family history was determined for each of the probands. Eyes with severe intraocular disease and/or extraocular extension were enucleated or exenterated and the tumor tissue stored in a -20^{0}C biofreezer. Specimens were obtained according to protocols approved by the hospital Institutional Scientific Review Committee and the Institutional Ethics Review Board after parental informed consents were obtained.

Histopathologic Examination

Histopathological examination was done on the enucleated or exenterated eyes stored in formalin by the SLMC Institute of Pathology. The diagnosis of retinoblastoma was confirmed in these samples. Degree of differentiation and choroidal or optic nerve invasion were characterized.

DNA Isolation

Genomic DNA was isolated from the tumor tissues using the QIAGEN QIAamp® DNA Mini Kit protocol as per manufacturer's instructions. DNA quantity and quality were assessed using spectrophotometry (Nanodrop® v.1000) measured by absorbance at 260nm/280nm.

DNA Amplification of RB1 Gene Exons by PCR

PCR amplification of exons 13-21 of the RB1 gene was done using genomic DNA with exon-specific primers. Primer sequences were based on the RB1 gene sequence published in National Center for Biotechnology Information (NCBI) GenBank, accession number L11910. Primers were designed using the UCSC Gene Browser (University of California Santa Cruz) website and TUM ExonPrimer (Technische Universitat Munchen). PCR was done using QIAGEN Taq Core Kit® and G-Storm GS1 thermocycler. The PCR conditions were optimized so that each exon was amplified quantitatively. Amplification products were electrophoresced in 2.0% agarose gel and stained with ethidium bromide.

DNA Mutation Analysis by Sequencing

PCR amplicons were purified using QIAGEN according to the manufacturer's instructions and sent to First Base Sequencing (Malaysia) for DNA sequencing. Sequences obtained were aligned to the RB1 reference sequence from NCBI GenBank using Bioedit® software for identification of mutations. Mutations were compared to known mutations in the RB1 mutation Leiden Open Variation Database (LOVD); URL: http://www.verandi.de/joomla/

RESULTS

Demographic data on the six patients included in the study are presented in Table 1. There were 3 males and 3 females. Five patients had unilateral tumors and one had bilateral tumors. The age at presentation ranged from 7 months to 5 years, and the mean age of presentation was 2 years and 7 months. There was no family history of retinoblastoma for all cases.

Tumor staging according to the clinical and pathologic TNM classification scheme for retinoblastoma are enumerated in Table 2. Specifics of each classification scheme are included in Appendix A. The need for either enucleation or exenteration was indicated in all cases. Clinically, five out of six cases had extraocular disease detected by imaging studies with invasion of the optic nerve and/or invasion into the orbit. One case (RB5) had severe intraocular disease with tumor-associated angle closure glaucoma. The bilateral case (RB4) had extraocular disease with invasion into the orbit in the left eye, and subretinal seeding and retinal detachment in the right eye. The tumors in all patients were too large and homogenous to properly assess the presence of multifocal disease. There were no cases with lymph node involvement or metastasis. Histopathologically, five out of six tumor samples had invasion of the optic nerve to resection line with extraocular extension identified. One case (RB5) had optic nerve invasion past the lamina cribrosa but not to surgical resection line.

Table 1. Patient Demographics

Patient Number	Gender	Laterality	Age at Presentation	Family History
RB2	Female	Left	5 years old	Negative
RB3	Male	Right	3 years old	Negative
RB4	Male	Bilateral	7 months old	Negative
RB5	Male	Right	2 years old	Negative
RB6	Female	Left	3 years old	Negative
RB7	Female	Left	2 years old	Negative

Despite full optimization, amplification of exon 20 in all samples produced multiple bands, instead of the desired single band representative of the expected PCR product. Primer design for exon 20 was deemed faulty and none of the samples were sequenced for this particular exon.

Mutations were detected in two out of the six samples. Summary of all identified mutations are listed in Table 3. In a patient with unilateral disease and no family history (RB3), two mutations were identified: a previously reported nonsense mutation in exon 18 and a novel C>A missense mutation in exon 19 (Figures 1 and 2). In another case of unilateral disease and no family history (RB7), none of the exons were adequately amplified and this may represent a large exonic deletion or a whole gene deletion.

Table 2. Tumor Staging according to TNM Classification Scheme for Retinoblastoma in the Study Sample (N=6)

Patient Number	Clinical Classification (cTNM)	Pathologic Classification (pTNM)
RB2	cT4bM0N0	pT4bMXNX
RB3	cT4bM0N0	pT4bMXNX
RB4 (Left Eye)	cT4bM0N0	pT4bMXNX
RB4 (Right Eye)	cT2aM0N0	pTXMXNX
RB5	cT3bM0N0	pT3aMXNX
RB6	cT4bM0N0	pT4bMXNX
RB7	cT4bM0N0	pT4bMXNX

Table 3. Summary of Identified Mutations in the Study Sample (N=6)

Patient No.	Exon No.	Position	Nucleotide Change	Altered Amino Acid	Consequence	Database
RB2	No mutations identified					
RB3	18	c.1735	CGA →TGA	Arginine → STOP	Substitution; Nonsense mutation	Reported 85 times
RB3	19	c.1861	CGT → AGT	Arginine → Serine	Substitution; Missense mutation	No reports yet
RB7	No exons amplified				Possible large exonic deletion	
RB4	No mutations identified					
RB5	No mutations identified					
RB6	No mutations identified					

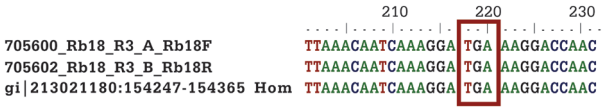

Figure 1. Alignment of tumor sample RB3 sequence to reference sequence for exon 18 showing CGA →TGA mutation.

The first two lines correspond to the forward and reverse complement sequences of the tumor sample. The last line corresponds to the normal RB1 sequence.

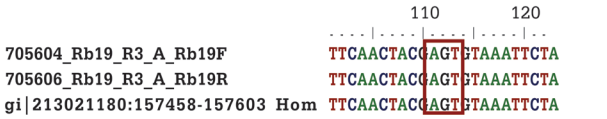

Figure 2. Alignment of tumor sample RB3 sequence to reference sequence for exon 19 showing CGT →AGT mutation.

The first two lines correspond to the forward and reverse complement sequences of the tumor sample. The last line corresponds to the normal RB1 sequence.

DISCUSSION

Most centers that offer genetic testing for retinoblastoma typically screen for mutations in all exons of the RB1 gene. In this study, we opted to initially screen for mutations in exons 13-21. These exons were chosen because they code for the binding site and functional pocket domain of the retinoblastoma protein pRB and mutations in these exons will lead to a corresponding functional change in the protein.[10] Most clinically significant mutations result in amino acid insertion, deletion, or substitution within the pocket domain.[11] Mutation analysis in this study was limited by the number of exons sequenced, and analysis of other RB1 exons should be done in samples wherein no mutations were detected.

In a review of 932 reported RB1 mutations, 42% corresponded to nonsense mutations.[21] Majority of germline mutations have been identified to be nonsense or frameshift mutations.[16] The specific C>T nonsense mutation found in position c.1735 within exon 18 identified in patient RB3 has been reported in other cases of non-familial, unilateral retinoblastoma.[13] However, heterozygous carriers of nonsense mutations almost invariably developed bilateral and multifocal tumors[17], unlike what was seen in our patient. Nonsense mutations resulting in a premature stop codon and truncated protein were associated with nearly complete penetrance because it resulted in complete inactivation of the retinoblastoma protein.[14]

The C>A point mutation identified in exon 19 of patient RB3 is a novel missense mutation. The codon change from CGT→AGT produced an amino acid change from arginine (polar basic amino acid) to serine (polar uncharged amino acid) which may contribute to functional changes in the resultant pRB protein. Missense mutations comprised 10% of reported RB1 mutations.[21] They were associated with a low penetrance phenotype with reduced expressivity resulting in unilateral non-familial retinoblastoma, similar to the clinical presentation of this patient.

Large exonic deletions are possibly present in patient RB7. Whole exon deletions have been identified in 10% of bilateral or familial retinoblastoma[22] and have not yet been seen in unilateral and non-familial cases. However, it is uncertain if this mutation was indeed a large deletion without doing other confirmatory tests.

In conclusion, we were able to identify both novel and known mutations in the RB1 gene of Filipino retinoblastoma cases using DNA sequencing techniques. These techniques may be applied not only to tumor tissue but may also be used for comparative analysis of peripheral blood. With this protocol, we can further characterize the genetic mutations of Filipino retinoblastoma cases and their families in the hope of developing a rational method of genetic testing for early diagnosis and counseling.

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APPENDIX A

TNM Classification for Retinoblastoma*

The TNM (Tumour, Node, Metastasis) classification is developed, monitored and enforced by the American Joint Commission on Cancer and the Union Internationale Contre le Cancer (AJCC/UICC). Many cancers have a unique TNM classification, but TNM has traditionally been used only for extension of retinoblastoma beyond the eye. The TNM classification for retinoblastoma was redeveloped in 2010 to incorporate the International Intraocular Retinoblastoma Classification which has been favoured by physicians worldwide in staging tumour within the eye. The system includes both

clinical (cTNM) and pathological (pTNM) findings. Primary retinoblastoma (T) is divided into four groups, with several sub- groups in each, and many additional descriptors. The stages from cT1a – cT3b closely reflect the five stages of the International Intraocular Retinoblastoma Classification. Stage cT3 (IIRC Group E) indicates high risk for invasion of tissues surrounding the eye, and the need for urgent removal of the eye. Stage pT2 and pT3 confirm invasion of surrounding tissues. Stages cT4, pT4 N and M involve significant disease invasion of tissues beyond the eye. From June 2010, researchers are required to state TNM classification alongside or in place of IIRC / REC in manuscripts submitted for journal publication or conference presentation. When both eyes are affected, each eye is staged independently. When only one stage is given, this refers to the stage of the worst eye, which is an indicator of the risk to the child's life.

* Sobin LH, Gospodarowicz MK, Wittekind C. TNM Classification of Malignant Tumors, 7th ed. Oxford: Wiley-Blackwell, 2010; 291-297.