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ORIGINAL ARTICLE

Linkage analysis and detection of somatic, postzygous *RB1* mutations in Serbian retinoblastoma patients

M. Kontic[1], I. Palacios[2], M. Kontic[3], J. Alonso[2], A. Pestana[2]

[1]Institute of Lung Diseases, Clinical Center of Serbia, Belgrade, Serbia; [2]Oncolab, Instituto de Investigaciones Biomedicas "Alberto Sols", Madrid, España; [3]Clinic for Eye Diseases, Military Medical Academy, Belgrade, Serbia

Summary

Purpose: *The etiology of retinoblastoma (RB) is mutational inactivation of two RB1 alleles, the prototype of tumor suppressor gene. The aim of this research was to reveal sporadic, postzygous RB1 gene mutations, in particular loss of heterozygosity (LOH), from formalin-fixed, paraffin-embedded tumor samples in RB patients, as well as tracking RB1 allele inheritance in 10 RB families.*

Methods: *The mutational studies were carried out in the peripheral blood lymphocytes' DNA of 4 bilateral and 12 unilateral RB patients and DNAs from tumors from 3 bilateral and 10 unilateral patients. Tumor samples were collected from the same patients whose blood was analyzed. DNA was extracted and linkage analysis and microsatellite markers method were performed. LOH for two RB1 intragenic markers was analyzed.*

Results: *Ten LOH were found in the area of two intragene microsatellite loci. Linkage analysis revealed inheritance of RB1 alleles in 10 families. LOH was found in 63.16% of tumors.*

Conclusion: *Peripheral blood lymphocytes' DNA gives better results as a control group for somatic mutations than DNA isolated from eye tissue outside the tumor. Linkage analysis is essential for identifying the individual risk, offering the possibility of an adequate genetic counseling in familial RB.*

Key words: linkage, mutations, *RB1,* Serbia, retinoblastoma

Introduction

Modern research in molecular genetics has made a great contribution in prevention, early diagnosis and prognosis of RB. The gene responsible for RB, *RB1* gene, is the prototype of tumor suppressor gene [1,2]. The *RB1* gene consists of 27 exons scattered over 180 kb at chromosome 13q14. The gene is transcribed into a 4.7 kb messenger RNA. The 2.7 kb open reading frame encodes the ubiquitously expressed phosphoprotein pRb.

Two mutational events in *RB1* alleles are required for the initiation of RB [2]. In the hereditary form of the disease, which accounts for 40% of the cases, the predisposing germline mutation is transmitted as an autosomal dominant trait with high penetrance (90%), resulting in a 45% risk of occurrence in the offspring. Most of these patients have bilateral RBs with a mean age at diagnosis of 12 months and a lifetime predisposition to other *RB1*-dependent tumors, such as osteosarcoma.

In the nonhereditary form of the disease, both inactivating events occur during the somatic development of retinal cells, resulting in the relatively late onset of a single tumor in only one eye [3].

Modern methods of molecular genetics allow for the detection of large number of mutations on DNA isolated from peripheral blood, and from DNA isolated from tumors of RB patients. Ability to detect mutations in *RB1* gene is the base for studying the genetics of RB and determining prenatal risk for developing this kind of tumor. Direct mutation analysis is the best approach to characterize most of the gene defects in RB, but its usefulness as a screening method is hampered by the fact that mutations are scattered among 27 exons and the promotor region of the *RB1* gene, and no single hotspot has been found. Linkage analysis remains a useful tool, as a rapid, simple and low-cost method, for the assessment of individuals at risk in families with RB.

The aim of this research was to detect sporadic, postzygous *RB1* gene mutations - the second mutational event, in particular LOH and big deletions in tumor

Correspondence to: Milica Kontic, MD, MSc. Institute of Lung Diseases, Clinical Center of Serbia, School of Medicine, 11000 Belgrade, Serbia.
Tel: +381 63 850 21 51, E-mail: milicakontic@yahoo.com

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DNAs from formalin-fixed, paraffin-embedded tumor samples in RB patients, as well as tracking *RB1* allele inheritance in 10 RB families.

This is the first molecular genetic research in Serbian RB patients.

Methods

The mutational studies were carried out on peripheral blood lymphocytes' DNA of 4 bilateral and 12 unilateral RB patients and 22 of their parents and siblings, referred to the Institute of Ophthalmology, Belgrade. One of the bilateral patients had a family history of RB. Written informed consent was obtained from the patients or their parents. Diagnosis of RB was established by standard ophthalmologic and histological criteria. Formalin-fixed and paraffin-embedded histological representative tumor samples from 3 bilateral and 11 unilateral patients were also available for study, as well as samples of healthy eye tissue from the same eyes used as a control group. Tumor samples corresponded to the same patients in which linkage analysis was carried out. In addition, a chorionic villous sample was provided for prenatal diagnosis.

Peripheral blood lymphocytes from patients and relatives was collected in standard EDTA blood collection tubes (5 ml) and the DNA was extracted as described elsewhere [1,4]. DNA was also extracted from paraffin-embedded tumor sample using reagents and protocols provided by QIAGEN (QIamp Kit, QIagen Inc, Valencia, CA, USA). The purity and quantity of DNA preparation was ascertained by spectrophotometric and agarose gel electrophoretic criteria. The laboratory research was carried out at the Institute for Biomedical Research "Alberto Sols", CSIC-UAM, Madrid, Spain.

DNA was PCR-amplified using high performance liquid chromatography (HPLC) purified and fluorochrome-labeled primers (PCR conditions and primer sequences with characteristics as previously described). Deletions involving intron 2 and/or intron 20 were analyzed in peripheral blood lymphocytes and tumor DNA samples, amplified with fluorescent-labeled primers specific for high polymorphic microsatellite markers RBi2 and RB1.20, coupled to fragment analysis. Microsatellite markers used (RBi2, a CA repeat locate in intron 2 and RB1.20, a CTTT[T] repeat in intron 20) were selected according to the heterozygosity noticed in tumor tissues. This way, a LOH and large deletions of *RB1* gene were detected in tumor DNA. The method used to calculate the percentage of LOH is described elsewhere [4].

Analysis of microsatellite markers RBi2 and RB1.20 of *RB1* gene was performed on the DNA from peripheral blood lymphocytes and fixed eye tissue that was not affected by the tumor, in the same patients on which the analysis of tumor DNA was performed. These results served as a control group for the tumor DNA samples.

Tracking the origin of patients' *RB1* alleles responsible for disease was possible in families where one of the parents also suffered from RB. That indirectly made possible the tracking of inheritance within the family [5,6]. Through this linkage analysis, carriers of alleles responsible for RB were detected and thus the tracking of mutations was enabled.

Results

Ten LOH were found in the area of two intragene microsatellite loci within *RB1* locus (RBi2 in intron 2 and RB1.20 in intron 20), among them, 8 LOH on tumor DNAs of unilateral and 2 LOH on tumor DNA of bilateral patients (Figures 1-3).

A total of 63.16% of the analyzed tumor DNA of RB patients had LOH (Table 1). In material from patients, absence of LOH in *RB1* locus was discovered in 9 tumor DNA: 5 of DNA samples were without LOH by RBi2 analysis and 4 were without LOH by RB1.20 (Table 1). For 2 unilateral patients it was impossible to determine LOH for the microsatellite marker 1.20 because they were homozygotes.

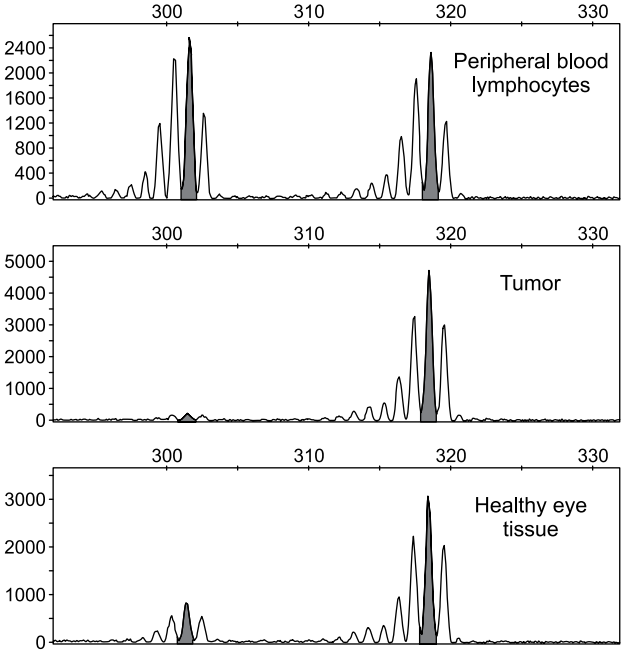

Figure 1. Analysis of microsatellite marker 1.20 in peripheral blood lymphocytes, tumor and healthy eye tissue in patient SR2 with unilateral retinoblastoma. LOH in tumor DNA. Allelic peaks are black. The horizontal coordinate represents the size of alleles (in base pairs) and the vertical height.

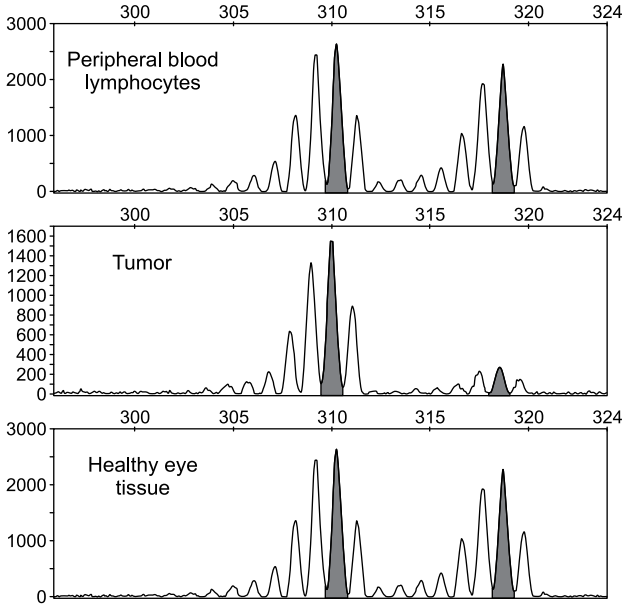

Figure 2. Analysis of microsatellite marker 1.20 in peripheral blood lymphocytes, tumor and healthy eye tissue in patient SR10 with bilateral retinoblastoma. LOH in tumor DNA. Allelic peaks are black. The horizontal coordinate represents the size of alleles (in base pairs) and the vertical height.

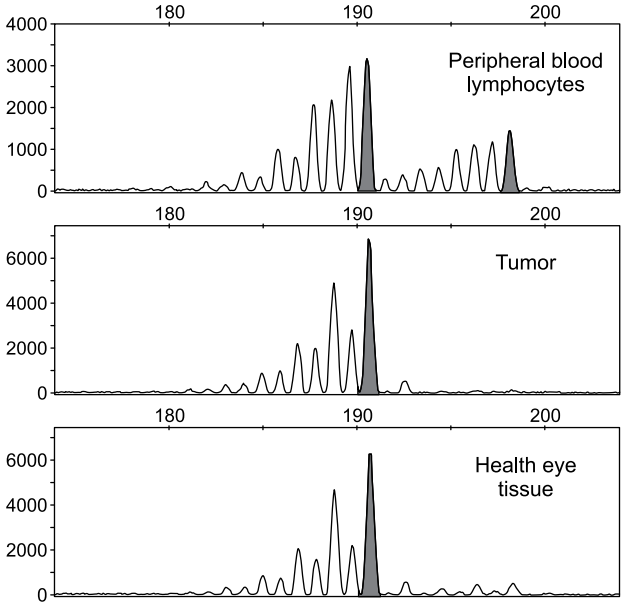

Figure 3. Analysis of microsatellite marker Rbi2 in peripheral blood lymphocytes, tumor and healthy eye tissue in patient SR26 with unilateral retinoblastoma. LOH in tumor DNA from eye tissue, macroscopically outside of tumor. It was probably due to spreading of tumor to the surrounding tissues that was not macroscopically visible or to contamination during the process of slicing. Allelic peaks are black. The horizontal coordinate represents the size of alleles (in base pairs) and the vertical height.

In 2 unilateral patients the isolated tumor DNA was not pure enough, so the collected amount of DNA available was not sufficient to enable further PCR analysis of microsatellite markers. This could be due to partial calcification of the tumor from which the DNA isolation was tried. Impossibility to obtain adequate PCR product led to no result on RB1.20 microsatellite analysis in one of the unilateral patients.

Linkage analysis detected inheritance of the alleles responsible for RB in our cases. Inheritance of alleles of *RB1* gene was traced in 10 pedigrees. It was determined which allele was received from the father and which originated from the mother (Figures 4-6). These 10 families included families of 4 bilateral and 6 unilateral patients.

Table 1. Results of microsatellite markers analysis (tumor samples were available for 13 out of 16 patients)

Patient ID	*Type of retinoblastoma*	*LOH by Rbi2 analysis in tumor DNA*	*LOH by RB1.20 analysis in tumor DNA*	*Linkage analysis in retinoblastoma families by tracking Rbi2 marker*	*Linkage analysis retinoblastoma families by tracking RB1.20 marker*
SR1	Unilateral RB	Failed	Failed	–	–
SR2	Unilateral RB	LOH 86%	LOH 100%	+	+
SR3	Unilateral RB	LOH 50%	Without LOH	–	–
SR4	Unilateral RB	LOH 59%	LOH 71%	+	+
SR7	Unilateral RB	Without LOH	Without LOH	+	+
SR8	Unilateral RB	Without LOH	Failed	+	+
SR9	Unilateral RB	Failed	Failed	–	–
SR10	Bilateral RB	LOH 70%	LOH 81%	+	+
SR15	Bilateral RB	Without LOH	Without LOH	+	+
SR16	Unilateral RB	–	–	–	–
SR20	Unilateral RB	Without LOH	Non informative	+	+
SR21	Bilateral RB	–	–	+	+
SR22	Unilateral RB	LOH 77%	Non informative	–	–
SR23	Bilateral RB	Without LOH	Without LOH	+	+
SR25	Unilateral RB	–	–	–	–
SR26	Unilateral RB	LOH 100%	LOH 100%	+	+

+: Linkage analysis in this retinoblastoma family has been done, –: Linkage analysis in this retinoblastoma family has not been done, LOH: loss of heterozygosity, RB: retinoblastoma, ID: identification number

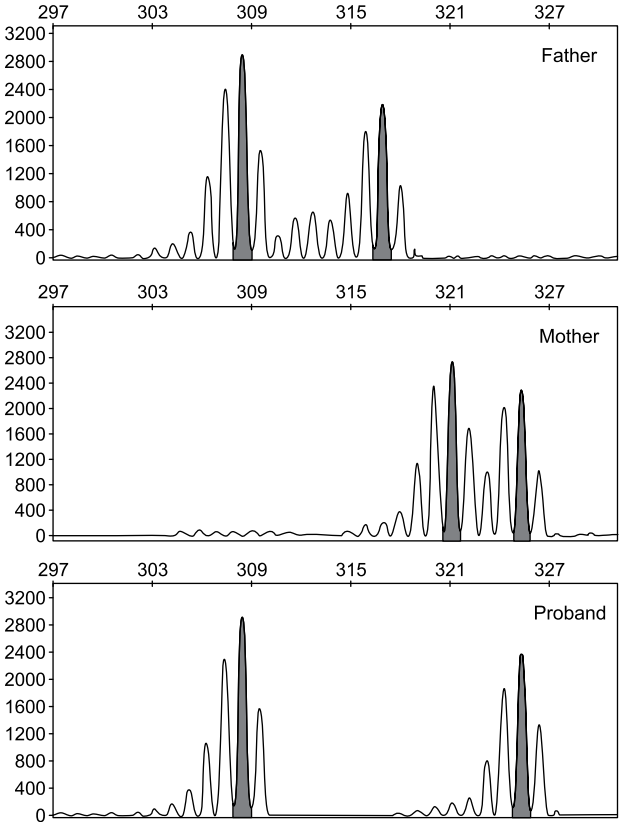

Figure 4. Genealogy of microsatellite marker 1.20. Father, mother and one of the patients with unilateral retinoblastoma (SR2). Proband's *RB1* allele on the left side is inherited from the father and the right one is inherited from the mother. Allelic peaks are black. The horizontal coordinate represents the size of alleles (in base pairs) and the vertical height.

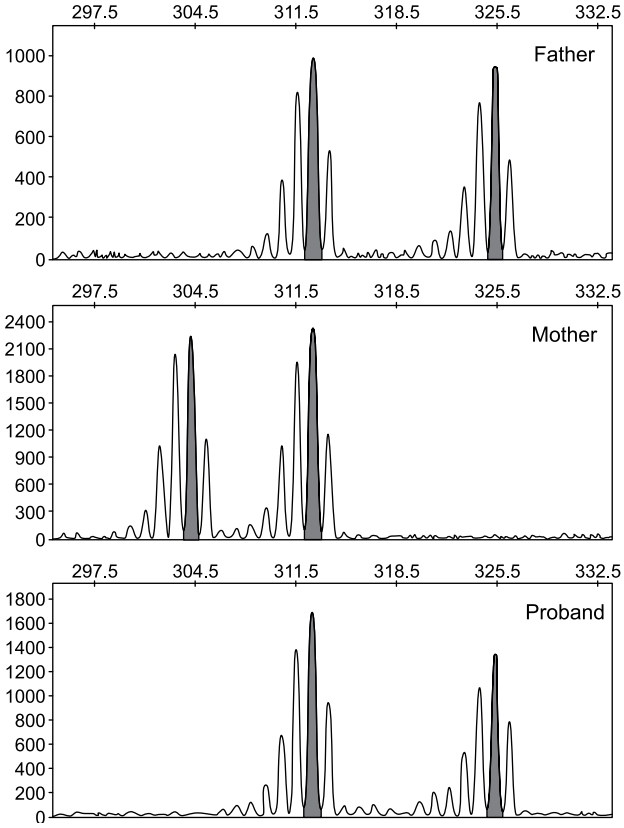

Figure 5. Genealogy of microsatellite marker 1.20. Father, mother and patient SR8 with unilateral retinoblastoma. Father is a healthy transmitter of mutated allele *RB1*. Proband's *RB1* allele on the right side is inherited from the father and the left one is inherited from the mother. Allelic peaks are black. The horizontal coordinate represents the size of alleles (in base pairs) and the vertical height.

Discussion

Linkage analysis is an easier, simpler, more efficient and low-cost way for determining the risk of RB for the relatives of a patient. By this analysis we determined the inheritance of alleles of *RB1* gene in 10 families by checking whether a relative has inherited one particular allele responsible for RB, instead of precise sequencing of all 27 exons of *RB1* gene.

In the case the relative of a patient has inherited an allele responsible for RB, he should undergo regular ophthalmologic examinations every 3 months until the age of 5, due to extremely high risk of developing RB because of a high penetration of mutations of *RB1* gene (more than 90%) [7].

Since this analysis has been performed in one family of a patient with bilateral RB with another member affected –father who survived unilateral RB– the allele responsible for RB has been undoubtedly determined in case of both microsatellite markers (Figure 6). In this way, prenatal diagnosis was particularly helpful for this family, since expensive and time-consuming sequencing of the whole *RB1* gene was not necessary any more [8]. In this study we have done the first case of prenatal diagnosis by linkage analysis of fetal DNA isolated from chorionic villous sample. In this case the allele responsible for RB was not present, and after a normal pregnancy a healthy child was born.

The healthy father of one unilateral RB patient had a germline mutation. This mutation was identified by the sequencing method. Based on this we discovered which allele was responsible for RB in his child (Figure 5). Prenatal diagnosis was also helpful for this family since the allele responsible for RB was found, and the risk for future offspring will be possible to be determined through linkage analysis instead of sequencing the whole gene [9,10]. By microsatellite and linkage analysis in *RB1* locus sporadic mutations (LOH) were detected. Due to LOH, only *RB1* alleles with inherited mutation remained in this locus [11,12]. This resulted in loss of tumor-suppressor function of *RB1* gene since both of its alleles lost their normal function. Deactivation of both *RB1* gene alleles lead to forming RB [13]. Analysis of *RB1* gene on inherited and sporadic forms

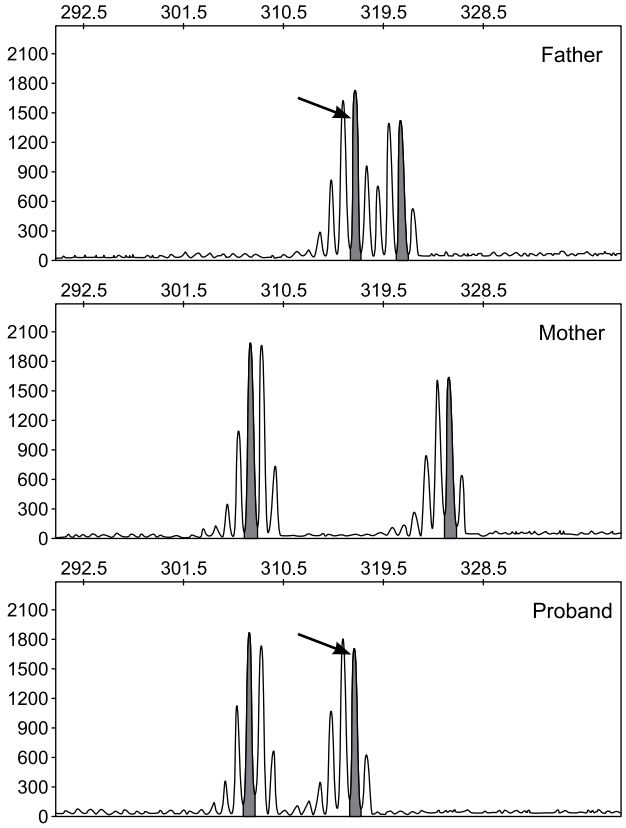

Figure 6. Genealogy of microsatellite marker 1.20. Father, mother and bilateral retinoblastoma patient SR21. Father was treated for unilateral retinoblastoma in his childhood and survived. *RB1* alleles responsible for retinoblastoma in this family are marked with arrows. Allelic peaks are black. The horizontal coordinate represents the size of alleles (in base pairs) and the vertical height.

of RB revealed a significant difference between normal and tumor cells. In normal cells there is heterozygosity and in tumor cells there is homozygosity on the same loci (LOH). This means that tumor DNA contains allele of just one homologue on chromosome 13. In the inherited form of RB the remaining chromosome 13 is the one inherited from the diseased parent - parent with mutated *RB1* allele [14].

We found LOH in 63.16% of the analyzed tumor DNAs of patients with RB which is in accordance with the data in the relevant literature, where this percentage is 66.66% [15].

LOH and presence of big deletions discovered in 5 patients in DNA isolated from healthy tissue could also be an artefact due to the spreading of tumor into the surrounding tissues that was not macroscopically visible or to the contamination of healthy tissue during the process of slicing.

In our experience, DNA isolated from whole blood is better as control group in detecting somatic mutations of *RB1* gene than tumor-free ocular tissues.

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