

Mutational screening of germline *RBI* gene in Vietnamese patients with retinoblastoma reveals three novel mutations

Ha Hai Nguyen,^{1,3} Hoa Thi Thanh Nguyen,^{1,3} Nhung Phuong Vu,^{1,3} Quynh Thuy Le,² Chau Minh Pham,² Thuong Thi Huyen Ma,¹ Hung Manh Do,¹ Hang Le Bich Pham,¹ Ton Dang Nguyen,^{1,3} Hien Thi Thu Le,^{1,3} Hai Van Nong^{1,3}

¹Institute of Genome Research, Vietnam Academy of Science and Technology, Hanoi, Vietnam; ²National Institute of Ophthalmology, Hanoi, Vietnam; ³Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam

Purpose: Retinoblastoma (Rb) is a rare and unique eye cancer that usually develops in the retinas of children less than 5 years old due to mutations in the *RBI* gene. About 40% of affected individuals have the heritable form making genetics testing of the *RBI* gene important for disease management. This study aims to identify germline mutations in *RBI* in a cohort of patients with Rb from northern Vietnam.

Methods: Genomic DNA was extracted from peripheral blood of 34 patients with Rb (nine unilateral and 25 bilateral cases) and their available parents. Twenty-seven exons, flanking sequences, and the promoter region of *RBI* gene were screened for mutations with direct PCR sequencing. Multiplex ligation-dependent probe amplification (MLPA) was applied for patients with negative sequencing results. In the mutation-positive patients, their available parental DNA was analyzed to determine the parental origin of the mutation.

Results: Germline mutations in *RBI* were identified in 25 (73.53%) of 34 patients (four unilateral and 21 bilateral cases). Of these mutations, 19 were detected, including seven nonsense, six frameshift, four splice-site (one was identified in two siblings), and one missense, with Sanger sequencing. Three novel frameshift mutations were discovered in one unilateral and two bilateral patients. MLPA detected mutations in the *RBI* gene in six bilateral cases, of whom five had a whole gene deletion (three familial cases) and one had a partial gene deletion (from exon 4 to exon 27) in one allele of the *RBI* gene. Parental testing showed five mutations originated from the fathers and one was inherited from a mother who was mosaic for the mutation.

Conclusions: This study provides a data set of germline mutations in the *RBI* gene in Vietnamese patients with retinoblastoma. Screening of mutations in the *RBI* gene can help to identify heritable Rb and contribute to clinical management and genetic counseling for affected families.

Retinoblastoma (Rb) is a rare and unique eye cancer that develops in the eyes of diagnosed children less than 5 years old, and it has an estimated incidence of 1:20,000 live births [1]. The most common clinical presentation of Rb is leukocoria or the abnormal whitish appearance of the pupil [2]. This disease is triggered mostly by inactivation mutations in both alleles of the *RBI* gene (Gene ID: 5925; OMIM 614041) in one or more retinal cells [3]. Heritable Rb (40% of patients) has one germline and one somatic mutation in *RBI* while non-heritable Rb (60% of patients) has both somatic mutations in *RBI* [4]. Germline mutations can be transmitted from parents or occur during gametogenesis or gestation. Children born to a person with a germline mutation have a high risk of pathogenic expression of Rb and other cancers later in life as this mutation is an autosomal dominant trait with an

average 90% penetrance [3,5]. About 40% of Rb cases are bilateral, and 60% are reported as unilateral. In certain cases, a pinealoblastoma cooccurs that is known as trilateral Rb. The majority of familial patients with Rb who have inherited the germline pathogenic variant develop multiple tumors in both eyes. However, nearly 10% of families with heritable Rb present as unilateral tumors due to a low-penetrance phenotype or incomplete penetrance [6].

In Vietnam, approximately 90–100 patients are diagnosed with Rb each year. The tumors are intraocular in more than 90% of cases. The majority of these patients (70%) are diagnosed with an advanced stage (group D or E) and are forced to enucleate. According to doctors, only 15–30% of cases can be treated with modalities such as cold laser or chemotherapy to preserve vision. In the present study, we used two conventional methods (direct PCR sequencing and multiplex ligation-dependent probe amplification, MLPA) to identify germline mutations in the *RBI* gene, elucidate their influences in the cohort of 34 patients with Rb, and thus, provide accurate genetic counseling.

Correspondence to: Ha Hai Nguyen, Institute of Genome Research, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam; Phone: +84.24.3756.2934; FAX: (+84.24.3791.7039); email: nguyenhaiha@igr.ac.vn

METHODS

Patients: Thirty-four patients with retinoblastoma were recruited between 2013 and 2017 at the National Institute of Ophthalmology in Hanoi, Vietnam. Diagnosis of Rb was established by standard ophthalmologic and histological criteria. The age at diagnosis was between 3 and 36 months old. Written and signed informed consent from a patient's parent was obtained before the genetic test for *RBI* was performed. A total of 5 ml peripheral blood samples from patients and their available parents were collected into a standard EDTA tubes and stored at -20°C until DNA extraction. Once a patient was identified as having a germline mutation, parental available samples were checked for the mutation at the same nucleotide position on the *RBI* gene. This study was approved by the Institutional Review Board (IRB) of the Institute of Genome Research, Vietnam Academy of Science and Technology. The study protocol was in accordance with the provisions of the Declaration of Helsinki and the Association for Research in Vision and Ophthalmology (ARVO) statement on human subjects.

DNA extraction: Genomic DNA was isolated from peripheral blood leukocytes using the Omega E.Z.N.A Blood DNA Mini Kit (Omega Bio-tek, Norcross, GA) according to the manufacturer's protocol. DNA quality was evaluated with agarose gel electrophoresis and spectrophotometry at 260 and 280 nm (BioSpectrometer Basic; Eppendorf, Hamburg, Germany).

PCR and sequencing: Screening of germline mutations in the *RBI* gene was preceded by Sanger sequencing for the entire 27 coding exons and their flanking regions using the primers as described by Abouzeid (2009) [7] with slight modifications using primer 3 software. PCR was amplified in a PCR Mastercycler pro S (Eppendorf) in a total volume of 25 µl containing 25–50 ng of genomic DNA, 0.5 pmol of each primer, and 12.5 µl of Taq 2X Master Mix (New England Biolab, Ipswich, MA). Dimethyl sulfoxide (DMSO) was added to the amplification reaction of the promoter–exon 1 fragments. PCR cycling was initiated with the denaturation step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, annealing at the specific temperature for 30 s, 68 °C for 30 s to 1 min, and a final extension step at 68 °C for 5 min. The amplicons were purified using a MultiScreen PCR filter plate (Merck Millipore, Darmstadt, Germany). The purified products were sequenced using the ABI Big Dye Terminator v 3.1 Sequencing Standard Kit (Applied Biosystems, Foster City, CA) and run on an ABI 3500 Genetic Analyzer (Applied Biosystems). The sequence data were analyzed by comparison with the standard sequence of the *RBI* gene (GenBank L11910.1) using BioEdit sequence alignment editor software. Additional information about mutations in the *RBI* gene

and polymorphisms was confirmed from the *RBI* variation database *rbi-lsdb* and The Human Gene Mutation Database (HGMD).

MLPA analysis: The SALSA MLPA probemix PO47-B1 *RBI* (MRC Holland) was used to detect single- to multiexon deletions or duplications at the *RBI* locus. The probemix contains probes for detecting 26 of 27 *RBI* exons without exon 15. The procedure was performed according to the manufacturer's instructions using 50 ng of genomic DNA from the participants' peripheral blood. PCR amplicons ranging between 100 and 500 nt were separated on Genetic Analyzer 3500 (Applied Biosystem). The results were analyzed using Coffalyser.Net Software. Coffalyser.Net uses 0.7 and 1.3 as cut-off values for heterozygous deletion or duplication, respectively (normal status is diploid).

RESULTS

A total of 34 samples from 12 (35.29%) male and 22 female (64.71%) Vietnamese patients with Rb and their available parents were subjected to molecular diagnosis. Twenty-four patients were affected bilaterally (70.59%), and ten were affected unilaterally (29.41%). Concerning inheritance, 25 were sporadic cases (73.53%) while nine were family cases (26.47%). Of these family patients, one case (RB15) was first diagnosed with sporadic bilateral retinoblastoma and then reclassified as a family history because her younger sister later developed Rb. The clinical data for the patients were summarized in Appendix 1.

Analyzing the DNA samples with the Sanger sequencing method yielded a pathogenic heterozygous mutation rate of 55.88% (19/34). Of these mutations, 15 cases (78.95%) were affected bilaterally, and four (21.05%) were affected unilaterally. In the spectrum of mutation types among the 19 mutations, 36.84% (7/19) were nonsense, 31.58% (6/19) were frameshift, 26.32% (5/19) were splicing alterations, and only 5.88% (1/19) were missense. The distribution of the mutations in the *RBI* gene is shown in Figure 1. Among the detected mutations, 78.95% (15/19) were located in the regions encoding two conserved domains A (exons 12–18) and B (exons 19–23) of the pRb pocket. Among these cases, 11 were sporadic (9/15 bilateral and 2/15 unilateral), and four were familial Rb (2/13 for each bilateral and unilateral). Only one missense mutation substituting arginine to tryptophan at codon 661 (p.Arg661Trp) in exon 20 was found in the cohort, and the mutation was relevant to the mild expression of sporadic unilateral Rb. Four substitution mutations were found in intron 2 (c.265–1G>T), intron 21 (c.2211+1G>A), intron 12 (c.1215+1G>A), and exon 19 (c.1960G>A) that altered canonical splice signals. In this study, one novel

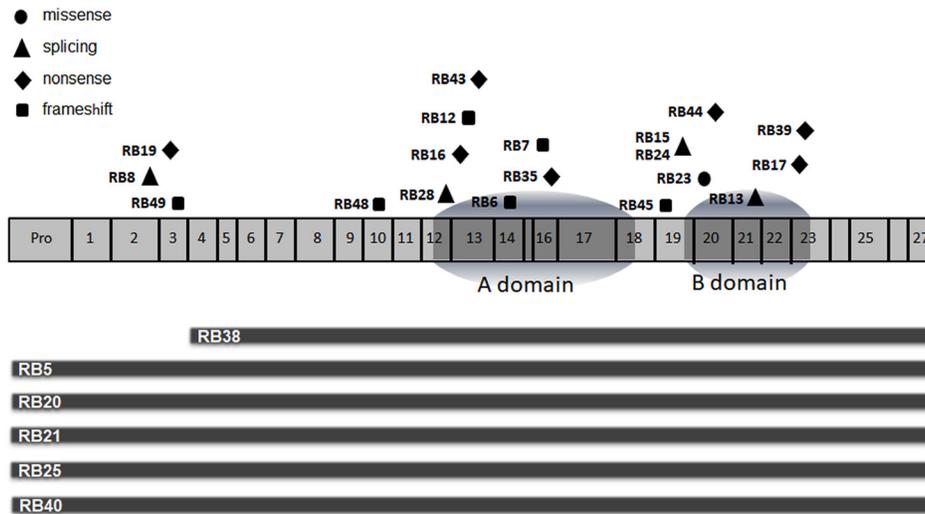


Figure 1. Schematic representation of sequence mutations detected in 25 patients across the *RB1* gene. Distribution of mutations in *RB1* is described by mutation type in the promoter region, 27 exons, and splice sites of the gene. The functional domains A and B are shown; the numbers in boxes refer to the exons. The symbols denote ● missense, ▲ splicing, ◆ nonsense and ■ frameshift mutations. A total of one partial and five whole gene deletions of *RB1* are presented as the shaded bars. All mutations and gene deletions in the patients were detected in the heterozygous state.

insertion and two novel deletions in the *RB1* gene were revealed in the cohort, representing 15.79% (3/19) of all mutations, including a familial bilateral, a sporadic unilateral, and a sporadic bilateral (patients RB7, RB6, and RB12, respectively). Noticeably, all of these novel mutations were located in the important protein-binding sites of domain A of the pRb pocket (c.1337insTA in exon 14, c. 1449–1450delTA in exon 16, and c.1312delT in exon 13; Figure 2). Further analysis of the parental origin of the detected mutations showed that two were inherited from the father of RB15 and RB24 (c.1960G>A; Figure 3A), one from the mother of RB35 (c.1494T>G; Figure 3B) and one from the father of RB17 (c.2939T>G; not shown).

MLPA of the negative cases from the PCR sequencing revealed 17.65% (6/34) of the patients had whole or a partial gene deletion in heterozygous state, of which all were bilateral. There were two-whole allele deletion cases for which the clinical diagnosis was heritability from the father (patients RB20 and RB21). In patient RB38, the gross deletion was identified from exon 4 to 27 of the *RB1* gene. The complete list of mutations in *RB1* and gene deletions in the cohort is summarized in Table 1.

No pathogenic mutation was detected in the remaining nine sporadic cases (five unilateral and four bilateral) either with Sanger sequencing or MLPA, accounting for 26.74%. Among these cases, unilateral patients RB10 and RB14 showed alterations in c.607+61C>A (non-reported) and c.540–23dup (reported twice/LOVD) in intron 6, respectively. The frequency of non-identified and identified mutations for the 34 patients is presented in Figure 4.

DISCUSSION

Retinoblastoma is a primary intraocular malignancy occurring in children and has a high risk of mortality. The incidence in Vietnam is one of the ten highest rates in Asia-Pacific countries [8]. To our knowledge, this is the first study to comprehensively screen germline mutations in the *RB1* gene in Vietnamese patients with retinoblastoma.

To date, more than 1,900 distinct mutations have been reported in the *RB1* gene worldwide [9-12], and they are scattered all over the gene. However, the point mutations found in this study were observed to be located mainly in two regions: One was around exon 3, and the other was the sequence encoding two pRb pockets, A and B (Figure 1). These domains are sufficient to interact and inhibit the E2F transcription factors that makes protein pRb play a vital role in cell cycle regulation, promoting G1/S arrest and growth restriction [13]. Thus, mutations located on mutational hotspot regions indicate a high frequency of tumorigenesis in many types of cancer, including retinoblastoma [14]. Out of 34 patients, mutations resulting in absent or premature chain termination due to gross insertions or deletions, nonsense, and frameshift associated with high-penetrance disease were found in 54.89% of the patients. In contrast, missense and splicing mutations related to low penetrance Rb were found in 17.65% of the patients.

In this study, 13 parental and 15 maternal samples of mutation-positive patients were examined to determine the inheritance of the mutations. Three point mutations originated from parents, and two large deletions were transmitted from fathers. A constitutional mutation was identified in

a 6-month-old male patient (RB17) and his father whose right eye was enucleated. Likewise, the splice-site mutant (c.1960G>A) leading to reduced penetrance was detected in two siblings (RB15, bilateral; RB24, unilateral) and in their father who was unaffected (Figure 3A). The only mosaic mutation was found in the mother of RB35 whose right eye was removed while her daughter’s mutation was in the heterozygous state (Figure 3B).

The novel mutations in the present study exhibited frameshift, one of the majority of germline mutations associated with bilateral Rb and high penetrance (>90%) [6]. This type of mutation has a great impact on the phenotype because it completely abolishes the activity of one allele. The affected patients, thus, will most likely develop tumors in both eyes [15]. Two patients (RB7 and RB12) were diagnosed bilaterally. However, the alteration of *RB1* open reading frame in the case of patient RB6 (female) expressed a unilateral

tumor only at the time of diagnosis at 3 months old. Thus, her remaining eye should be monitored for tumorigenesis.

It is obvious that the precise diagnosis of mutations would contribute to prenatal and preimplantation genetic testing to manage disease and provide appropriate therapy for the infant or child with retinoblastoma. In the case of patient RB45, as she is the first patient with bilateral retinoblastoma in her family and her frameshift mutation was de novo, her pregnant mother can expect to have a third child with a decreased fetal risk (Figure 3C). Such a risk has been reported to range from 2.5% to 0.007% [16]. Therefore, molecular genetic monitoring for patients with Rb is extremely important for giving accurate strategies in genetic counseling.

MLPA recently has been believed to be a sensitive and sequence-specific technique for detecting cytogenetic and subcytogenetic abnormalities of single exons in small amounts of human DNA samples [17]. Approximately 15–25% of Rb cases due to large rearrangements has been

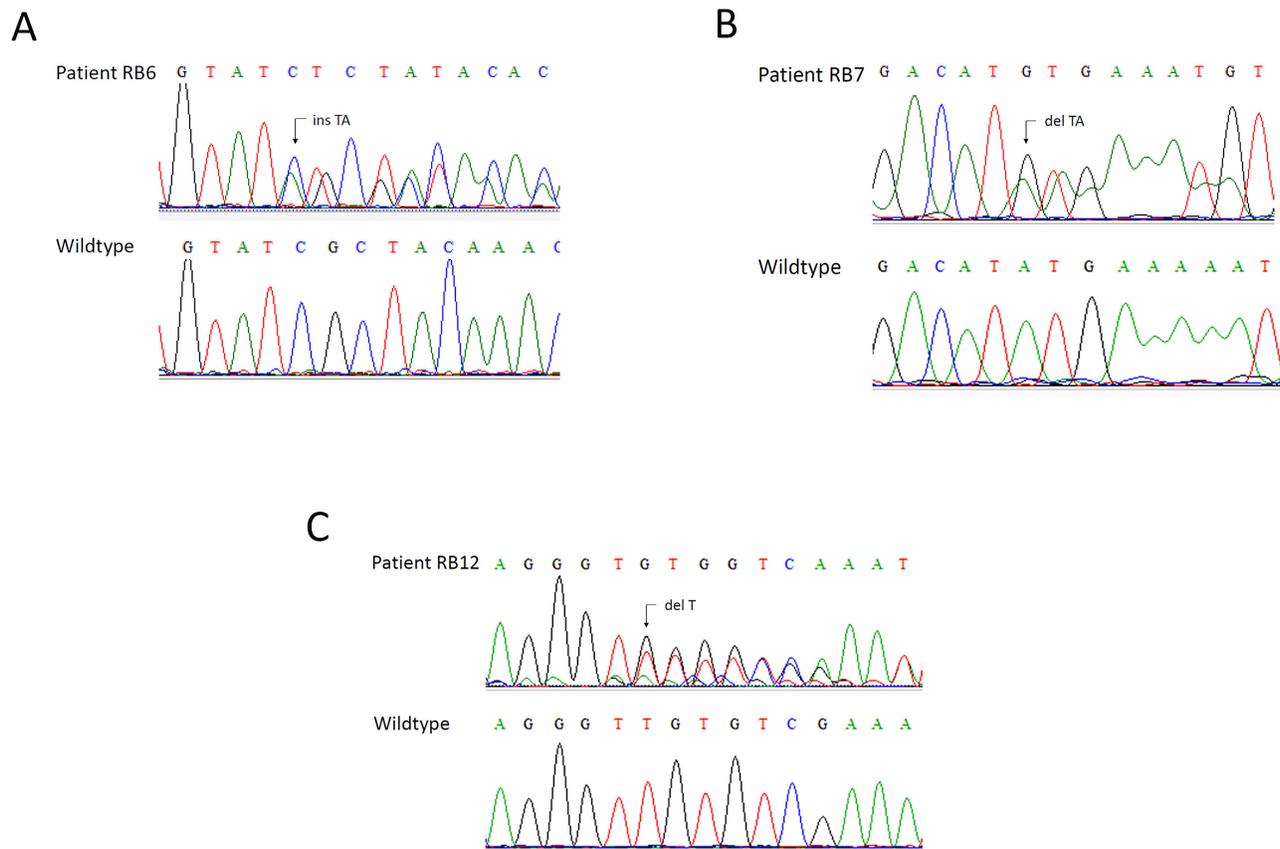


Figure 2. Sequencing chromatograms illustrating the novel mutations detected in patients. A: c.1337insTA heterozygous mutation in patient RB6 (reverse direction). B: c.1449–1450delTA heterozygous mutation in patient RB7. C: c.1312delT heterozygous mutation in patient RB12.

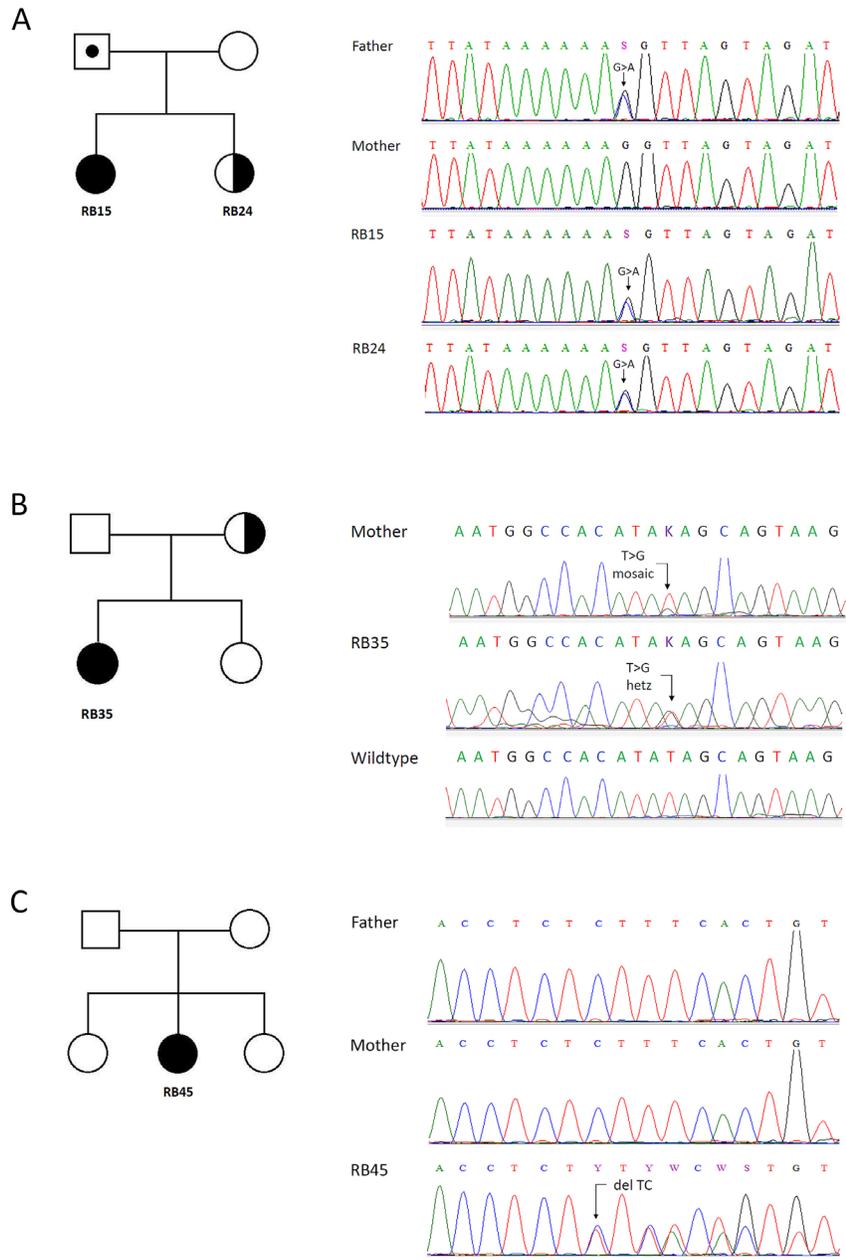


Figure 3. Pedigrees of the Rb family cases. **A:** c.1960G>A mutation causing a splice-site variation in the siblings patient RB15 and patient RB24. **B:** c.1494T>G heterozygous mutation of patient RB35 and c.1494T>G mosaic mutation of patient RB35’s mother. **C:** c.1939–1940delTC causing a frameshift mutation in patient RB45. Solid black and half-black symbols represent bilateral and unilateral Rb, respectively. The symbol with a black dot represents an unaffected mutation carrier.

reported in many previous investigations [18-20]. In the present study, the combined method of DNA sequencing and MLPA enhanced the diagnostic yield for discovering the mutation in *RBI* clearly (73.53%) while the detection proportion by using single DNA sequencing was only 55.88%. Thus,

we recommend that both methods (PCR direct sequencing and MLPA) be used to perform genetic testing for the *RBI* gene in Vietnamese patients with Rb.

In nine cases in the cohort, we did not find a mutation in the *RBI* gene in their blood samples. They may have somatic

TABLE 1. *RB1* GENE MUTATIONS IDENTIFIED IN RETINOBLASTOMA PATIENTS BY SANGER SEQUENCING AND MLPA METHODS.

Patient ID	gDNA position	Exon/or intron	cDNA position	Consequence	Present in mother/father	Reference
RB5	Whole gene deletion				NY/NY	NA
RB6	g.76434insTA	Exon 14	c.1337insTA	Frameshift	No/NA	Novel
RB7	g.77028-77029delTA	Exon 16	c.1449-1450delTA	Frameshift	No/NA	Novel
RB8	g.39445G>T	Intron 2	c.265-1G>T	Splicing	No/NA	LOVD
RB12	g.73849delT	Exon 13	c.1312delT	Frameshift	No/NA	Novel
RB13	g.160835G>A	Intron 21	c.2211+1G>A	Splicing	No/NA	LOVD
RB15*	g.153353G>A	Exon 19	c.1960G>A	Splicing	No/Yes	LOVD
RB16	g.73843C>T	Exon 13	c.1306C>T	p.Gln436X	No/NA	LOVD
RB17	g.162317T>G	Exon 23	c.2439T>G	p.Tyr813X	No/Yes	LOVD
RB19	g.39478G>A	Exon 3	c.297G>A	p.Trp99X	No/NA	LOVD
RB20	Whole gene deletion				NY/Yes	NA
RB21	Whole gene deletion				NY/Yes	NA
RB23	g.156713C>T	Exon 20	c.1981C>T	p.Arg661Trp	No/NA	LOVD
RB24*	g.153353G>A	Exon 19	c.1960G>A	Splicing	No/Yes	LOVD
RB25	Whole gene deletion				NA/NY	NA
RB28	g.70330G>A	Intron 12	c.1215+1G>A	Splicing	No/NA	LOVD
RB35	g.77073T>G	Exon 16	c.1494T>G	p.Tyr498X	Yes, mosaicism/No	LOVD
RB38	Exon 4-27 deletion				NA/NY	NA
RB39	g.162237C>T	Exon 23	c.2359C>T	p.Arg787X	No/No	LOVD
RB40	Whole gene deletion				NY/NY	NA
RB43	g.73867C>T	Exon 13	c.1330C>T	p.Gln444X	No/No	LOVD
RB44	g.156761G>T	Exon 20	c.2029G>T	p.Glu667X	No/NA	LOVD
RB45	g.153332-153333delTC;	Exon 19	c.1939-1940delTC	Frameshift	No/No	LOVD
RB48	g.64341-64344delTCCTT	Exon 10	c.951-954delTCCTT	Frameshift	NA/NA	LOVD
RB49	g.39551-39552delTA	Exon 3	c.371-372delTA	Frameshift	No/No	LOVD

Nucleotide numbering for complete coding sequences of DNA and cDNA deposited in the GenBank under accession numbers L11910.1 and NM_000321.2 respectively. Amino acid numbering refers to the reference sequence for pRB protein, NP-000312.2 *; sibling, NA: not available; NY: sample has not yet checked. All detected mutations were heterozygous as described in the legend to the Figure 1.

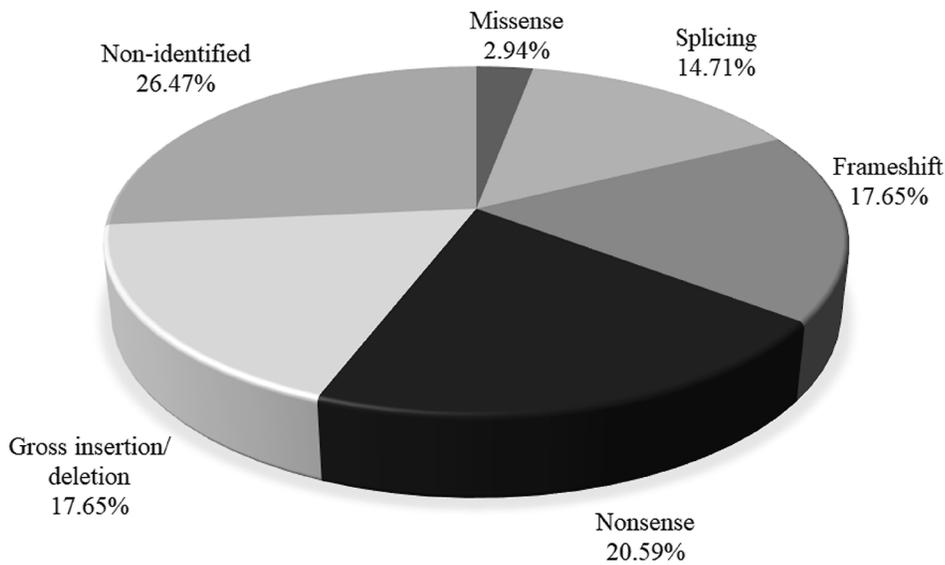


Figure 4. Distribution of non-identified and identified mutations in *Rb1* by type. The frequency of nonsense, frameshift, splice site, missense, and gross insertion/deletion mutations identified in the cohort of patients with Rb.

mutations in *Rb1* or other factors, such as an amplification of the *MYCN* oncogene (Gene ID: 4613; OMIM 164840) [21], altered pRB phosphorylation by *PIN1* gene (Gene ID: 5300; OMIM 601052) [22], viral infection [23], or hypermethylation of the CpG-rich islands in the promoter region of the *Rb1* gene, which is normally unmethylated [24]. Typically, two cases (patient RB10 and patient RB14) revealed alterations recorded as unknown and a rare single nucleotide polymorphism (SNP) from the *Rb1* Gene Mutation Database, respectively. Although these mutations have not been confirmed to be causative Rb yet, their influences might be noted and clarified by another method. Therefore, further studies on screening other elements should be examined to find out the causes of pathogenicity. However, these patients probably carry low-level mosaic mutations or mutations that reside deep in introns, which were undetected with current screening methods. In these situations, another approach for uncovering such mutations such as next-generation sequencing is desired [25].

APPENDIX 1. CLINICAL FEATURES OF PATIENTS

To access the data, click or select the words “[Appendix 1](#)” *: sibling, F: female, M: male, B: bilateral, U: unilateral, Fa: family, S: sporadic, L: left eye, R: right eye, NA: not available

ACKNOWLEDGMENTS

We thank all patients and families that participated in this study. This work was supported by the Institute of Genome Research (Grant No.24aQDNCHG), Vietnam Academy of Science and Technology.

REFERENCES

1. Kivelä T. The epidemiological challenge of the most frequent eye cancer: retinoblastoma, an issue of birth and death. *Br J Ophthalmol* 2009; 93:1129-31. [PMID: 19704035].
2. Shields JA, Augsburger JJ. Current approaches to the diagnosis and management of retinoblastoma. *Surv Ophthalmol* 1981; 25:347-72. [PMID: 7017985].
3. Knudson AG. Mutation and Cancer: Statistical Study of Retinoblastoma. *Proc Natl Acad Sci USA* 1971; 68:820-3. [PMID: 5279523].
4. Serrano ML, Yunis JJ. Identification of three new mutations in the *Rb1* gene in patients with sporadic retinoblastoma in Colombia. *Biomedica* 2013; 33:53-61. [PMID: 23715307].
5. Vogel F. Genetics of retinoblastoma. *Hum Genet* 1979; 52:1-54. [PMID: 393614].
6. Lohmann DR, Gallie BL. Retinoblastoma: Revisiting the model prototype of inherited cancer. *Am J Med Genet C Semin Med Genet* 2004; 129C:23-8. [PMID: 15264269].
7. Abouzeid H, Schorderet DF, Balmer A, Munier FL. Germline mutations in retinoma patients: relevance to low-penetrance and low-expressivity molecular basis. *Mol Vis* 2009; 15:771-7. [PMID: 19390654].

8. Usmanov RH, Kivelä T. Predicted Trends in the Incidence of Retinoblastoma in the Asia-Pacific Region. *Asia Pac J Ophthalmol (Phila)* 2014; 3:151-7. [PMID: 26107585].
9. Rodríguez M, Salcedo M, González M, Coral-Vazquez R, Salamanca F, Arenas D. Identification of novel mutations in the gene in Mexican patients with retinoblastoma. *Cancer Genet Cytogenet* 2002; 138:27-31. [PMID: 12419581].
10. Alonso J, Frayle H, Menéndez I, López A, García-Miguel P, Abelairas J, Sarret E, Vendrell MT, Navajas A, Artigas M, Indiano JM, Carbone A, Torrenteras C, Palacios I, Pestaña A. Identification of 26 new constitutional RB1 gene mutations in Spanish, Colombian, and Cuban retinoblastoma patients. *Hum Mutat* 2005; 25:99-[PMID: 15605413].
11. Abidi O, Knari S, Sefri H, Charif M, Senechal A, Hamel C, Rouba H, Zaghoul K, El Kettani A, Lenaers G, Barakat A. Mutational analysis of the RB1 gene in Moroccan patients with retinoblastoma. *Mol Vis* 2011; 17:3541-7. [PMID: 22219649].
12. Thirumalairaj K, Abraham A, Devarajan B, Gaikwad N, Kim U, Muthukkaruppan V, Vanniarajan A. A stepwise strategy for rapid and cost-effective RB1 screening in Indian retinoblastoma patients. *J Hum Genet* 2015; 60:547-[PMID: 26084579].
13. Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell* 1995; 81:323-30. [PMID: 7736585].
14. Dyson NJ. RB1: a prototype tumor suppressor and an enigma. *Genes Dev* 2016; 30:1492-502. [PMID: 27401552].
15. De Falco G, Giordano A. pRb2/p130: a new candidate for retinoblastoma tumor formation. *Oncogene* 2006; 25:5333-[PMID: 16936755].
16. Skalet AH, Gombos DS, Gallie BL, Kim JW, Shields CL, Marr BP, Plon SE, Chévez-Barrios P. Screening children at risk for retinoblastoma: consensus report from the American Association of Ophthalmic Oncologists and Pathologists. *Ophthalmology* 2018; 125:453-8. [PMID: 29056300].
17. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res* 2002; 30:e57-57. [PMID: 12060695].
18. Richter S, Vandezande K, Chen N, Zhang K, Sutherland J, Anderson J, Han L, Panton R, Branco P, Gallie B. Sensitive and Efficient Detection of RB1 Gene Mutations Enhances Care for Families with Retinoblastoma. *Am J Hum Genet* 2003; 72:253-69. [PMID: 12541220].
19. Ahani A, Behnam B, Khorram Khorshid HR, Akbari MT. RB1 Gene mutations in Iranian patients with retinoblastoma: report of four novel mutations. *Cancer Genet* 2011; 204:316-22. [PMID: 21763628].
20. He M-y, An Y, Gao Y-j, Qian X-w, Li G, Qian J. Screening of RB1 gene mutations in Chinese patients with retinoblastoma and preliminary exploration of genotype-phenotype correlations. *Mol Vis* 2014; 20:545-52. [PMID: 24791139].
21. Rushlow DE, Mol BM, Kennett JY, Yee S, Pajovic S, Thériault BL, Prigoda-Lee NL, Spencer C, Dimaras H, Corson TW, Pang R, Massey C, Godbout R, Jiang Z, Zacksenhaus E, Paton K, Moll AC, Houdayer C, Raizis A, Halliday W, Lam WL, Boutros PC, Lohmann D, Dorsman JC, Gallie BL. Characterisation of retinoblastomas without RB1 mutations: genomic, gene expression, and clinical studies. *Lancet Oncol* 2013; 14:327-34. [PMID: 23498719].
22. Rizzolio F, Lucchetti C, Caligiuri I, Marchesi I, Caputo M, Klein-Szanto AJ, Bagella L, Castronovo M, Giordano A. Retinoblastoma tumor-suppressor protein phosphorylation and inactivation depend on direct interaction with Pin1. *Cell Death Differ* 2012; 19:1152-61. [PMID: 22322860].
23. Palazzi MA, Yunes JA, Cardinalli IA, Stangenhaus GP, Brandalise SR, Ferreira SA, Sobrinho JSP, Villa LL. Detection of oncogenic human papillomavirus in sporadic retinoblastoma. *Acta Ophthalmol Scand* 2003; 81:396-8. [PMID: 12859268].
24. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 2002; 21:5427-[PMID: 12154405].
25. Li WL, Buckley J, Sanchez-Lara PA, Maglinte DT, Viduetsky L, Tatarinova TV, Aparicio JG, Kim JW, Au M, Ostrow D, Lee TC, O’Gorman M, Judkins A, Cobrinik D, Triche TJ. A rapid and sensitive Next-Generation Sequencing method to detect RB1 mutations improves care for retinoblastoma patients and their families. *J Mol Diagn* 2016; 18:480-93. [PMID: 27155049].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 17 March 2018. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.