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Screening of *RB1* gene mutations in Chinese patients with retinoblastoma and preliminary exploration of genotype–phenotype correlations

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Purpose: Retinoblastoma (RB) sets the paradigm for hereditary cancer syndromes, for which medical care can change depending on the results of genetic testing. In this study, we screened constitutional mutations in the *RB1* gene via a method combining DNA sequencing and multiplex ligation-dependent probe amplification (MLPA), and performed a preliminary exploration of genotype–phenotype correlations.
Methods: The peripheral blood of 85 retinoblastoma probands, including 39 bilateral and 46 unilateral, was collected, and genomic DNA was extracted. DNA sequencing was conducted first. MLPA analysis was applied for patients with bilateral RB with negative sequencing results and unilateral probands whose age at diagnosis was less than 1 year old.
Results: Thirty-four distinct mutations were identified in 40 (47.1%) of the 85 probands (36 bilateral and four unilateral), of which 20% (8/40) was identified by MLPA. The total detection rate in bilateral cases was 92.3% (36/39). Of the total mutations identified, 77.5% (31/40) probands with a mean age of 10.7 months at diagnosis had null mutations, and 22.5% (9/40) with a mean age of 13.5 months at diagnosis had in-frame mutations. Of the 31 probands with null mutations, bilateral RB accounted for 96.8% (30/31). Of the nine probands with in-frame mutations, 66.7% had bilateral RB. There were seven new mutations of *RB1* identified in this report, including six null mutations and one missense mutation. Clinical staging of the tumor did not show obvious differences between patients with null mutations and in-frame mutations.
Conclusions: Our results confirm that the type of mutation is related to age of onset and the laterality, but not staging of the retinoblastoma tumor. MLPA is a reliable method for detecting gross deletion or duplication of the *RB1* gene. The combination of sequencing and MLPA improves the clinical diagnosis of RB.

Retinoblastoma (RB; OMIM 180200) is the most frequent primary intraocular malignant tumor in children, probably arising from cone precursor cells [1]. RB mainly affects children under 6 years old, with an incidence rate of 1 case per 15,000 to 20,000 live births [2,3]. According to the "two-hit" hypothesis, RB includes hereditary and nonhereditary forms, resulting from the mutation of both alleles of the *RB1* gene (Gene ID: 5925) [4,5]. Approximately 40% of the cases (including all bilateral and 15% of unilateral RB) are heritable, carrying a germ-line *RB1* mutation transmitted as an autosomal dominant trait with 90% penetrance [6,7]. The other 60% of cases are non-heritable RB (85% unilateral RB), caused by the inactivation of both alleles in the developing retina [8].

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The *RB1* gene was the first tumor-suppressor gene found, located in 13q14.2, and the whole DNA length is 183 kb. Mutations in the *RB1* gene are highly heterogeneous and scattered in the promoter and the 27 coding exons. To date, more than 1,600 distinct mutations, ranging from small mutations to large deletions, have been registered in the *RB1* Gene Mutation Database. In developed countries, *RB1* gene testing has already been applied as a routine examination in RB probands [9,10], since the detection of *RB1* mutations provides evidence for genetic counseling and clinical management. However, molecular diagnosis is still in its early stages in China. A search of the PubMed and Chinese databases, including CNKI (China National Knowledge Infrastructure) and WanFang data, revealed several studies regarding *RB1* alterations in Chinese patients with RB [11-21]. However, these studies mainly focus on pedigree or have a small sample size, with no definitive analysis of mutational characteristics and the correlation between genotype and phenotype. Therefore, our cooperative group, which is based on encouraging treatment outcomes of patients with RB reported in a

previous article [6], developed the molecular detection project to further enhance the level of clinical management of retinoblastoma. We have already reported several *RB1* alterations by using DNA sequencing with a detection rate of 78.6% in bilateral RB [22], which had not been capable of screening all *RB1* variations. Here, we demonstrate that combining DNA sequencing and multiplex ligation-dependent probe amplification (MLPA) for detecting the mutation spectrum of *RB1* allows for the preliminary exploration of genotype–phenotype correlations.

METHODS

Patients: This study recruited a total of 85 unrelated RB probands, including 37 (43.5%) boys and 48 (56.5%) girls, who were diagnosed between January 2012 and October 2013 in a Chinese cooperative group consisting of the Children's Hospital of Fudan University and the Eye & ENT Hospital of Fudan University. The genetic testing results, using Sanger sequencing, of 35 of these patients were reported in a previous paper published in Chinese [22], but new findings emerged using the complementary MLPA method. The combined results are discussed in the present paper. The patients included 39 cases of bilateral RB and 46 cases of unilateral RB. Clinical features, including laterality, age at diagnosis, and International Classification of Intraocular Retinoblastoma (ICRB) staging at diagnosis (Appendix 1), were recorded. After the study protocol was approved by the Institutional Review Board of Fudan University, informed consent for genetic testing was obtained from the guardians of children affected with RB. The study protocol was in accordance with the provisions of the Declaration of Helsinki and the ARVO (the Association for Research in Vision and Ophthalmology) statement on human subjects. Peripheral blood samples (3 ml) were collected from the probands in EDTA (EDTA) anticoagulant tubes. Blood samples were stored at −20 °C until DNA was extracted.

RB1 gene sequencing: Genomic DNA was isolated from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Düsseldorf, Germany), according to the manufacturer's instructions. Extracted DNA was measured with spectrophotometry (NanoDrop 2000; Thermo Scientific, Wilmington, DE) to ensure a concentration of 40 ng/µl.

Primers were designed according to a previous study by Abouzeid et al., with some modifications, using the Oligo software (National Biosciences, Plymouth, MN) [23]. Twenty-four pairs of primers were generated, which covered all coding exons and at least 50 bp flanking intronic sequences of each exon. PCR reactions were performed in a thermal cycler (ABI 2700), in a total volume of 10 µl containing 40 ng of genomic DNA, 2 picomoles of each primer, 0.2 µl of Taq DNA polymerase, and 1 µl of 10× buffer. Dimethyl sulfoxide (DMSO, 10%) was added to the exon 1 amplification system. Reactions were performed for the first 14 cycles of denaturation at 95 °C for 30 s, annealing at a specific temperature gradient (65 °C, −0.5 °C per cycle) for 30 s, extension at 72 °C for 40s; the second 20 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 40 s, and a final extension step at 72 °C for 5 min. The PCR products were qualified with agarose gel electrophoresis.

After the unincorporated deoxynucleoside triphosphates and primers were removed using Exonuclease I and shrimp alkaline phosphatase, the PCR products were directly sequenced using the ABI BigDye Terminator v 3.1 Sequencing Standard Kit (Carlsbad, CA) and run on an ABI 3500 Genetic Analyzer. The sequence data were analyzed by comparison to the consensus sequence of the *RB1* gene (NM_000321), using Mutation Surveyor (V 4.0) software (SoftGenetics; State College, PA). Additional information was obtained from the literature, the RB1 Gene Database (LOVD, 2010), the Human Gene Mutation Database (HGMD, 2007), and the Thousand Genomes Project (Thousand Genome Consortium, 2010). The functional consequences of the mutations were predicted by bioinformatics tools, SIFT, and PolyPhen-2.

Multiplex ligation-dependent probe amplification analysis: MLPA analysis was applied in bilateral probands who did not show germ-line mutations in DNA sequencing and in unilateral probands whose guardians demanded additional genetic examinations, considering the earlier age at diagnosis, which was less than 1 year old, although the DNA sequencing results had been negative. Gross deletion and duplication of *RB1* were examined using methylation-specific (MS)-MLPA analysis, the commercially available SALSA MLPA P047 RB1 probemix (MRC-Holland, Amsterdam, The Netherlands). This P047-C1 RB1 probemix contains probes for 26 of the 27 *RB1* exons and 13 reference probes. This method was used to detect loss of heterozygosity (LOH) status of the *RB1* gene located on 13q14. The procedure was based on the manufacturer's protocol. Each MS-MLPA reaction was subjected to further analysis on an ABI 3730 automated sequencer (Applied Biosystems). The data were analyzed with GeneMarker v1.91 (SoftGenetics) according to the manufacturer's instructions. The relative value of probes compared to the reference control was calculated. Threshold ratios for deletion and duplication were set at 0.75 and 1.35, respectively. The peak ratios for all target loci in a 1:1 ratio indicated normal copy numbers. The reduced or amplified

signal of probes with <0.75 or >1.35 indicated loss or gain, respectively, of the copy number in these probe loci.

Statistical analysis: Data were analyzed using SPSS software (version 16.0, Chicago, IL). Statistical analysis was performed with one-way ANOVA, the chi-square test, and Fisher's exact test. $p<0.05$ was considered to indicate statistical significance.

RESULTS

Patients included 39 (45.9%) bilateral and 46 (54.1%) unilateral cases, none of whom had a family history of RB. The age at diagnosis ranged from 1 to 72 months, with a median age of 11.1 months for patients with bilateral RB and 27.1 months for patients with unilateral RB. By combining the two different approaches, we discovered 34 distinct mutations (47.1%) in 40 probands (36 bilateral and four unilateral), of which 20% (8/40) was identified by MLPA. The ability to identify a germ-line mutation from blood samples of patients with bilateral RB was 92.3% (36/39). Of the total mutations identified (Table 1) in 40 patients, eight (20%) had gross deletions involving multiple exons or the whole gene, 17 (42.5%) had nonsense mutations, four (10%) had small deletions or insertions, two (5%) had splice mutations, and nine (22.5%) of patients had missense mutations, all of which were heterozygous mutations, except one homozygous missense mutation, c.2455C>G. The details of each group are as follows.

Null mutation: A null mutation is a pathogenic mutational type, which refers to those with mRNA harboring a premature translational-termination codon, including whole allele deletions, deletions involving one or more exons, and splice mutations, small frame-shifting intraexonic insertions and deletions, and nonsense mutations [24,25]. Our group identified 25 distinct null mutations in 31 (36.5%) of the 85 patients (Table 1). Of these mutations, the c.1735C>T mutation was a recurrent nonsense mutation identified in three patients. Six null mutations had not been reported before. The 31 patients with RB with an average age of 10.7 months at diagnosis included one patient (3.2%) with unilateral RB and 30 patients (96.8%) with bilateral RB (Table 2). Of the 61 eyes studied in this group, 20 (32.8%) presented with group E ICRB followed by groups C, D, B, and A ICRB, and the other four (6.6%) presented with extraocular extension at diagnosis (Table 3).

In-frame mutations: In-frame mutations stand for those not truncating based on prediction, such as indels (small insertions and deletions) in frame, and missense mutations [24]. We identified nine missense mutations in nine probands, of which one was a new mutation. Predicting the effects of these non-synonymous variants on protein function indicated 'damaging' designations for six patients with bilateral RB and 'tolerated' for three patients with unilateral RB (Table 1). The nine probands with an average age of 13.5 months at diagnosis involved 66.7% (6/9) bilateral and 33.3% (3/9) unilateral patients (Table 2). Of the 15 eyes, eight (53.3%) presented with group E ICRB, followed by groups D, A/B, and C ICRB, and one (6.7%) presented with extraocular extension (Table 3).

Negative genetic testing results: This group represented patients with negative genetic testing results. There were 45 cases, including three (6.67%) bilateral and 42 (93.3%) unilateral. The mean age at diagnosis was 27.3 months. Of the 48 eyes, 23 (47.9%) presented with group E ICRB, followed by groups D/C, B, and A ICRB. Sixteen of the 48 (33.3%) presented with extraocular extension (Table 3).

DISCUSSION

Genotype–phenotype correlations and implications for genetic counseling: In our study, all mutations were classified based on the prediction whether the transcripts harbored a premature translational-termination codon. Transcripts from alleles of the *RB1* gene with null mutations have been shown to be subject to degradation by nonsense-mediated decay (NMD) [25-27], resulting in no detectable protein. Therefore, a null mutation is a comparatively more severe mutation than an in-frame mutation. Of the 31 probands with null mutations, 96.8% had bilateral RB, while only 66.7% of the probands with in-frame mutations developed bilateral RB (Table 2). Early onset retinoblastoma was seen more with null mutations (average age at diagnosis was 10.7 months) than in-frame mutations (average age at diagnosis was 13.5 months). These results are consistent with reports from Canada and India [24,28], and confirm that null mutations tend to the severe phenotype (early onset and bilateral RB). However, ICRB staging, an additional clinical feature, did not seem to be correlated with genotype. Group E was the most frequent staging at diagnosis in our study, which had no remarkable differences between patients with null mutations and in-frame mutations. We believe that tumor staging might be more related to access to care rather than the type of mutation.

Interestingly, cases with negative genetic testing results, which were mostly non-hereditary (42 unilateral and 3 bilateral probands), seemed to present with more serious clinical staging at diagnosis, with 16 (33.3%) extraocular extension tumors. The finding that advanced tumors tend to occur in non-hereditary cases might be explained by two hypotheses. First, most of the hereditary cases were bilateral RB, and the growth of the retinoblastoma tumors in the two eyes was not completely synchronous. The first-discovered eye disease in bilateral probands likely contributed to early diagnosis of

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Table 1. Summary of variants of RB1 in patients with retinoblastoma.

mutation group	mutational type	cDNA change	protein	location	prediction (SIFT/ PolyPhen-2)	status #
null mutations	nonsense	c.179T>A	p.L60X	Exon 2	-	novel, pathogenic
	nonsense	c.224G>A	p.W75X	Exon 2	-	pathogenic
	nonsense	c.751C>T	p.R251X	Exon 8	-	pathogenic
	nonsense	c.963C>G	p.Y321X	Exon 10	-	pathogenic
	nonsense	c.967G>T	p.E323X	Exon 10	-	pathogenic
	nonsense	c.1072C>T	p.R358X	Exon 11	-	pathogenic[a]
	nonsense	c.1183C>T	p.Q395X	Exon 12	-	pathogenic
	nonsense	c.1333C>T	p.R445X	Exon 14	-	pathogenic[a]
	nonsense	c.1363C>T	p.R455X	Exon 14	-	pathogenic[a]
	nonsense	c.1399C>T	p.R467X	Exon 15	-	pathogenic[a]
	nonsense	c.1689G>A	p.W563X	Exon 17	-	pathogenic
	nonsense	c.1654C>T	p.R552X	Exon 17	-	pathogenic
	nonsense	c.1735C>T (3)	p.R579X	Exon 18	-	pathogenic
	nonsense	c.2359C>T	p.R787X	Exon 23	-	pathogenic
	nonsense	c.2501C>A	p.S834X	Exon 24	-	pathogenic[a]
	frameshift	c.142_170del	p.E48YfsX52	Exon 2	-	novel[a], pathogenic
	frameshift	c.1954_1959dupA	p.V654MfsX14	Exon 19	-	novel, pathogenic
	frameshift	c.1947delG	p.L649LfsX9	Exon 19	-	novel[a], pathogenic
	frameshift	c.2403delA	p.G801GfsX9	Exon 23	-	novel[a], pathogenic
	splice	c.607+1G>C	-	Exon 6	-	pathogenic
	splice	c.2490–1G>A	-	Exon 25	-	novel, pathogenic
	gross deletion	-	-	Whole gene deletion (5)	-	pathogenic
	gross deletion	-	-	Del exons 7–17	-	pathogenic
	gross deletion	-	-	Del exons 7–9	-	pathogenic
	gross deletion	-	-	Del exons 13–16	-	pathogenic
in-frame mutations	missense	c.920C>T	p.T307I	Exon 9	tolerated/benign	pathogenic[a]
	missense	c.584G>T	p.W195L	Exon 6	damaging/probably damaging	novel, pathogenic
	missense	c.1468G>A	p.A490T	Exon 16	damaging/probably damaging	probably pathogenic [a] MAF0.001 (A)
	missense	c.1861C>A	p.R621S	Exon 19	tolerated/benign	Probably pathogenic[a]
	missense	c.2455C>G	p.L819V	Exon 23	tolerated/benign	unknown significant
	missense	c.1346G>A	p.G449E	Exon 14	damaging/probably damaging	pathogenic[a]

mutation group	mutational type	cDNA change	protein	location	prediction (SIFT/ PolyPhen-2)	status #
	missense	c.1981C>T	p.R661W	Exon 20	damaging/probably damaging	pathogenic
	missense	c.1332G>C	p.Q444H	Exon13	damaging/probably damaging	probably pathogenic somatic
	missense	c.1994T>C	p.L665P	Exon20	damaging/probably damaging	pathogenic

* Arabic numbers in the parentheses show the occurrence times for each mutation. # Interpretation of mutations which refer to HGMD, LOVD and thousand genomes. Novel, pathogenic: Sequence variation is previously unreported and is of the type which is expected to cause the disorder; Unknown significant: Sequence variation is a missense mutation that alters an amino acid that is not evolutionarily conserved predicted by bioinformatics tools but is of the type which may or may not be causative of the disorder. MAF: minor allele frequency supported by 1000 genome database. [a]: Reported in our previous study published in Chinese;

Table 2. Clinical profile of retinoblastoma probands (age and laterality).

Groups	probands (no. and %)	mean age (months)	P value	laterality (no. and %)		P value
				bilateral	unilateral	
null mutation	31 (34.5%)	10.7		30 (96.8%)	1 (3.2%)	0.008**
in-frame mutation	9 (10.6%)	13.5	<0.001*	6 (66.7%)	3 (33.3%)	
negative genetic testing results	45 (52.9%)	27.3		3 (6.67%)	42 (93.3%)	
total	85	19.8		39 (45.9%)	46 (54.1%)	

*ANOVA was applied (F=22.97); there were significant difference between two groups (null versus in-frame, null versus neg, in-frame versus neg). **Chi-Square test was applied between null and in-frame ($\chi 2$=7.0251);

another eye disease. Second, the growth processes of retinoblastoma tumors might have certain differences between patients with and without germ-line *RB1* mutation, resulting in faster tumor growth in probands with negative genetic testing. A recent study reported that a previously unrecognized subtype of retinoblastoma tumor with intact *RB1* alleles had high-level v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN) oncogene amplification, which might initiate retinoblastoma with intact *RB1* alleles [29]. This suggests that the underlying mechanism involved in the initiation or development of retinoblastoma tumor could be investigated in future studies.

Distribution of the RB1 gene mutation: Our *RB1* mutational distribution results revealed that *RB1* mutations were scattered along the genomic sequence (Figure 1), lacking hot spots clustered in the important functional domains—A/B pocket regions. Richter et al. reported that 13 of 15 missense mutations of the *RB1* gene gathered in the A/B pocket and the intervening spacer region, confirming that in-frame mutations favored the critical regions [24]. However, only five of the nine missense mutations appeared in the A/B pocket domains in our study. All five missense mutations that appeared in the A/B pocket domains were identified in bilateral probands, while only one of the four missense mutations that appeared in other regions were identified in bilateral probands, suggesting that in-frame mutations in A/B pocket domains could be considered to have more complete penetrance. Mutations occurring in different regions along the *RB1* gene still need to be investigated in a study with an expanded sample size.

Incomplete detection rate: We reported a detection rate of 78.6% in our previous paper by using single DNA sequencing. Here, a method combining DNA sequencing and MLPA increased the detection rate to 92.3% in bilateral RB, which is consistent with an earlier study that reported that the *RB1* mutation detection rate was 92.6% via a combination of full sequencing and deletions/duplication analysis of *RB1* [30]. The remarkable enhancing of the effectiveness of detecting gross deletions attributable to MLPA was 20% (8/40)), which is slightly higher than in previous studies [31]. However, *RB1* defects can also result from hypermethylation of the promoter region and different levels of mosaic mutations, as evidenced by reports that highly sensitive allele-specific PCR increased the sensitivity from 92.6% to 94.8% for detecting mosaic *RB1* mutations [30]. Detecting methylation and mosaic mutations of *RB1* would be interesting in future studies.

In conclusion, we developed the molecular detection project to optimize the clinical management of all patients with retinoblastoma. This study reports our results involving

Table 3. Clinical profile of retinoblastoma probands (staging).

Groups	total no. of eyes	staging (no. and %)					
		A	B	C	D	E	Extra-ocular
null mutation	61	3 (4.9%)	8 (13.1%)	16 (26.2%)	10 (26.4%)	20 (32.8%)	4 (6.6%)
in-frame mutation	15	1 (6.7%)	1 (6.7%)	0	4 (26.7%)	8 (53.3%)	1 (6.7%)
negative genetic testing results	48	1 (2.1%)	2 (4.2%)	3 (6.3%)	3 (6.3%)	23 (47.9%)	16 (33.3%)
total	124	5 (4.0%)	11 (8.9%)	19 (15.3%)	17 (13.8%)	51 (41.1%)	21 (16.9%)

*Fisher's exact was applied between null and in-frame (p=0.261); Additionally, mutations (including null and in-frame) were compared with negative testing. Chi-Square test was applied between the intraocular tumor (including staging A, B, C, D, E) and extra-ocular tumor ($\chi 2$=14.9696, p=0.000):

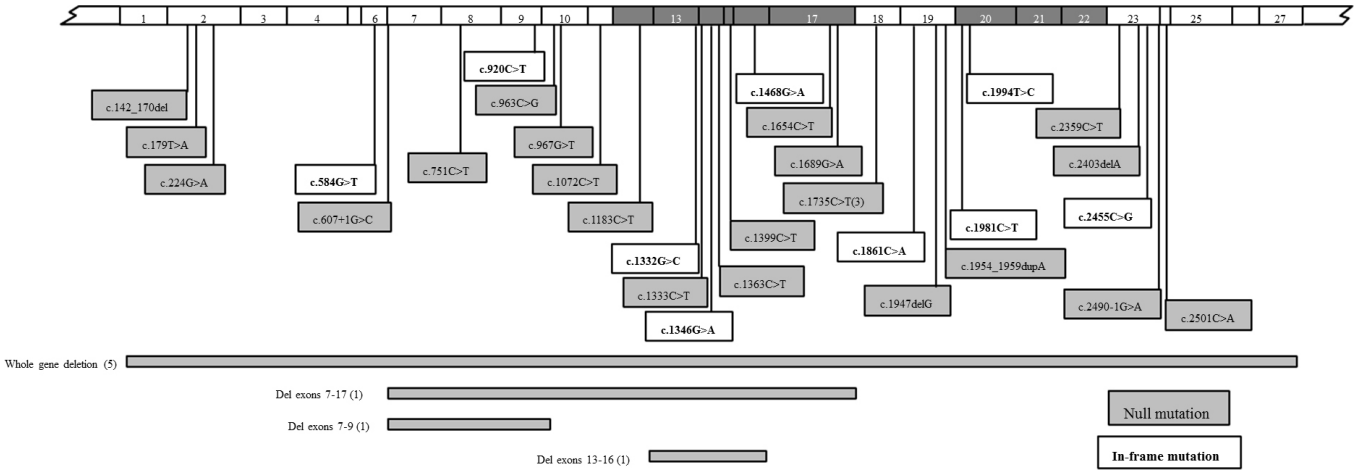

Figure 1. Schematic representation of *RB1* exonic mutation found in this study. Arabic numbers in the parentheses show the occurrence times for each mutation. All gray bars represent null mutations, and the bars represent in-frame mutations. The dark gray regions on the *RB1* gene show the pocket domains.

31 patients who had been identified with null mutations and nine patients identified with in-frame mutations. The different groups had different clinical outcomes, and the type of mutations was related to age of onset and laterality, but not related to staging of the tumor, which has implications for genetic counseling. In addition, 20% of the total mutations were detected with MLPA, which confirms that combining sequencing and MLPA is a convenient, reliable, and powerful method for evaluating *RB1* gene mutations in patients with retinoblastoma.

APPENDIX 1. RESULTS OF GENETIC TESTING AND CLINICAL FEATURES COVERING LATERALITY, AGE AT DIAGNOSIS, STAGING AT DIAGNOSIS.

To access the data, click or select the words "Appendix 1."

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