Basic Medical Research

Novel point mutation and intronic mutations of *RB1* gene in retinoblastoma patients in Indonesia

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pISSN: 0853-1773 • eISSN: 2252-8083
https://doi.org/10.13181/mji.oa.236544
Med J Indones. 2022;31:218–24

Received: October 22, 2022
Accepted: March 10, 2023
Published online: March 29, 2023

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ABSTRACT

BACKGROUND Retinoblastoma (RB) is an inherited disorder caused by the *RB1* gene mutation in retinal cells or germline mutation. Identifying the specific mutation is crucial for prognosis, inheritance risk assessment, and treatment planning. This study aimed to identify the germline mutation in the *RB1* gene in patients with RB and their parents from the eastern part of Indonesia.

METHODS This observational analytic study recruited patients with RB and their parents between 2016 and 2018 at Dr. Wahidin Sudirohusodo Hospital, Makassar, Indonesia. The normal control subjects were children from the outpatient clinic at the Department of Ophthalmology, Universitas Hasanuddin Hospital. Ophthalmic examinations and peripheral blood tests were performed in RB patients, their parents, and control subjects. Genomic DNA was isolated from blood leukocytes and amplified using conventional PCR. Hotspot exons 8, 10, 14, 17, and 22 were screened for mutations using the Sanger method.

RESULTS There were 21 patients with RB (16 unilateral and 5 bilateral) and 14 normal subjects. Of the 184 variations detected in RB patients, 164 were also found in normal subjects. 19 intronic mutations in introns 10, 16, 17, and 21, and 1 novel missense mutation in exon 17 were identified. Parental testing revealed 8 substitutions in exon 17 and 5 intronic mutations in introns 16 and 17 of the parents. None of the variations in exons were passed to their children.

CONCLUSIONS This study found a novel missense mutation in exon 17 of the *RB1* gene.

KEYWORDS germline mutation, *RB1* gene, retinoblastoma

Retinoblastoma (RB) is the most common eye neoplasm in childhood,[1] with approximately 50% of cases being heritable due to a mutation of the *RB1* gene that predisposes the development of retinal tumors. The *RB1* gene mutation occurs in retinal cells (somatic mutation) or gametes (germline mutation) in 98% of RB cases.[2] This gene comprises 27 exons spanning 178,143 kb genomic DNA at chromosome 13q14[3,4] and encodes RB protein (pRB), which is a nuclear phosphoprotein and crucial for regulating cell cycle progression.[4,5] More than 1,900 mutations in the *RB1* gene have been reported globally,[6] and identifying these mutations in RB patients is crucial for developing mutational analysis procedures and understanding the molecular mechanism underlying disease penetrance and expressivity.

Familial RB mutations exhibit variable expressivity and phenotypic variability.[6–10] The RB1 mutations associated with low penetrance include promoter mutations, missense mutations, and in-frame

deletions/insertions.[11,12] These mutations are autosomal dominant; thus, children whose parents have the *RB1* gene mutation are at a higher risk of developing RB and other cancers such as osteosarcoma, small cell lung carcinoma, bladder tumor, and breast carcinoma.[6] Identifying mutation type in patients with RB and their parents is essential for assessing short-term prognosis (intraocular tumor in the same eye or fellow eye), long-term prognosis (the risk of non-ocular malignancies), inheritance risk, and treatment strategy.[13,14] Mutational screening of the *RB1* gene can contribute to clinical management and provide accurate genetic counseling to patients and their families. Despite the importance of *RB1* gene mutational analysis, there is a lack of data about germline *RB1* mutations in RB patients in Indonesia. This study aimed to identify the *RB1* gene mutations in patients with RB in the eastern part of Indonesia.

METHODS

Subjects

All subjects were recruited between 2016 and 2018 at Dr. Wahidin Sudirohusodo Hospital, Makassar, Indonesia, which included patients diagnosed with RB and their parents. The diagnosis of RB was established through standard ophthalmological and histological examinations. Patients with a history of radiotherapy or other malignancies were excluded from the study. The control group was composed of children at the outpatient clinic at the Department of Ophthalmology, Universitas Hasanuddin Hospital, who had no abnormal ophthalmological findings except for mild refractive error and had neither history nor family history of neoplasm.

Sample collection and DNA extraction

A total of 5 ml peripheral blood samples were collected from patients and their parents into standard ethylenediaminetetraacetic acid (EDTA) tubes, and the DNA sample was stored at –20°C. The available parental samples were examined for the *RB1* gene mutation at the same nucleotide position as their child who had a germline mutation. The blood samples were analyzed at Hasanuddin University Medical Research Center laboratory. DNA was isolated from peripheral blood leukocytes using gSYNC DNA Extraction Kit (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's protocol.[15]

Sample preparation

Transfer up to 200 µl of whole blood to a 1.5 ml microcentrifuge tube. Adjust the volume to 200 µl with phosphate buffered saline. Add 20 µl of proteinase K then mix by pipetting. Incubate at 60°C for 5 min.

Cell lysis

Add 200 µl of gel sample buffer, then mix by shaking vigorously. Incubate at 60°C for 5 min, add proteinase, then incubate again for 5 min.

DNA binding

Add 200 µl of absolute ethanol to the sample lysate and mix immediately by shaking vigorously for 10 sec. Transfer all of the mixture to the GS Column. Centrifuge at 14–16,000 × *g* for 1 min. Transfer the GS Column to a new 2 ml collection tube.

Wash

Add 400 µl of W1 buffer to the GS Column. Centrifuge at 14–16,000 × *g* for 30 sec. Add 600 µl of wash buffer (make sure absolute ethanol was added) to the GS Column. Centrifuge at 14–16,000 × *g* for 30 sec. Centrifuge for 3 min at 14–16,000 × *g*.

Elution

Transfer the dried GS Column to a 1.5 ml microcentrifuge tube. Add 100 µl of pre-heated elution buffer, Tris-EDTA buffer. Let stand for at least 3 min. Centrifuge at 14–16,000 × *g* for 30 sec. DNA quality was evaluated with 2% agarose gel electrophoresis at 100 V (Bio-Rad, USA); the DNA quantity was not calculated.

Polymerase chain reaction (PCR) and sequencing

Screening of germline mutations in the *RB1* gene was performed by direct Sanger sequencing for exons 8, 10, 14, 17, and 22. The primer sequences followed the previously published primer sequences for the *RB1* gene, as presented in Table 1.[3] The DNA was amplified in a PCR DNA thermal cycler (Bio-Rad, USA) in a total volume of 50 µl. The PCR component was KAPPA2G fast enzyme, forward and reverse *RB1* gene primer (Table 1), nuclease-free water, and DNA template. PCR cycling was initiated with the denaturation step at 95°C for 15 min, then 94°C for 1 min, annealing at 55°C for 30 sec, extension at 72°C for 1.5 min as much as 45 cycles, continued with the last extension at 72°C for 10 min and 12°C for 30 min for storage. The PCR product was sequenced to detect mutation in

Table 1. Sequence of primers used to amplify exons of the *RB1* gene in Indonesian patients with RB

Primer name	Forward primer	Reverse primer	Length (bp)
RB1 exon 8	5′-GCAGAGTAGAAGAGGGATGGC-3′	5′-TGATTCCAGAGTGAGGGAGC-3′	558
RB1 exon 10	5′-TTTATATTGCATGCGAACTCAG-3′	5′-GGTAACTGTTATAGGACACACAATTC-3′	469
RB1 exon 14	5′-GTGATTTTCTAAAATAGCAGG-3′	5′-TGCCTTGACCTCCTGATCTG-3′	191
RB1 exon 17	5′-TCAAAATTGGAAGGCTATTTCC-3′	5′-TTAGATGGTTTAGGGTGCTCG-3′	567
RB1 exon 22 + 23	5′-TCCTTTATAATATGTGCTTCTTACCAG-3′	5′-TTCTTGGATCAAAATAATCCCC-3′	577

bp=base pair; RB=retinoblastoma

the *RB1* gene, then analyzed using BioEdit Sequence Alignment Editor version 7.0.5.1. software (Tom Hall; North Carolina State University, USA). The results were then compared with the data in Gene Bank of National Center for Biotechnology Information GRCh37 database using basic local alignment search tool. Additional information about mutations in the *RB1* gene was confirmed from the *RB1* variation database *RB1*-lsdb and the Human Gene Mutation Database.

Statistical analysis

The demographic data between normal subjects and patients with RB were compared using the Mann–Whitney *U* test. A *p*-value of <0.05 was considered statistically significant. The statistical analyses were performed using the JMP software version 13.0 (SAS Institute Inc., USA).

Ethical considerations

The procedures adhered to the tenets of the Declaration of Helsinki and were approved by the Ethics Committee of the Faculty of Medicine, Universitas Hasanuddin (No. 555/UN4.6.4.5.31/PP36/2019). Written and signed informed consent from the parents was obtained before the examinations.

RESULTS

A total of 21 patients with RB and 14 normal subjects were included in this study. RB patients were mostly diagnosed at a later stage, with 81% of patients exhibiting tumor extension. Additionally, 14% of patients were classified as Group V according to the Reese-Ellsworth classification, and 5% had a regressed type of RB. Of the patients, 76% had unilateral RB, and 24% had bilateral RB. Table 2 presents the demographic data for these groups.

Ten patients with unilateral RB and one with bilateral RB had the *RB1* gene variations. The mean paternal age was 36 years old, and the mean maternal age was 32 years old. Ten out of 11 fathers had a smoking habit, whereas none of the mothers smoked. Furthermore, the parents had no history of radiation exposure or a family history of RB.

In this study, 184 variations were discovered in RB patients, with 164 of which were also found in normal subjects. Nineteen intronic mutations in introns 10, 16, 17, and 21, and one missense mutation in exon 17 were identified; these mutations were not found in normal subjects. One patient (5%) (R33) had a germline mutation among the 21 patients. The heterozygous mutation identified in exon 17 of R33 is presented in Figure 1, which caused an amino acid change from tryptophan to glycine. No family members of R33 had a history of RB. The mutation list of the *RB1* gene in RB patients is shown in Table 3.

The analysis of the parental origin of the detected mutations revealed the *RB1* gene variations in seven out of 21 parents whose children tested positive for the *RB1* gene mutations. The variations included eight substitutions in exon 17 and five intronic mutations in introns 16 and 17, as shown in Table 3. However, none of the variations in exons were passed to their children who had RB.

DISCUSSION

This study conducted a comprehensive screening of germline mutations of the *RB1* gene in Indonesian patients with RB and found a 5% germline RB mutation rate. The prevalence of the *RB1* gene germline mutation varies worldwide, ranging from 37% in Italy to 51% in India among patients with RB. Germline mutation occurs in 51.06, 37.14, and 42.4% of patients with RB in India, Italy, and Singapore, respectively.[8,9,16]

Table 2. Demographic data of patients with RB and normal subjects

Parameter	RB, n (N = 21)	Normal, n (N = 14)	*p**
Patient's characteristics			
Age (years), mean (SD)	3.90 (4.07)	4.07 (4.09)	0.057
Male sex	10	5	0.484
Laterality			-
Unilateral	10	-	
Bilateral	1	-	
Parent's characteristics			
Paternal age (years), mean (SD)	33.76 (9.34)	31.78 (5.67)	0.829
Maternal age (years), mean (SD)	32.33 (7.36)	32.71 (4.78)	0.855
Paternal smoking habit			0.414
Yes	17	9	
No	4	5	
Maternal smoking habit			0.727
Yes	0	1	
No	21	13	
Paternal history of radiation exposure			0.293
Yes	0	3	
No	21	11	
Maternal history of radiation exposure			0.727
Yes	0	1	
No	21	13	
Paternal family history of RB			-
Yes	0	0	
No	21	14	
Paternal family history of RB			-
Yes	0	0	
No	21	14	

RB=retinoblastoma; SD=standard deviation. *Mann–Whitney *U* test

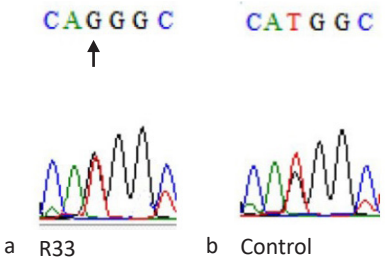

Figure 1. Sequencing and pedigree of patient with novel exon mutation. (a) Sequencing result of a patient (R33) with heterozygous mutation of exon 17, c.1687T>G (arrow); (b) sequencing result of normal subjects

Moreover, the rate is higher in Vietnam (73.53%).[6] The prevalence of germline mutation of the *RB1* gene in various countries are presented in Table 4.[8–10,16–22]

Notably, this study found a novel missense mutation in exon 17 in patients with a unilateral tumor, but no germline mutation was detected in patients with bilateral RB. A study in Singapore found that 71% of patients with bilateral RB and 29% with unilateral RB had a *RB1* gene germline mutation.[16] Meanwhile, these mutations were detected in 44% of Vietnamese patients with a unilateral tumor and 84% with a bilateral tumor.[6]

Exon 17 is crucial as it encodes the domains A and B of the pRB pocket, necessary for repressor activity that blocks cell growth by binding to transcriptional factors E2F/DP.[4,23] Mutations in this repressor domain may disturb growth suppression and cause loss of cell growth control. The identified *de novo* missense mutation in exon 17 induced amino acid changes from tryptophan to glycine. Tryptophan is an essential amino acid with amphipathic properties, exhibiting weak hydrophilic and hydrophobic characteristics.[24–26] Consequently, it tends to compose the inner core of soluble proteins.[27] Meanwhile, glycine is a non-

Table 3. Data of the *RB1* gene mutations in patients with RB

Patient ID	Exon/intron No	g. position	c. position	Type of mutation	Present in mother/father
R6	Intron 17	g.78390A>T	c.1695+110C>T	Intronic	No/No
R6	Intron 17	g.78425T>A	c.1695+145T>A	Intronic	No/No
R6	Intron 17	g.78426C>A	c.1695+146C>A	Intronic	No/No
R6	Intron 17	g.78430T>G	c.1695+150T>G	Intronic	No/No
M6	Intron 17	g.78301G>A	c.1695+21G>A	Intronic	-
M6	Exon 17	g.78176T>G	c.1592T>G	Missense	-
R8	Intron 17	g.78393G>A	c.1695+113G>A	Intronic	No/No
M8	Intron 16	g.77969DelA	c.1499-113DelA	Intronic	-
M8	Exon 17	g.78108T>G	c.1524T>G	Silent mutation	-
M8	Exon 17	g.78143T>G	c.1559T>G	Missense	-
R10	Intron 17	g.78367C>T	c.1695+87C>T	Intronic	No/NA
R13	Intron 17	g.78301G>A	c.1695+21G>A	Intronic	No/No
R15	Intron 10	g.64575InsC	c.1049+135InsC	Intronic	No/No
M15	Exon 17	g.78130T>G	c.1546T>G	Missense	-
R16	Intron 10	g.64570T>C	c.1049+130T>C	Intronic	No/No
R16	Intron 10	g.64576InsC	c.1049+136InsC	Intronic	No/No
R16	Intron 10	g.64449T>C	c.1049+9T>C	Intronic	No/No
R17	Intron 10	g.64599InsA	c.1049+159InsA	Intronic	No/No
R32	Intron 21	g.161917A>T	c.2212-79A>T	Intronic	No/No
R32	Intron 21	g.161917InsC	c.2212-79InsC	Intronic	No/No
R32	Intron 21	g.161937InsT	c.2212-59InsT	Intronic	No/No
M32	Exon 17	g.78271T>G	c.1687T>G	Missense	-
M32	Intron 17	g.78301G>A	c.1695+21G>A	Intronic	-
M32	Exon 17	g.78108T>G	c.1524T>G	Silent mutation	-
M32	Exon 17	g.78130T>G	c.1546T>G	Missense	-
R33	Intron 16	g.77969DelA	c.1499-113DelA	Intronic	NA/No
R33	Exon 17	g.78271T>G	c.1687T>G	Missense*	NA/No
R33	Intron 17	g.78301G>A	c.1695+21G>A	Intronic	NA/Yes
F33	Intron 17	g.78301G>A	c.1695+21G>A	Intronic	-
R35	Intron 16	g.77969DelA	c.1499-113DelA	Intronic	Yes/No
M35	Intron 16	g.77969DelA	c.1499-113DelA	Intronic	-
R36	Intron 16	g.77969DelA	c.1499-113DelA	Intronic	No/No
M36	Exon 17	g.78130T>G	c.1546T>G	Missense	-

Del=deletion; ID=identity; Ins=insertion; NA=not available; RB=retinoblastoma. All patients had unilateral RB except R8 and had intronic mutation except R33. *Novel mutation

essential hydrophobic amino acid with unique features, including its backbone consisting of only one hydrogen atom, which makes it more flexible than other amino acids.[25–28] Due to this characteristic, glycine is often located at the interface where two polypeptides come into contact. In the *RB1* gene, even a single amino acid substitution may result in a dysfunctional pRB, compromising its role as a tumor suppressor gene.[29] However, additional analysis such as minigene assay or *in silico* analysis may be necessary to confirm the pathogenicity of a mutation.[30,31] This was another limitation of this study; we did not perform additional assay and pathogenicity assessment. This low prevalence of germline mutation might be due to the inability to identify mutational change in all *RB1* gene exons, as some "hot-spot" exons (8, 10, 14,

Table 4. Prevalence of the *RB1* gene germline mutation in various countries

First author, year	Germline mutation prevalence (%)	Country	Year
Alonso,[17] 2001	69	Spain	2001
Kiran,[8] 2003	14.8 (tumor) and 65 (peripheral blood alone)	India	2003
Sampieri,[9] 2006	37	Italy	2006
Kontic,[18] 2006	43.8	Serbia	2006
Macias,[19] 2008	64.3	Mexico	2008
Ali,[20] 2010	66.2	Saudi	2010
Barbosa,[21] 2013	86.2	Brazil	2013
Ayari-Jeridi,[10] 2015	18.9	Tunisia	2015
Tomar,[16] 2017	19.5	Singapore	2017
Parma,[22] 2017	20	Argentina	2017

17, and 22) are more commonly affected.[16] Another possibility was due to the small sample size.

This study compared sequencing results between the RB patients and normal subjects (control) to reduce the possibility of normal gene variants or polymorphism. DNA exon primers were used,[3] as well as DNA sequence to read the adjacent intronic parts. Of the 20 mutations discovered, 90% were intronic mutations, with deep intronic mutations accounting for 66.67%. Although only a few bases deep in introns are known to be effective targets for oncogenic mutations,[32] point mutations deep within introns (>100 bp from a splice junction) may alter normal splicing, transcription regulatory motifs, and non-coding RNA genes.[33–35] Some promoters also collaborate with regulatory sequences within the intron.[36]

In most cases, RB was unilateral and sporadic, and the parents appeared genetically normal. In some families, carriers had no tumor (reduced penetrance) or only unilateral RB or benign retinocytoma (reduced expressivity).[11] These variations in the phenotype may be caused by various factors such as immunologic factors, epigenetic mechanisms, delayed mutation, host resistance, and modulatory genes.[37–41]

Our findings have implications for prognosis and genetic counseling for the patients and their families. We provided genetic counseling to one pregnant patient with unilateral regressed RB (R3), and no germline mutation was detected. However, we advised her about the possibly inheriting the condition on to her offspring, even though the risk was smaller than for patients with a germline mutation. Since most parents were of their active reproductive age, comprehensive genetic counseling about RB is crucial.

In conclusion, one novel missense mutation was identified in exon 17 of the *RB1* gene, which was not detected in normal subjects. This finding has implications for prognosis, genetic counseling, and risk prediction for affected families. Additional studies are needed to confirm the pathogenicity of the mutation and explore other mutational changes in the entire *RB1* gene sequencing.

Conflict of Interest

The authors affirm no conflict of interest in this study.

Acknowledgment

This study was supported by the Universitas Hasanuddin Research Grant (Grant No.2006/UN4.1/KEP/2019). We thank Handayani Halik and Asep M. Ridwanulloh who provided insight and expertise that greatly assisted the research. English language editing and review were done by John Ellis, MD, Ph.D from University of Dundee, United Kingdom.

Funding Sources

This study was funded by Universitas Hasanuddin Research Grant (Grant No.2006/UN4.1/KEP/2019).

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