Clinical evaluation of RB1 genetic testing reveals novel mutations in Vietnamese patients with retinoblastoma

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Abstract. Clinical evaluation of the genetic testing strategy is essential for ensuring the correct determination of mutation carriers. The current study retrospectively analyzed genetic and clinicopathological data from 62 Vietnamese patients with retinoblastoma (RB) referred to the Vinmec Hi-Tech Center for RB transcriptional corepressor 1 (*RB1*) genetic testing between 2017 and 2019. The present study aimed to evaluate the sensitivity of the Next Generation Sequencing (NGS) method to identify novel *RB1* mutations, and to consider using age at diagnosis as a risk factor. Genomic DNA was analyzed with custom panel based targeted NGS. NGS was performed on the Beijing Genomics Institute (BGI) sequencing platform, and pathogenic or likely pathogenic variants were confirmed by Sanger sequencing, quantitative PCR (qPCR) or Multiplex Ligation-dependent Probe Amplification assay (MLPA). Constitutional *RB1* variants were identified in 100% (25/25) of the bilateral cases, while several common previously reported *RB1* mutations were also recorded. In addition, in Vietnamese patients with RB, nine novel *RB1* mutations were identified. Children aged between 0-36 months were more likely to be *RB1* carriers compared with those aged >36 months. The current findings indicated that the NGS method implemented in the Vinmec Hi-Tech Center was highly accurate, and age at diagnosis may be used to assess the risk of hereditary RB. Furthermore, the newly identified *RB1* mutations may provide additional data to improve the current understanding of the mechanisms underlying *RB1* inactivation and the development of rapid assays for detecting *RB1* mutations. Overall, the present study suggested that NGS may be applied for detecting germline *RB1* mutations in routine clinical practice.

Introduction

Retinoblastoma (RB) is the most common intraocular malignancy, characterized by high mortality if not detected early and treated promptly. Early diagnosis and intervention play a key role in the successful treatment of RB (1). Delayed diagnosis of RB for >6 months from the first clinical sign has been reported to be associated with a mortality rate of ~70% (2). Therefore, although in patients with RB diagnosed at stage A of the disease the eyes or vision can be salvaged, currently, there is not an effective treatment approach for those diagnosed at stage E (3,4).

RB is considered as a monogenetic hereditary type of cancer since 97% of RB cases are caused by the disruption of the RB transcriptional corepressor 1 (*RB1*) tumor suppressor gene (5). The *RB1*-encoded protein (pRB) acts as a scaffold protein, which interacts with other proteins to regulate multiple cellular processes essential for cell fate and function. Consequently, *RB1* deficiency may predispose cells to tumorigenesis. In fact, it has been reported that *RB1* inactivation is detected in several types of cancer. Since pRB interacts with other proteins through cyclin folds in the N-terminus and pocket domain, and intrinsically disordered structures in the C-terminus, a wide spectrum of mutations dispersed throughout the *RB1* gene has been identified in patients with cancer (6).

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It has been also reported that 45% of patients with RB are suffering from the inherited type of the disease (*RB1* carriers), where the first allele of *RB1* is mutated during preconception or shortly after conception, predisposing the child to retinal tumorigenesis (7). The remaining *RB1* allele is functionally lost during retinal development, promoting the initiation of RB in either both eyes (bilateral RB) or one eye (unilateral RB) with multifocal tumors. 'Non-heritable or sporadic RB' (non-*RB1* carriers) is commonly referred to as patients without germline *RB1* mutations, who usually present with unifocal tumors in one eye (8). Patients with hereditary RB exhibit a worse prognosis, are sensitive to certain treatments, have a high risk of developing second primary malignancies and can pass the mutations on to their offspring (3,9). Therefore, identifying germline *RB1* mutations is of great importance for implementing the appropriate treatment approach, and assessing the risk of developing second primary malignancies, both secondary RB and other primary malignancies, in patients with RB, and the risk of RB onset in the patient's relatives.

Genetic testing and counseling (GTC) are recommended for all patients with RB and are integrated in the management of RB in developed countries (10), thus resulting in high survival rate of patients with RB and cost-effective medical treatments for RB (11,12). GTC for patients with bilateral or familial RB is straightforward. However, for unilateral, non-familial RB cases, GTC is not as easy due to the low risk of *RB1* mutation carriers, and the inefficient detection of germline *RB1* mosaicism (13). Genetic testing for *RB1* is a time-consuming and expensive procedure given the large size of the *RB1* gene, which can be inactivated by multiple mutations, and the absence of mutational hotspots (14,15). Direct sequencing is widely applied for detecting *RB1* mutations, however, this method is not recommended for identifying low allelic-fraction variants (16). For the detection of these types of variants, PCR can be applied only when the mutations are already known. In addition, Multiplex Ligation-dependent Probe Amplification (MLPA), quantitative PCR (qPCR) or array Comparative Genomic Hybridization (aCGH) can identify large *RB1* rearrangements. The combination of the aforementioned methods is essential for detecting all possible *RB1* mutations (17-19). Recently, Next Generation Sequencing (NGS) has been implemented as rapid and effective strategy for identification of *RB1* mutations since all variations can be detected in a single test, thus providing a number of advantages, including high sensitivity and cost-effectiveness (20-25). In Vietnam, Sanger sequencing coupled with MLPA (SS-MLPA) could detect germline *RB1* mutations in 82-84% of bilateral cases (26,27). Nevertheless, the sensitivity of NGS in a routine clinical practice remains unknown.

The two-hit hypothesis suggests that patients with bilateral or unilateral multifocal RB, and/or diagnosed at an early age are more likely to carry germline *RB1* mutations (28). This hypothesis is supported by clinical data. Therefore, a study demonstrated that up to 100% of patients with bilateral carried germline *RB1* mutations, and their age at diagnosis was 10 months younger compared with that of the unilateral cases (13). Bilateral and unilateral eye diseases account for 40 and 60% of all RB cases (7), respectively. All bilateral RB cases are considered heritable, whereas ~15% of unilateral cases carry constitutional *RB1* mutations (29,30). Since age at diagnosis is younger in bilateral cases, previous studies have associated age at diagnosis with germline *RB1* status in order to predict age associated with increased risk of patients with unilateral disease being *RB1* carriers. Unfortunately, these studies yielded conflicting results (31-34).

The current study retrospectively analyzed the clinicopathological and genetic data of patients with RB to evaluate the sensitivity of NGS for detecting constitutional *RB1* variants, to detect novel germline *RB1* mutations, and to consider age at diagnosis as a risk factor for patients being *RB1* carriers.

Materials and methods

Patients and samples. In the current retrospective study, a total of 62 patients with RB were included, who were referred to Department of Cancer Research, Vinmec Research Institute of Stem Cell and Gene Technology and the Department of Medical Genetics, Vinmec Hi-Tech Center for genetic testing between 2017 and 2019. Signed informed consents were obtained from parents/caregivers of all subjects and the study was approved by the Vinmec's Institutional Review Board. All data, including age at diagnosis, sex, tumor stages, laterality and family history were retrieved from the patients' medical records. Genetic testing for germline *RB1* variants was performed at the Beijing Genomics Institute (BGI), HongKong. The workflow for analyzing germline *RB1* variants is illustrated in Fig. 1.

Preparation of tissue samples, NGS and variant calling. Genomic DNA (gDNA) was extracted from peripheral blood mononuclear cells and its concentration was quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Inc.). Subsequently, gDNA was fragmented, indexed and amplified. The *RB1* promoter and all its exons, plus 20 nucleotides proximal to either 5' or 3' of each exon, were captured by a BGI chip. Library size and quantity were verified using Qubit® 2.0 (Thermo Fisher Scientific, Inc.) and an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). Sequencing was performed on the BGIseq platform. All samples identified with pathogenic or likely pathogenic variants were confirmed by Sanger Sequencing, qPCR or MLPA.

Bioinformatics analysis. The sequence reads were aligned to the human reference genome (GRCh37/Hg19) using the Burrows-Wheeler Aligner. Single nucleotide variants, insertion/deletion variants (InDel) and copy number variations were identified using the BGI internal NGS software (BGISEQ-500).

Variant annotation. Interpretation of germline variants followed the American College of Medical Genetics and Genomics (ACMG) standards and guidelines (35). The variants were designated according to nomenclature and the recommendations of the Human Genome Variation Society (36). Public databases, including Clinvar (37), Universal Mutation Database (38), Leiden Open Variation Database (LOVD) (39) and ARUP (40) were used for data analysis. The SIFT (41), POLYPHEN2 (42) and Mutation taster (43) tools were applied to predict deleterious mutations, while variants were classified using the Varsome (44) and InterVar (45) classification tools.

Identification of novel variants. All variants identified by the BGI laboratory were annotated with the Ensemble Variant Effect
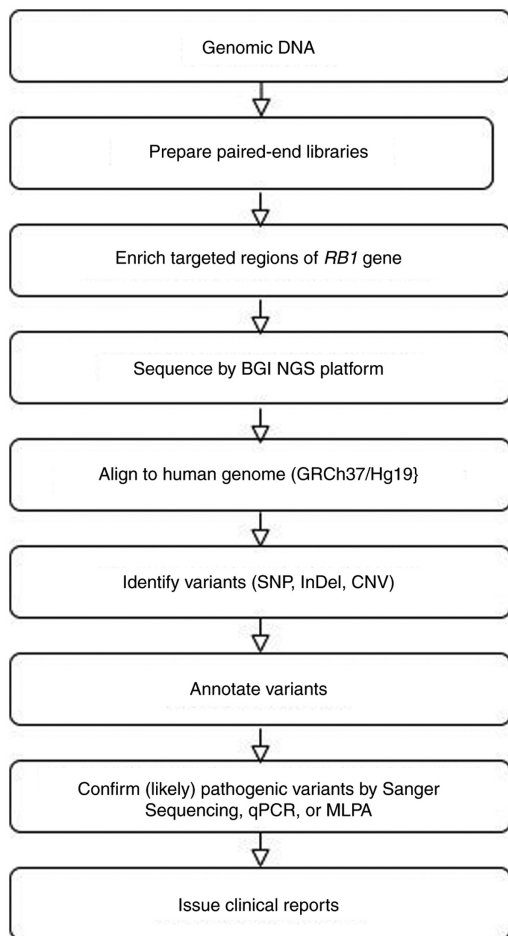
Figure 1. Workflow for analyzing *RB1* germline variants. RB1, RB transcriptional corepressor 1; NGS, next generation sequencing; NSP, single nucleotide polymorphism; InDel, insertion/deletion; CNV, copy number variant; qPCR, quantitative PCR; MLPA, Multiplex Ligation-dependent Probe Amplification; BGI, Beijing Genomics Institute.

Predictor (EVEP) tool (46). Variants with existing ID were checked on corresponding databases. The unmatched variants and those without existing ID were screened on PubMed. Variants not previously reported in public databases and PubMed were considered as novel ones. The allele frequency of the identified novel variants was investigated in four databases, including the 1000 Genomes Project (browser.1000genomes.org), the Exome Sequencing Project (esp.gs.washington.edu/drupal), the Genome Aggregation Database and the Vietnamese Genetic Variation Database (47). Deleterious novel variants were evaluated by Combined Annotation Dependent Depletion (CADD) (48) and EVEP, while their pathogenicity was interpreted using VarSome.

Statistical analysis. The differences between the characteristics of *RB1* and non-*RB1* carriers were compared using a χ^2 test, Fisher's exact test or Student's t-test. Fisher's exact test and odds ratio (OR) were calculated using calculators provided by the Social Science Statistics website (49) and MedCalc statistical software (50), respectively.

Results

Characteristics of RB1 and non-RB1 carriers. A total of 25 bilateral and 37 unilateral RB cases were included in the present study. There was no statistically significant difference in the total number of *RB1* and non-*RB1* carriers, as well as in sex distribution between the two groups. However, the mean age at diagnosis of *RB1* carriers was significantly younger compared with that of non-*RB1* carriers (22.14 vs. 32.66 months). Although 80% of all patients were diagnosed with stage E RB, the distribution of the RB stages was not statistically different between *RB1* and non-*RB1* carriers. Additionally, the proportion of *RB1* carriers was notably higher in patients with bilateral RB and significantly decreased in the unilateral cases. Constitutional *RB1* mutations were detected in 100% (25/25) and 27% (10/37) of patients with bilateral and unilateral RB, respectively (Tables I and II), suggesting a sensitivity rate of 100% for detecting germline *RB1* variants using the NGS technology.

Novel RB1 germline mutations. A total of 28 distinct variants, including four recurrent and 24 non-recurrent ones, were identified in 56% (35/62) of patients with. RB. In addition, the four recurrent mutations were found in 33% (12/35) of *RB1* carriers. Point and small InDel mutations in *RB1* were dispersed along the gene. However, large rearrangements were only identified in or nearby the pocket domain. The majority of mutations (26/28) were located in the N-terminus or pocket domain of pRB (Fig. 2). The EVEP tool predicted that all these variations, except one (c.83C>G), exerted a highly disruptive effect in pRB and were classified as pathogenic variants using the Clinvar, Cosmic or Varsome databases. The c.83C>G mutation was concurrently found with an exon 12 duplication in one patient with bilateral RB. Nonsense and slice mutations were the two most frequent mutations, identified in 34 (12/35) and 31% (11/35) of all *RB1* carriers, respectively. Additionally, large rearrangements, frameshift and missense mutations were detected in 17 (6/35), 11.4 (4/35) and 8.6% (3/35) of *RB1* carriers, respectively (Tables I and III).

The novel variants were defined by screening 28 *RB1* mutations into the EVEP tool. Among them, 16 *RB1* alterations exerted Variation ID on the Clinvar, Cosmic or LOVD databases. For the remaining 12 unidentified variants, screening on the PubMed platform was carried out. The analysis revealed three large rearrangement, including Ex1_27 DEL, Ex24 DEL and Ex13_18 DUP. Therefore, nine variants were considered as novel. These nine variations, including one large rearrangement, one nonsense, four slice and three frameshift mutations, were all null mutations, identified in seven patients with bilateral and two with unilateral RB. These variants were located at either the pocket (5/9) or N-terminus (4/9) domain. Furthermore, the EVEP tool predicted that these variants could have a significant disruptive effect on pRB, and all, except EX12 DUP, were also predicted to be among the top 1% of the most deleterious variants in the human genome by CADD. Finally, these mutations were not found in polymorphism databases, and were classified as pathogenic by VarSome (Table III; Fig. 2).

Association between age at diagnosis and the genetic status of patients with RB. The proportion of *RB1* and non-*RB1* carriers with an age at diagnosis of 0-36 and >36 months is presented in Table IV. It was found that the proportion of *RB1* carriers with age at diagnosis of 0-36 months was notably higher compared with those of >36 months. Consequently, the relative risk of children aged between 0-36 months being *RB1* carriers was significantly higher than that of children >36 months

Table I. Summary of germline *RB1* mutations identified in Vietnamese patients with retinoblastoma.

Case ID	Family history	Months at diagnosis	Laterality	Sex	Staging	*RB1* germline mutations	Molecular consequences	Interpretation	Exon/Intron	Previously reported (Refs.)
VinRB01	Yes	48	Bil	F	C/E	Ex1_27 Del	Large rearrangement	Pathogenic	Exon 1-27	(53,61-63)
VinRB02	No	38	Bil	M	A/E	Ex1_27 Del	Large rearrangement	Pathogenic	Exon 1-27	(53,61-63)
VinRB03	No	25	Bil	F	E/C	Ex1_27 Del	Large rearrangement	Pathogenic	Exon 1-27	(53,61-63)
VinRB04	No	10	Bil	F	E/A	Ex18_23 Dup	Large rearrangement	Pathogenic	Exon 18-23	(64)
VinRB05	No	30	Bil	F	D/E	Ex12_dup & c.83 C>G	Large rearrangement	Pathogenic & VUS	Exon 12 & Exon 1	Novel & rs776175164
VinRB06	No	3	Bil	M	E/B	c.210_211insAG	Frameshift	Pathogenic	Exon 2	Novel
VinRB07	No	31	Bil	M	A/E	c.515_516insA	Frameshift	Pathogenic	Exon 5	Novel
VinRB08	No	6	Bil	M	B/E	c.380G>A	Missense	Pathogenic	Exon 3	COSV57302882
VinRB09	No	18	Bil	M	E/C	c.1072C>T	Nonsense	Pathogenic	Exon 11	rs121913301
VinRB10	No	36	Bil	M	D/E	c.1403_1404insA	Frameshift	Pathogenic	Exon 15	Novel
VinRB11	No	7	Bil	F	E/B	c.1735C>T	Nonsense	Pathogenic	Exon 18	rs121913305
VinRB12	Yes	6	Bil	M	B/B	c.1735C>T	Nonsense	Pathogenic	Exon 18	rs121913305
VinRB13	No	5	Bil	F	B/E	c.2359C>T	Nonsense	Pathogenic	Exon 23	rs137853293
VinRB14	No	1	Bil	M	B/B	c.306T>A	Nonsense	Pathogenic	Exon 3	Novel
VinRB15	No	26	Bil	F	E/E	c.958C>T	Nonsense	Pathogenic	Exon 10	rs121913300
VinRB16	No	29	Bil	F	E/E	c.958C>T	Nonsense	Pathogenic	Exon 10	rs121913300
VinRB17	No	31	Bil	M	B/E	c.1953_c.1960+1delTAAAAAGG	Splice	Likely pathogenic	Exon 19	Novel
VinRB18	No	19	Bil	F	B/E	c.1128-1G>A	Splice	Pathogenic	Intron 11	COSV57294096
VinRB19	No	20	Bil	M	B/E	c.1215+1G>A	Splice	Pathogenic	Intron 12	rs587776783
VinRB20	No	2	Bil	F	B/B	c.1696-1G>A	Splice	Pathogenic	Intron 17	COSV57329272
VinRB21	No	3	Bil	F	B/B	c.2520+1_2520+4delGTGA	Splice	Pathogenic	Intron 24	rs1131690858
VinRB22	No	22	Bil	M	E/B	c.607+1G>A	Splice	Pathogenic	Intron 6	COSV57310480
VinRB23	No	14	Bil	M	D/D	c.607+1G>T	Splice	Pathogenic	Intron 6	COSV57310480
VinRB24	No	54	Uni	M	/E	c.2107-2A>C	Splice	Likely pathogenic	Intron 20	Novel
VinRB25	No	21	Bil	M	E/B	c.719-6_719-2del TTACA	Splice	Likely pathogenic	Intron 7	Novel
VinRB26	No	30	Uni	F	E	c.1981C>T	Missense	Pathogenic	Exon 20	rs137853294
VinRB27	No	19	Uni	F	E/	Ex24 Del	Large rearrangement	Pathogenic	Exon 24	(65)
VinRB28	No	14	Uni	M	E/	c.1953_1954insA	Frameshift	Pathogenic	Exon 19	rs1566234123
VinRB29	Yes	2	Uni	M	/B	c.958C>T	Nonsense	Pathogenic	Exon 10	rs121913300
VinRB30	No	74	Uni	F	/D	c.1072C>T	Nonsense	Pathogenic	Exon 11	rs121913301
VinRB31	No	17	Uni	F	E/	c.1303G>T	Nonsense	Likely pathogenic	Exon 13	COSV57313162
VinRB32	Yes	34	Uni	M	E/	c.1735C>T	Nonsense	Pathogenic	Exon 18	rs121913305

Table I. Continued.

Case ID	Family history	Months at diagnosis	Laterality	Sex	Staging	RB1 germline mutations	Molecular consequences	Interpretation	Exon/Intron	Previously reported (Refs.)
VinRB33	No	21	Bil	F	D/E	c.958C>T	Nonsense	Pathogenic	Exon 10	rs121913300
VinRB34	No	24	Uni	F	/E	c.2106+1G>C	Splice	Likely pathogenic	Intron 20	Novel
VinRB35	No	35	Uni	M	D/	c.264+1G>C	Splice	Pathogenic	Intron 2	COSV57317171
VinRB36	No	14	Uni	F	E/	No				
VinRB37	No	10	Uni	F	/E	No				
VinRB38	No	11	Uni	M	E/	No				
VinRB39	No	22	Uni	F	E/	No				
VinRB40	No	30	Uni	M	E/	No				
VinRB41	No	24	Uni	F	/E	No				
VinRB42	No	25	Uni	F	E/	No				
VinRB43	No	57	Uni	M	/D	No				
VinRB44	No	25	Uni	M	E/	No				
VinRB45	No	17	Uni	M	E	No				
VinRB46	No	31	Uni	M	/E	No				
VinRB47	No	22	Uni	M	E/	No				
VinRB48	No	108	Uni	F	E/	No				
VinRB49	No	59	Uni	F	E/	No				
VinRB50	No	84	Uni	F	/E	No				
VinRB51	No	8	Uni	M	E/	No				
VinRB52	No	10	Uni	M	D/	No				
VinRB53	No	8	Uni	F	E/	No				
VinRB54	No	44	Uni	M	E/	No				
VinRB55	No	23	Uni	M	E/	No				
VinRB56	No	49	Uni	F	D/	No				
VinRB57	No	21	Uni	F	D/	No				
VinRB58	No	56	Uni	M	E/	No				
VinRB59	No	41	Uni	F	E/	No				
VinRB60	No	33	Uni	M	E/	No				
VinRB61	No	42	Uni	M	E/	No				
VinRB62	No	8	Uni	F	/E	No				

RB stages were classified according to the International Classification of Intraocular Retinoblastoma (66) as follows: A, small post-equatorial tumor confined to the retina; B, foveal tumor confined to the retina; C, retinal tumor surrounded by a cuff of subretinal seeds; D, diffuse vitreous seeding; E, large tumor touching the crystalline lens, resulting in elevated intraocular pressure and expanded globe. F, female; M, male; Bil, bilateral; Uni, unilateral; RB1, RB transcriptional corepressor 1; Ex, exon; Ins, insertion; Del, deletion; Dup, duplication; VUS, variants of uncertain significance.

Table II. Clinicopathological distribution of *RB1* (n=35) and non-*RB1* (n=27) carriers by family history, laterality, sex, age at diagnosis and stage of RB tumorigenesis.

Characteristics	RB1 carriers (n=35)	Non-RB1 carriers (n=27)	Both (n=62)	P-value
Family history, n (%)	4 (100.0)	0 (0.0)	4 (100.0)	
Laterality, n (%)				0.001[a]
Bilateral RB	25 (100.0)	0 (0.0)	25 (100.0)	
Unilateral RB	10 (27.0)	27 (73.0)	37 (100.0)	
Sex, n (%)				0.87[b]
Female	17 (57.0)	13 (43.0)	30 (100.0)	
Male	18 (56.0)	14 (44.0)	32 (100.0)	
Mean age, months	22.14	32.67		0.04[c]
RB stage, n (%)				0.80[a]
A	0 (0.0)	0 (0.0)	0 (0.0)	
B	4 (11.4)	1 (3.7)	5 (8.0)	
C	0 (0.0)	0 (0.0)	0 (0.0)	
D	3 (8.6)	4 (14.8)	7 (11.3)	
E	28 (80.0)	22 (81.5)	50 (80.6)	

[a]Calculated using Fisher's exact test. [b]Calculated using χ^2 test. [c]Calculated using Student's t-test. RB stages were classified according to the International Classification of Intraocular Retinoblastoma (66) as follows: A, small post-equatorial tumor confined to the retina; B, foveal tumor confined to the retina; C, retinal tumor surrounded by a cuff of subretinal seeds; D, diffuse vitreous seeding; E, large tumor touching the crystalline lens, resulting in elevated intraocular pressure and expanded globe. RB, retinoblastoma; RB1, RB transcriptional corepressor 1.
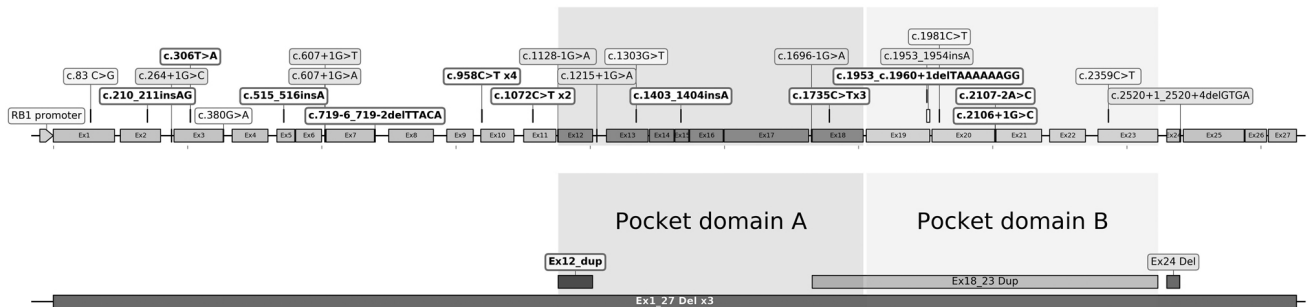

Figure 2. Distribution of 28 distinctly constitutional *RB1* variants, including point and InDel alterations (top) and large rearrangements (bottom). Bold variations in white boxes indicate newly identified and recurrent mutations. Recurrent mutations were marked with numbers of carriers (x n). RB1, RB transcriptional corepressor 1; InDel, insertion/deletion; Ex, exon; Dup, duplication.

(63.3 vs. 36%; OR=3.88; 95% CI=1.04-14.4; Table IV). In terms of unilateral cases only, although the number of *RB1* carriers was reduced compared with the non-*RB1* ones, the difference between the two groups was not statistically significant. Accordingly, the relative risk of children aged between 0-36 months suffering from the inherited form of the disease was not markedly different compared with children >36 months of age (30.8 vs. 18%; OR=2; 95% CI=0.35=11.4; Table IV).

Discussion

Integrated *RB1* genetic testing for the management of RB may reduce RB-associated mortality and treatment costs. However, the test sensitivity rate should be ≥90% to ensure a negative result would indicate a low risk of hereditary RB. In the current study, patients who were diagnosed with bilateral RB were used as a positive control group to evaluate the sensitivity of NGS in routine clinical practice. The detection rate of NGS was 100%

for patients with bilateral RB, while all common *RB1* mutation types were detected. Additionally, nine novel pathogenic mutations were identified. The present study also supported the potential use of age at diagnosis as a risk factor for inherited RB.

Recently, NGS is considered a highly sensitive and efficient approach for the detection of *RB1* mutations due to increasing use of NGS in gene mutation analysis of RB; especially screening and identification of *RB1* mutations with NGS substantially benefits the prepotency, early diagnosis and treatment of retinoblastoma (20-25). Furthermore, the sensitivity rate of NGS in the present study was similar with that reported to previous studies. For example, Li *et al* (51) detected germline *RB1* mutations in 100% (19/19) of patients with bilateral RB, which was consistent with previously reported laboratory data. Additionally, Singh *et al* (52) demonstrated a detection rate of 100% (22/22) for bilateral cases, following validation of data using the TruSight Cancer Sequencing Panel (Illumina, Inc.), with a sensitivity rate of 98.2%, specificity of 100%, and reproducibility of 99.5%.

Table III. A total of 28 distinct *RB1* germline alterations, including 9 novel ones, were identified in 35 patients with retinoblastoma.

RB1 germline variants	Molecular consequences	Amino acid alterations	Clinical significance	Existing variations	No. of carriers	Subunits
c.210_211insAG	Frameshift	p.(Ala74Glufs*4)	Pathogenic	Newly identified	1	N-terminus
c.306T>A	Nonsense	p.(Cys102Ter)	Pathogenic	Newly identified	1	N-terminus
c.515_516insA	Frameshift	p.(Tyr173Ilefs*12)	Pathogenic	Newly identified	1	N-terminus
c.719-6_7192 delTTACA	Splice	-	Pathogenic	Newly identified	1	N-terminus
Ex12 Dup	Large rearrangement	-	Pathogenic	Newly identified	1	Pocket
c.1403_1404insA	Frameshift	p.(Ser469Ilefs*6)	Pathogenic	Newly identified	1	Pocket
c.1953_c.1960+1 delTAAAAAAGG	Splice	-	Pathogenic	Newly identified	1	Pocket
c.2106+1G>C	Splice	-	Pathogenic	Newly identified	1	Pocket
c.2107-2A>C	Splice	-	Pathogenic	Newly identified	1	Pocket
c.83C>G (concurrent with Ex12dup)	Missense	p.(Pro28Arg)	VUS	rs776175164	1	N-terminus
c.1215+1G>A	Splice	-	Pathogenic	rs587776783	1	Pocket
c.1953_1954insA	Frameshift	p.(V654Sfs*14)	Pathogenic	rs1566234123	1	Pocket
c.1981C>T	Missense	p.(Arg661Trp)	Pathogenic	rs137853294	1	Pocket
c.2359C>T	Nonsense	p.(Arg787Ter)	Pathogenic	rs137853293	1	Pocket
c.1735C>T	Nonsense	p.(Arg579Ter)	Pathogenic	rs121913305	3	Pocket
c.1072C>T	Nonsense	p.(Arg358Ter)	Pathogenic	rs121913301	2	N-terminus
c.958C>T	Nonsense	p.(Arg320Ter)	Pathogenic	rs121913300	4	N-terminus
c.2520+1_2520+4delGTGA	Splice	-	Pathogenic	rs1131690858	1	C-terminus
Ex1_27 Del	Large rearrangement	-	Pathogenic	(53,61-63)	3	Whole gene
Ex18_23 Dup	Large rearrangement	-	Pathogenic	(64)	1	Pocket
Ex24 Del	Large rearrangement	-	Pathogenic	(65)	1	C-terminus
c.1696-1G>A	Splice	-	Pathogenic	COSV57329272	1	Pocket
c.264+1G>C	Splice	-	Pathogenic	COSV57317171	1	N-terminus
c.1303G>T	Nonsense	p.(Gly435Ter)	Pathogenic	COSV57313162	1	Pocket
c.607+1G>A	Splice	-	Pathogenic	COSV57310480	1	N-terminus
c.607+1G>T	Splice	-	Pathogenic	COSV57310480	1	N-terminus
c.380G>A	Missense	p.(Ser127Asn)	Pathogenic	COSV57302882	1	N-terminus
c.1128-1G>A	Splice	-	Pathogenic	COSV57294096	1	Pocket

RB1, RB transcriptional corepressor 1; Ex, exon; Ins, insertion; Del, deletion; Dup, duplication.

Furthermore, Grotta *et al* (53) showed a determination rate of 96% (28/29) for patients with bilateral RB when combining NGS and aCGH. Herein, the detected *RB1* variants had common characteristics with *RB1* alterations commonly observed in patients with RB. For instance, mutations were not identified in the exons 26 and 27 of the *RB1* gene and were scattered along the *RB1* gene. Another study revealed that null mutations, including large rearrangements, nonsense, splicing and frameshift mutations were detected at high frequency, whereas missense mutations exhibited a low frequency (6). Overall, the aforementioned data indicated that NGS could be considered as an accurate method for detecting germline *RB1* mutations.

The detection rate of germline *RB1* mutations in Vietnamese patients with RB using SS-MLPA has been previously reported.

Therefore, Kiet *et al* (26) and Nguyen *et al* (27) detected germline *RB1* mutations in 84 (21/25) and 82% (9/11) of patients with bilateral RB, respectively. However, the detection rates were lower compared with those reported in other Asian studies applying the same approach. For example, Tomar *et al* (18) and He *et al* (54) revealed a detection rate of 94 (17/18) and 92% (36/39) in Singaporean and Chinese patients with bilateral RB, respectively. In addition, Rojanaporn *et al* (55) and Mohd Khalid *et al* (56) also reported a detection rate of germline *RB1* mutations of 92 (25/27) and 100% (7/7) in Thai and Malaysian patients with bilateral RB, respectively.

In the present study nine novel *RB1* mutations were identified in the cyclin fold-contained subunits of the pRB. The allele frequencies of these null mutations were <0.0001% in the public

Table IV. Distribution of *RB1* and non-*RB1* carriers diagnosed at 0-36 vs. >36 months.

A, All patients with RB

Characteristic	*RB1* carriers, n=35	Non-*RB1* carriers, n=27	Total	P-value
Age, n (%)				0.036[a]
0-36 months	31 (63.3)	18 (36.7)	49 (100.0)	
>36 months	4 (36.0)	9 (64.0)	13 (100.0)	
OR, 0-36 vs. >36 months	3.88 (95% CI, 1.04-14.40)	0.26 (95% CI, 0.07-0.96)		0.040

B, Patients with unilateral RB only

Characteristic	*RB1* carriers, n=10	Non-*RB1* carriers, n=27	Total	P-value
Age, n (%)			0.62	0.043[a]
0-36 months	8 (30.8)	18 (69.2)	26 (100.0)	
>36 months	2 (18.0)	9 (82.0)	11 (100.0)	
OR, 0-36 vs. >36 months	2 (95% CI, 0.35-11.40)	0.5 (95% CI, 0.09-2.86)		0.043

[a]Calculated using Fisher's exact test. OR, odds ratio; RB, retinoblastoma; RB1, RB transcriptional corepressor 1.

databases, and had not been recorded in the genetic polymorphism database consisting of 400 healthy Vietnamese individuals (47). The low frequency of these variations in the general population indicated that these mutations could be eliminated during evolution due to their disadvantages. Additionally, the CADD tool predicted that all these alterations, except one (EX12 DUP), were among the top 1% of deleterious variants in the human genome. CADD integrates multiple annotations, including genomic features, gene-annotation models, evolutionary and epigenetic features, and gene functional predictions to generate a single, quantitative scoring system. These scores are then used to rank the deleterious effect of a given variant (48,57). For example, a CADD score of 20 or 30 suggests that a variant is among the top 1% or 0.1% of deleterious variants in the human genome, respectively. Consequently, VarSome (44), a tool for implementing the ACMG standards, is used to classify variants as pathogenic ones when they meet very strong evidence of pathogenicity (PSV1). The PSV1 criterion assumes that certain null mutations, such as nonsense, frameshift, splice sites of +/-1 or 2, initiation codon and exon deletions, can lead to a complete absence of the gene product due to impaired transcription or nonsense-mediated decay of an altered transcript (35). The predicted null variants in a gene, whose loss of function is the known mechanism underlying the development of a particular disease, such as *RB1* for RB, can be considered as the common cause of the disease. Devarajan *et al* (58) also applied the stringent criteria for defining pathogenic variants and reported no false-positive results during the detection of constitutional *RB1* variants.

The two-hit hypothesis suggests that in patients with hereditary RB the tumors are formed at younger ages compared with sporadic cases (28). In 1998, Zajaczek *et al* (31) reported four patients with unilateral RB, who were diagnosed with germline *RB1* mutations at the age of <19 months. This initial evidence supported the hypothesis that age at diagnosis could differentiate the hereditary from the sporadic form of unilateral

RB. However, further studies with larger sample sizes did not reveal any association between age at diagnosis and germline *RB1* status in patients with unilateral RB (32-34). In addition, Tomar *et al* (18) showed that Singaporean individuals diagnosed with RB at 0-36 months of age had a 53% risk of being *RB1* carriers, whereas those >36 months had 8% risk of suffering from hereditary RB. The results of the present study were consistent with those of the previous one, suggesting that children diagnosed with RB at 0-36 months of age were more likely to be *RB1* carriers compared with those aged >36 months. Nevertheless, similar analyses in patients with unilateral RB did not reveal significant difference in the risk of hereditary RB.

The present study revealed three clinical implications. Firstly, the results supported that the NGS method implemented at the Vinmec Hi-Tech Center was highly reliable in detecting germline *RB1* variants. Secondly, this study could provide novel insights into the mechanisms underlying *RB1* inactivation. Finally, the current findings suggested that age at diagnosis could be considered as a risk factor for hereditary RB.

Nevertheless, there are some limitations in the present retrospective study, including the small sample sizes and delay in diagnosis. Therefore, the results could not be generalizable to all patients with RB. The number of patients with bilateral RB, who underwent *RB1* testing in each of the aforementioned studies was <30. Furthermore, the small sample size and different detection strategies used to identify germline *RB1* mutations could result in variations in the detection rate between NGS and SS-MPLA. However, previous studies revealed that NGS-based methods could detect germline *RB1* mutations in patients with RB, whose constitutional *RB1* mutations could not be detected by either Sanger sequencing nor MPLA (59,60). In addition, the lack of statistical significance in the relative risk of patients with unilateral RB diagnosed at the age of 0-36 and those

>36 months being carriers of RB1 could be also due to the small number of unilateral cases.

In conclusion, the results of the present study indicated that NGS could be considered a reliable method for screening for constitutional *RB1* mutations, and age at diagnosis could be used to assess the risk of hereditary RB. Furthermore, the newly identified *RB1* mutations could provide useful information for an in-depth understanding of the mechanisms underlying *RB1* inactivation, and for the development of rapid assays for detecting *RB1* mutations. Altogether, the current study suggested that NGS could be used for detecting germline *RB1* mutations in routine clinical practice.

Availability of data and materials

The FASTQ data that support the findings of this study are available from the Beijing Genomics Institute (BGI) laboratory (Hong Kong) but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of the BGI laboratory.

Authors' contributions

CQH and HQD conceived and designed the study. BDN, LTP, DTN, CTMP and TLD recruited and referred patients to the study and helped with their follow-up. HQD, CQH, NTN, SAHN, CN, BDN, LTP, DTN, CTMP, TLD and MHT performed the experiments. HQD, CQH, NTN, SAHN, CN and MHT analyzed and interpreted the results. CQH and HQD wrote the manuscript. HQD and CQH are responsible for confirming the authenticity of the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Signed informed consents were obtained from parents/caregivers of all subjects, and the study was approved by the Vinmec's Institutional Review Board (Vinmec Healthcare System, Hanoi, Vietnam).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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