Int J Clin Exp Pathol 2016;9(2):2120-2126
www.ijcep.com /ISSN:1936-2625/IJCEP0018683

Original Article

Identification of five novel *RB1* gene mutations in Chinese patients with retinoblastoma

Tianqi Li[1,2,3*], Qingyu Meng[1,2,3*], Jianhong Liang[1,2,3*], Yujing Bai[1,2,3*], Lvzhen Huang[1,2,3], Xiaoxin Li[1,2,3]

[1]Department of Ophthalmology, People's Hospital of Peking University, Beijing, China; [2]Key Laboratory of Vision Loss and Restoration, Ministry of Education, Beijing, China; [3]Beijing Key Laboratory of Diagnosis and Therapy of Retinal and Choroid Diseases, Beijing, China. [*]Equal contributors and co-first authors.

Received October 28, 2015; Accepted December 25, 2015; Epub February 1, 2016; Published February 15, 2016

Abstract: In order to identify the spectrum of *RB1* gene mutation in Chinese retinoblastoma (RB) patients, a total of 38 RB patients, including 15 bilateral cases and 23 unilateral cases were included in this study. DNA samples were extracted from patients' peripheral blood and we performed PCR direct sequencing of the promoter and the 27 coding exons of the *RB1* gene to screen *RB1* mutation. In total, we identified nine causative *RB1* mutations in ten of 38 RB patients, and the global mutation rate is 26.3%. Five mutations have not been reported previously, including 1 nonsense mutation (p.169Cys > Term), 2 small deletions of one two base pairs (c.2047delC and c.871-872delGT) and 2 missense mutations (p.470Ile > Phe and p.Val654Leu). The other four mutations include 2 nonsense mutations (p.344Gln > Term and p.685Gln > Term), 1 small deletion of 2 base pair causing a frameshift and premature termination (c.371-372delTA) and 1 nonsense mutation (p.Gln344Ter). We reported 5 novel germline *RB1* mutations in Chinese RB patients in this study. Our results make contributions to the molecular diagnosis, risk prediction and early management in the proband and extended family.

Keywords: Retinoblastoma, gene mutation, *RB1* gene, nonsense mutation, small deletion, missense mutation

Introduction

Retinoblastoma (RB; OMIM 180200) is the most common primary intraocular malignancy among children. It originates from the primitive stem cells in the nuclear layer of the retina. Its prevalence is approximately 1:15,000 to 1:18,000, with 95% of cases occurring before the age of 5 years [1]. The main symptoms of retinoblastoma are leukokoria and strabismus. The *RB1* gene in chromosome 13 is associated with retinoblastoma. The *RB1* gene, which produces the RB protein, has an important role in regulating and controlling the cell cycle [2]. Loss-of function mutations in *RB1* disrupts the cell cycle, and has been shown to be a key initiating event prior to the development of retinoblastoma [3]. In the present study, the RB could be divided into hereditary and non-hereditary forms. In the hereditary cases, the predisposing-germline mutation in *RB1* could be transmitted as an autosomal dominant trait with high penetrance (90% or more), and the other mutation occurs somatically in the retina [4]. In non-hereditary RB, representing 60% of all cases, mutations in both *RB1* alleles affect retinal cell that will develop to form the tumor. Non-hereditary RB is usually unilateral and does not induce increased lifetime risk of non-ocular tumors [5].

It is known that germinal mutations in the *RB1* gene are also implicated in increased mortality associated with other tumors. As previously reported, additional tumors develop in 4.4% of cases during the first 10 years of follow-up, in 18.3% after 20 years, and in 26.1% after 30 years [6]. Therefore, it is clinically important to determine whether a RB proband carries a germline mutation, and to identify the causative mutation in germline cases of RB [7, 8]. Identification of *RB1* mutations is challenging because the mutations in the *RB1* gene are highly heterogeneous and scattered in the promoter and the 27 coding exons.

To identify the spectrum and the effect of germline mutations, we performed a genetic analysis of the *RB1* gene in Chinese RB patients.

Table 1. The primers sequences used to analysis of RB1 gene

Primer	Sequence (5'→3')	Primer	Sequence (5'→3')
RB1_ex1F	ACGCCAGGTTTCCCAGTTTA	RB1_ex13R	GCAGCATACACAGGCAGCAG
RB1_ex1R	CTCGCCCAAGAACCCAGAAT	RB1_ex14F	TGAGCCCAGGAGTGTGAAGG
RB1_ex2F	TCCTTATTTTGGAATGACCATGAAA	RB1_ex14R	ACCTCCTGATCTGCCCACCT
RB1_ex2R	GAGCTCAGGCAATCCACCTG	RB1_ex15-16F	CAATGCTGACACAAATAAGGTTTCA
RB1_ex3F	GCCATCAGAAGGATGTGTTACAA	RB1_ex15-16R	TATCCCCAAGATGGCCTCAA
RB1_ex3R	ACGGCTCCATGAGAGAATGG	RB1_ex17F	CACTCAAAATTGGAAGGCTATTTCC
RB1_ex4F	TTTGATGTAGAGCTGATAATCTTTTGA	RB1_ex17R	GGGTGCTCGATTAAAGCTCCA
RB1_ex4R	TTTTCCAGGAAGCATTCAGA	RB1_ex18F	CCACTGTCAATTGTGCCTAAAA
RB1_ex5F	TGTGAGATGTCATAAATTGGGAAAA	RB1_ex18R	GCCAACTCTGCCATGAAAAC
RB1_ex5R	TGCTGTCCTGAATCAATTCCAC	RB1_ex19F	AATCCCCAGGAAAAGCCATT
RB1_ex6F	GTAAATTATGCAATTAAAATGGACTGC	RB1_ex19R	CCACCGGTCACAGAGGTTTC
RB1_ex6R	GAAAGGGAGGGAAGATGGAATA	RB1_ex20F	ATGCCTTGCCCTCTGCATT
RB1_ex7F	AAAGAAAGAAAATCTTTACCATGCTG	RB1_ex20R	GGGAGGAGAGAAGGTGAAGTGC
RB1_ex7R	CATTTGTTTCCATGTCTTATCTTTCC	RB1_ex21F	TGAGCCTTGGTGATTTGCATT
RB1_ex8F	GGGAGCAGAGTAGAAGAGGGATG	RB1_ex21R	TTTCATAATTACCCTTATCTTTCCAA
RB1_ex8R	TGATTCCAGAGTGAGGGAGCTA	RB1_ex22-23F	GAAGAGCAGCTATAATCCAAGCCTA
RB1_ex9F	GCCCAAGCATTTGAAGCTGT	RB1_ex22-23R	TATTCGGCCATCTTGCGTTG
RB1_ex9R	TTTCACCACAATTCTACTTGGCTAGA	RB1_ex24F	TGATTAGACGGGCACTGTTAGAA
RB1_ex10F	TTGCATGCGAACTCAGTGTATATT	RB1_ex24R	TTTGAAGTTCACCAATTAGGAGTATGA
RB1_ex10R	GCAAAAAGGTAACTGTTATAGGACACA	RB1_ex25F	GCTGCATGGGAAAAGACAGG
RB1_ex11F	AAGCAGCAGCTGGGTCATCT	RB1_ex25R	TAAGCCAGGAGCAGTGCTGA
RB1_ex11R	CACCACACCTGGCCTTCAAT	RB1_ex26F	CCACTGTATTTTGTGAGAACCACTG
RB1_ex12F	GAGACAAGTGGGAGGCAGTG	RB1_ex26R	TCCTGCATGAAAGATCATAGAAAGG
RB1_ex12R	GCAAGAAAAGATTATGGATAACTACA	RB1_ex27F	GTCCTGAGCGCCATCAGTTT
RB1_ex13F	TGTCTGCTTATGTTCAGTAGTTGTGG	RB1_ex27R	GGTGAATGGGCAGTCAATCA

Materials and methods

This study was approved by the ethics committee of Peking University and Institutional Review Board of Peking University People's Hospital. Written informed consents were obtained for each case, with conforming to the tenets of the Declaration of Helsinki. Because all patients are without or with limited capacity for civil conduct (by Local laws and regulations), consents were obtained from the next of kin, caretakers, or guardians on behalf of the minors/juveniles.

Patients

The study included 38 related RB patients with different clinical presentations, recruited between August 2012 and December 2014. Of these, 15 had heritable disease and 23 had sporadic unilateral RB. The patients, 13 girls and 25 boys with age at diagnosis ranging from 2 to 84 months, were examined and treated at the Department of ophthalmology, Peking University People's Hospital. Blood samples were obtained in standard EDTA blood collection tubes (BD Vacutainer®, K2 EDTA 3.6 mg REF 367841, Lakes NJ USA), which were stored at -20°C until DNA was extracted.

DNA isolation

DNA was extracted from peripheral blood leukocytes from patients and available relatives (parents) by using FlexiGene DNA Kit (QIAGEN, Germany). DNA integrity was evaluated using Nanodrop 2000/2000C (Thermo Scientific) by spectrophotometry at 260 and 280 nm [9].

PCR amplification and sequence analysis of the DNA: RB1 gene 27 coding exons were PCR amplified with primer pairs shown in **Table 1**, and directly sequenced to analysis germline mutations. PCR reactions amplifications were performed in 25 μl mixes containing 1 μl genomic DNA (50-100 ng/μl), 1 μl of each

Table 2. Clinical Feature of all patients

Patient's ID	Age at diagnosis (month)	Gender	Type of disease	Exon mutation
RB1	7	Female	Unilateral	Yes
RB2	60	Male	Unilateral	No
RB3	13	Male	Unilateral	No
RB4	12	Male	Unilateral	No
RB5	24	Female	Unilateral	No
RB6	4	Female	Unilateral	No
RB7	9	Male	Unilateral	No
RB8	84	Male	Unilateral	Yes
RB9	12	Male	Unilateral	No
RB10	8	Female	Unilateral	Yes
RB11	18	Male	Unilateral	No
RB12	10	Female	Unilateral	No
RB13	23	Male	Unilateral	No
RB14	24	Female	Unilateral	No
RB15	15	Female	Unilateral	No
RB16	4	Male	Unilateral	No
RB17	4	Male	Unilateral	No
RB18	5	Male	Unilateral	No
RB19	12	Male	Unilateral	No
RB20	48	Female	Unilateral	No
RB21	16	Male	Bilateral	Yes
RB22	4	Male	Bilateral	No
RB23	4	Male	Bilateral	No
RB24	24	Male	Bilateral	Yes
RB25	13	Male	Bilateral	Yes
RB26	24	Female	Bilateral	No
RB27	18	Male	Bilateral	No
RB28	12	Male	Bilateral	Yes
RB29	2	Male	Bilateral	Yes
RB30	8	Male	Bilateral	Yes
RB31	5	Male	Bilateral	No
RB32	9	Female	Bilateral	Yes
RB33	36	Female	Bilateral	No
RB34	25	Female	Unilateral	No
RB35	12	Male	Unilateral	No
RB36	24	Female	Unilateral	No
RB37	12	Male	Bilateral	No
RB38	12	Male	Bilateral	No

primer (5 μmol/L), 12.5 μl of 2× Goldstar Taq Mix, and ultrapure water 9.5 μl. The protocol consisted of an initial denaturation at 95°C for 10 min, followed by 34 cycles consisting of denaturation at 94°C for 30 sec, annealing at 60°C (65°C for exon 8 and exon 20) for 30 sec, and extension at 72°C for 45 sec, and then a final extension step at 72°C for 5 min (S1000 Thermal Cycler, BIO-RAD, USA). The PCR products were directly sequenced using the ABI BigDye Terminator v 3.1 Sequencing Standard Kit (Carlsbad, CA) and run on an ABI 3730XL Genetic Analyzer. The sequence data were analyzed by comparison to the consensus sequence of the Human Gene Mutation Database (HGMD), using Mutation Surveyor V5.0.0 software.

Statistical analysis

We analyzed all data by using SPSS software (version 19.0).

Results

A total of 38 patients with sporadic retinoblastoma, including 15 (39.5%) bilateral and 23 (60.5%) unilateral cases, were analyzed in this study. The age at diagnosis ranged from 2 to 84 months, and mean age was 17.3 months (**Table 2**). In total, we identified nine causative *RB1* mutations in ten of 38 patients, and the global mutation rate is 26.3%. Among these patients, three had unilateral RB, one had familial bilateral RB and five had sporadic bilateral RB. Five mutations were novel, including 1 nonsense mutation (p.169Cys > Term), 2 small deletions of one two base pairs (c.2047delC and c.871-872delGT) and 2 missense mutations (p.470Ile > Phe and p.Val654Leu) (**Figure 1**). The other four mutations, which have been reported in the HGMD, include 2 nonsense mutations (p.344Gln > Term and p.685Gln > Term), 1 small deletion of 2 base pair causing a frameshift and premature termination (c.371-372delTA) and 1 nonsense mutation (p.Gln344Ter).

Furthermore, genetic analyses of parents were also performed among the ten families. For the RB28 family, the patient's father also had unilateral RB, and they showed the presence of the same *RB1* mutation. For the RB29 family, the patient's father carried a missense mutation, which was different from the proband, but without clinic symptoms. For other families, the causative mutations were absent in the parents, suggesting that these mutations are de novo (**Table 3**).

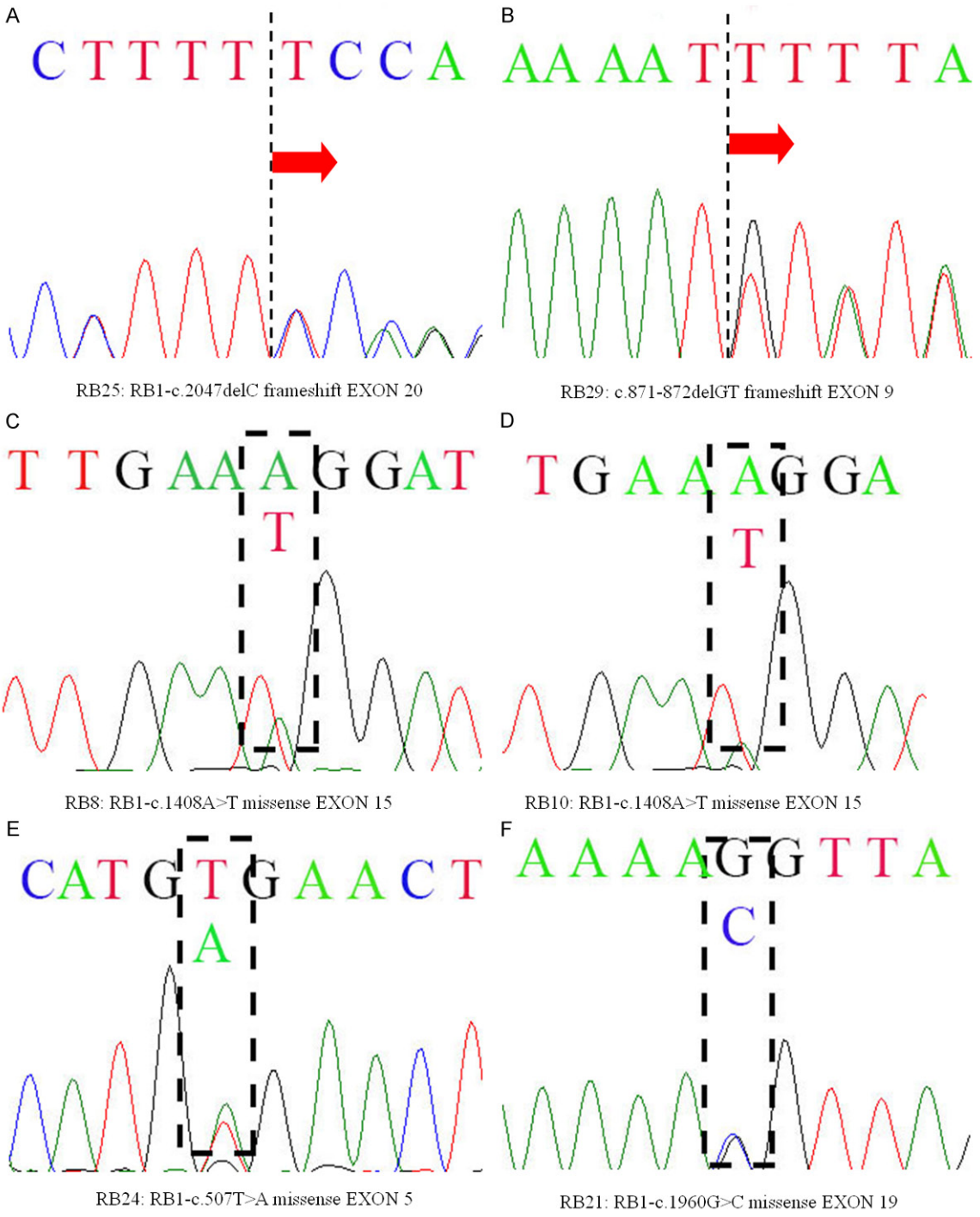

Figure 1. The 5 kinds of novel mutations in 6 patients. The genetic analyses of *RB1* gene (A) RB25: RB1-c.2047delC frameshift EXON 20 (B) RB29: RB1-c.871-872delGT frameshift EXON 9 (C) RB8: RB1-c.1408A > T missense EXON 15 (D) RB10: RB1-c.1408A > T missense EXON 15 (E) RB24: RB1-c.507T > A missense EXON 5 (F) RB21: RB1-c.1960G > C missense EXON 19.

Discussion

In our study, we identified 9 mutations in ten patients, with bilateral RB at about 46.7% (7/15) detection rate and in unilateral RB with a detection rate of 13.0% (3/23). The global mutation rate in our study is 26.3%. The heterogeneity of the mutation detection rate varied

Table 3. Summary of exon mutations of RB1 in patients and their parents

Patient's ID	Age at diagnosis (month)	Gender	Type of disease	Family history	Exon	Mutation	Amino acid change	HGMD inclusion	Parents' mutation
RB1	7	Female	Unilateral	No	10	c.1030C > T	p.Gln344Ter	Yes	No
RB8	84	Male	Unilateral	No	15	RB1-c.1408A > T	p.470Ile > Phe	No	No
RB10	8	Female	Unilateral	No	15	RB1-c.1408A > T	p.470Ile > Phe	No	No
RB21	16	Male	Bilateral	No	19	RB1-c.1960G > C	p.Val654Leu	No	No
RB24	24	Male	Bilateral	No	5	RB1-c.507T > A	p.169Cys > Term	No	No
RB25	13	Male	Bilateral	No	20	RB1-c.2047delC	-	No	No
RB28	12	Male	Bilateral	Father: Unilateral Mother: No	10	RB1-c.1030C > T	p.344Gln > Term	Yes	Father: c.1030C > T Mother: No
RB29	2	Male	Bilateral	No	9	RB1-c.871-872delGT	-	No	Father: c.920C > T Mother: No
RB30	8	Male	Bilateral	No	3	RB1-c.371-372delTA	-	Yes	No
RB32	9	Female	Bilateral	No	20	c.2053C > T	p.685Gln > Term	Yes	No

from 19% to 72% according to the technique diversity used and the size of the cohorts studied [7, 10-12]. In the patients who were *RB1* negative, 20 were unilateral sporadic cases, and 8 were bilateral sporadic case. The absence of mutation could be explained by the inactivation of *RB1* through mutation in non-coding regions situated outside the explored sequences. Epigenetic alteration, high mutational heterogeneity of the disease or the quality of the sequencing method may also be invoked to explain the absence of mutation in the remainder of the patients [12, 13].

Of the 9 mutations identified, there were 3 nonsense mutations, 4 missense mutations and 2 frameshift mutations and 5 mutations have not been reported previously. While the frequency of mutations detected varies over a wide range, the pattern of mutations is very similar in various populations [14-18], the most predominant categories being nonsense and frameshift mutations. Our result is comparable to those reported in these studies with about most of mutations detected being nonsense or frameshift. Even if we found novel mutations, confirming that the mutations causing RB have occurred independently in the majority of cases in the *RB1* gene database.

Additionally, we screened mutations in family members of probands. In RB family, proband's father, diagnosed unilateral RB, shared the same mutation with proband. However, in RB29 family, proband's father was identified a missense mutation, differing from proband's frameshift mutation. The father had no clinic symptoms or signs of RB. This could be explained by the low penetrance and variable expressivity of the disease, which was believed to be related to modified factors including MDM2 gene [19-21]. Furthermore, it is also reported that most families showing incomplete penetrance have distinct *RB1* mutations such as missense and in-frame mutations that do not result in premature termination codons. For others, the mutations were absent in the parents, suggesting that these germline mutations are de novo.

In conclusion, we screened and identified 5 novel germinal mutations in *RB1* gene in China. Our work helps to characterize the spectrum of germline mutations present in RB in China and provide information to improved molecular diagnosis, risk prediction and early management in the proband and extended family.

Acknowledgements

We would thank all the patients and their families for their invaluable assistance. Without them, this study cannot be finished. This study was supported by the Research Fund for Science and Technology Program of Beijing (No. Z121100005312006).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lvzhen Huang, Department of Ophthalmology, People's Hospital of Peking University, Key Laboratory of Vision Loss and Restoration, Ministry of Education, Beijing Key Laboratory of Diagnosis and Therapy of Retinal and Choroid Diseases, Xicheng District, 11 Xizhimen South Street, Beijing 100044, China. Tel: +86 10 88325413; Fax: +86 10 88325413; E-mail: dr_huanglvzhen@sina.com

References

[1] Bishop JO, Madson EC. Retinoblastoma. Review of the current status. Surv Ophthalmol 1975; 19: 342-66

[2] Knudson AG. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 1971; 68: 820-3.

[3] Naumova A, Sapienza C. The genetics of retinoblastoma, revisited. Am J Hum Genet 1994; 54: 264-73.

[4] Janey W, Magnus N, David Y, Joyce R, Valerie G, Marie J, Barbro W, Robert P, Alan C, Klaus R, Ruth L, David W, William W, Thaddeus PD. Prediction of the risk of hereditary retinoblastoma, using DNA polymorphisms within the retinoblastoma gene. N Engl J Med 1988; 318: 151-7.

[5] Harbour JW. Overview of RB gene mutations in patients with retinoblastoma. Implications for clinical genetic screening. Ophthalmology 1998; 105: 1442-7.

[6] Roarty JD, McLean IW, Zimmerman LE. Incidence of second neoplasms in patients with bilateral retinoblastoma. Ophthalmology 1988; 95: 1583-7.

[7] Houdayer C, Gauthier-Villars M, Laugé A, Pagès-Berhouet S, Dehainault C, Caux-Moncoutier V, Karczynski P, Tosi M, Doz F, Desjardins L, Couturier J, Stoppa-Lyonnet D. Comprehensive screening for constitutional

RB1 mutations by DHPLC and QMPSF. Hum Mutat 2004; 23: 193-202.

[8] Noorani HZ, Khan HN, Gallie BL, Detsky AS. Cost comparison of molecular versus conventional screening of relatives at risk for retinoblastoma. Am J Hum Genet 1996; 59: 301-7.

[9] Ayari-Jeridi H, Moran K, Chebbi A, Bouguila H, Abbes I, Charradi K, Benammar-Elgaaïed A, Ganguly A. Mutation spectrum of *RB1* gene in unilateral retinoblastoma cases from Tunisia and correlations with clinical features. PLoS One 2015; 10: e0116615.

[10] 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.

[11] Parsam VL, Kannabiran C, Honavar S, Bouguila H, Abbes I, Charradi K, Benammar-Elgaaïed A, Ganguly A. A comprehensive, sensitive and economical approach for the detection of mutations in the *RB1* gene in retinoblastoma. J Genet 2009; 88: 517-27.

[12] Rushlow D, Piovesan B, Zhang K, Prigoda-Lee NL, Marchong MN, Clark RD, Gallie BL. Detection of mosaic *RB1* mutations in families with retinoblastoma. Hum Mutat 2009; 30: 842-51.

[13] Abidi O, Knari S, Sefri H, Charif M, Senechal A, Hamel C, Rouba H, Zaghloul 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.

[14] Sugano K, Yoshida T, Izumi H, Umezawa S, Ushiama M, Ichikawa A, Hidaka A, Murakami Y, Kodama T, Suzuki S, Kaneko A. Outpatient clinic for genetic counseling and gene testing of retinoblastoma. Int J Clin Oncol 2004; 9: 25-30.

[15] Lohmann DR, Brandt B, Höpping W, Passarge E, Horsthemke B. The spectrum of *RB1* germline mutations in hereditary retinoblastoma. Am J Hum Genet 1996; 58: 940-9.

[16] 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.

[17] Choy KW, Pang CP, Yu CB, Wong HL, Ng JS, Fan DS, Lo KW, Chai JT, Wang J, Fu W, Lam DS. Loss of heterozygosity and mutations are the major mechanisms of *RB1* gene inactivation in Chinese with sporadic retinoblastoma. Hum Mutat 2002; 20: 408.

[18] Sampieri K, Hadjistilianou T, Mari F, Speciale C, Mencarelli MA, Cetta F, Manoukian S, Peissel B, Giachino D, Pasini B, Acquaviva A, Caporossi A, Frezzotti R, Renieri A, Bruttini M. Mutational screening of the *RB1* gene in Italian patients with retinoblastoma reveals 11 novel mutations. J Hum Genet 2006; 51: 209-16.

[19] Harbour JW. Molecular basis of low-penetrance retinoblastoma. Arch Ophthalmol 2001; 119: 1699-704.

[20] Taylor M, Dehainault C, Desjardins L, Doz F, Levy C, Sastre X, Couturier J, Stoppa-Lyonnet D, Houdayer C, Gauthier-Villars M. Genotype-phenotype correlations in hereditary familial retinoblastoma. Hum Mutat 2007; 28: 284-93.

[21] Castéra L, Sabbagh A, Dehainault C, Michaux D, Mansuet-Lupo A, Patillon B, Lamar E, Aerts I, Lumbroso-Le Rouic L, Couturier J, Stoppa-Lyonnet D, Gauthier-Villars M, Houdayer C. MDM2 as a modifier gene in retinoblastoma. J Natl Cancer Inst 2010; 102: 1805-8.