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A molecular study of first and second *RB1* mutational hits in retinoblastoma patients

Ana Flávia Belchior de Andrade[a,e], Raquel da Hora Barbosa[a,f], Fernando Regla Vargas[a], Sima Ferman[b], Ana Lúcia Eisenberg[c], Luisa Fernandes[d], Cibele R. Bonvicino[a,*]

[a]*Genetics Division,* [b]*Paediatric Service,* [c]*Pathology Division, Instituto Nacional de Câncer, André Cavalcanti St. 37, 20231-050 Rio de Janeiro, Brazil*
[d]*Ophtalmology Service, Hospital dos Servidores, Sacadura Cabral St. 178, 20221-903, Rio de Janeiro, Brazil*
[e]*Programa de Pós – Graduação em Biologia Celular e Molecular, Oswaldo Cruz Institute, Av. Brasil 4365, 21040-360 Rio de Janeiro, Brazil*
[f]*Programa de Pós – Graduação em Oncologia, Instituto Nacional de Câncer, André Cavalcanti St. 37, 20231-050, Rio de Janeiro, Brazil*

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Abstract *RB1* mutations accountable for biallelic inactivation are crucial events in the development of retinoblastoma because a first mutation (M1) predisposes to retinoblastoma while a second mutation (M2) is required for tumor development. Mutational analyses of this gene showed a wide spectrum of genetic alterations (single base substitutions, insertions, or deletions, as well as small and large deletions). The most frequent second hit in retinoblastoma patients is loss of heterozygosity (LOH) followed by promoter methylation. Molecular analyses of *RB1* mutations were conducted in 36 patients (20 unilateral and 16 bilateral) using polymerase chain reaction–mediated single-strand conformation polymorphism (SSCP) analysis, sequencing, and LOH analysis. Sixty-four amplified fragments showing abnormal SSCP patterns were sequenced, and mutations were confirmed in five patients (13.89%). Four mutations were located at coding regions, and a fifth one was found at an exon–intron junction. Two mutations were C→T transitions, two were small-length deletions, and one was a G→A transition. A total of 47.05% patients showed LOH. In one patient, the parental origin of the mutated allele was detected: the allele retained in the tumor was the paternal one. This work helps to characterize the spectrum of mutations in the Brazilian population, and to confirm that formaldehyde-fixed paraffin tissue can provide valuable information on the *RB1* status in retinoblastoma patients.

1. Introduction

RB1 mutations accountable for biallelic inactivation are crucial events in the development of retinoblastoma because a first mutation (M1) predisposes to this condition while a second mutation (M2) is required for tumor development [1]. In familial cases, frequently resulting in bilaterally affected patients, a constitutive mutation, transmitted as an autosomal dominant trait, is followed by an additional somatic mutational hit. Conversely, in sporadic cases, both mutations usually occur as independent somatic events [2].

Since 1986, when Friend et al. [3] cloned the *RB1* gene, mutational analyses showed a wide spectrum of genetic alterations [4–11]. Single base substitutions are the most frequent M1 events, accounting for approximately 40% of confirmed mutations, followed by short and large deletions [4,5,10]. Second hits might be associated with loss of heterozygosity (LOH), promoter hypermethylation, or even to a second independent base substitution. Most M2 events account for LOH that is present in approximately 60% of tumors [12–14] resulting from mitotic recombination in most cases (46%), followed by nondisjunction and reduplication (39%) and small deletions (8%) [13].

Molecular analyses of *RB1* mutations by polymerase chain reaction (PCR)-mediated single-strand conformation polymorphism (SSCP) analysis and sequencing have been reported in 28 Brazilian patients [15], and further studies have now been carried out, including LOH analyses in another 36 patients. In this new sample, four mutations were detected and identified.

* Corresponding author. Tel.: +55-21-3233-1460; fax: +55-21-3233-1423.
E-mail address: cibelerb@inca.gov.br (C.R. Bonvicino).

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2. Materials and methods

2.1. Patients and samples

We studied 36 patients, 20 with unilateral and 16 with bilateral retinoblastoma, who were admitted into two public institutions in Rio de Janeiro, Instituto Nacional de Câncer (INCA) and Hospital dos Servidores (HS). We analyzed formaldehyde-fixed paraffin tissue (FFP) and peripheral blood in 14 patients, FFP only in 2 patients, fresh tumor tissue and peripheral blood in 6 patients, and peripheral blood only in 14 patients. In patients carrying an *RB1* constitutive mutation, both parents were also screened. All participating families signed an informed consent for screening and identification of *RB1* mutations. Genomic DNA from FFP, fresh tissues, and peripheral blood was extracted by established procedures [16–18].

2.2. SSCP analysis

All 27 *RB1* exons were PCR-amplified with primer pairs used in a previous study [15]. Amplification of genomic DNA was performed in 50-μL reaction mixes containing 1× Mg-free buffer (Promega, Madison, WI), 3 mmol/L $MgCl_2$, 0.15 mmol/L of each dNTP, 0.4 pmol of each primer (forward and reverse), and 1 unit of *Taq* DNA polymerase (Promega). PCR was performed with 40 cycles at 94°C (1 minute), 52°C (40 seconds), and 72°C (35 seconds) in a programmable thermocycler (MJ Research, Inc., Waltham, MA). A 5-μL aliquot of amplified product was diluted with 2.5 μL of 0.05% bromophenol blue and 0.05% xylene cyanol and 2.5 μL of 95% formamide. Diluted samples were heat-denatured at 95°C for 5 minutes and subsequently loaded on 4% MDE (Cambrex Bio Science Rockland Inc., Rockland, ME) nondenaturing polyacrylamide gels with or without 10% glycerol. Electrophoresis was performed at room temperature with power ranging from 60 to 140 W for 18 hours, depending of the length of the fragments analyzed.

2.3. Sequence analysis

Sequence reactions were carried out with dideoxy terminator nucleotides labeled with fluorescent dyes for automatic detection using the DYEnamic ET dye terminator kit MegaBACE (Amersham Biosciences, Piscataway, NJ). Direct sequencing analyses were performed with an ABI Prism 377 automatic DNA sequencer (Applied Biosystems, Foster City, CA) or a MegaBACE 1000 DNA Analysis System (Amersham Biosciences). Sequences were edited using Sequence Navigator software (Applied Biosystems, 1994).

2.4. LOH analysis

Three 13q microsatellite markers (D13S153, D13S262, and D13S284) were used for LOH analysis in 17 patients. Products were amplified using labeled primers as described previously [19]. PCR-amplified products were diluted in water (1:9), and this solution was further diluted (2:8) in a solution containing 7.75 μL Tween (0.1%) and 0.25 μL MegaBACE ET400-R Size Standard (Amersham Biosciences). Samples were denatured at 95°C for 5 minutes, cooled immediately on ice, and subsequently run on a MegaBACE 1000 DNA automatic sequencer (Amersham Biosciences). Data were analyzed with the MegaBACE Genetic Profiler (Amersham Biosciences).

3. Results

3.1. SSCP and sequence analyses

Sixty-four amplified fragments showing abnormal SSCP patterns (any band shift on the gel in relation to control sample) were sequenced, and five patients (13.89%) carried mutations (Table 1). Four mutations were found at coding regions, and a fifth one was found at an exon–intron junction.

A single base substitution (g.63326C→T) in exon 11, generating a stop codon (TGA), was identified in tumor DNA of the bilaterally affected patient 1; the electropherogram showed clear evidence of heterozygosity. Analysis of blood DNA showed that this mutation was constitutional. Neither of his parents, however, carried this mutation.

A 4–base pair (bp) deletion (g.147968→147972del TCAA) in exon 18, resulting in a presumptive serine deletion of the translated protein and the appearance of a stop codon at position 1,824, downstream from the transcription initiation site, was identified in tumor DNA of the unilaterally affected patient 4. The electropherogram did not show evidence that the patient was a heterozygous carrier of this mutation, while analysis of blood DNA showed that this mutation was constitutional. Neither of his parents, however, carried this mutation.

Table 1
Mutations found in the *RB1* gene in this study

Patient	Sex	Form	Samples	Mutation	Location	Effect	LOH
RB1	M	Bi	FFP/B	g.63326C→T	E11	R358X	No
RB4	M	Uni	FFP/B	g.147968-14797del.	E18	S576fsX	Yes
RB9	F	Bi	FFP/B	g.147978-147988del.	E18	R607X	NI
RB18	F	Uni	FFP	g.57623C→T	E8	R251X	NT
RB20	M	Bi	FFP/B	g.68270G→A	I12	Abnormal splicing	No

Abbreviations: Bi, bilateral; Uni, unilateral; B, blood; E, exon; I, intron; NI, non informative; NT, not tested.

A 10-bp deletion (g.147978→147988delGAGAAGGACC) in exon 18, generating a stop codon at position 1,818, was identified in tumor DNA of the bilaterally affected patient 9. Analysis of blood DNA showed that this mutation was constitutional. Neither of her parents, however, carried this mutation.

A single base substitution (g.57623C→T) in exon 18, which changed a CGA codon to a stop codon (TGA), was found in tumor DNA of the unilaterally affected patient 8. The electropherogram did not show evidence of heterozygosity. Blood DNA could not be analyzed because this patient was not alive at the time of study.

A single base substitution (g.68270G→A) occurring at the first (5′) position of intron 12, at the junction with exon 12, was detected in tumor DNA of the bilaterally affected patient 20. The electropherogram showed clear evidence of heterozygosity. This mutation might be responsible for abnormal splicing, resulting in a nonfunctional protein. Analysis of blood DNA showed that this mutation was constitutional. Neither of his parents, however, carried this mutation.

3.2. LOH analysis

Eight of the 17 patients showed LOH (47.05%), 8 did not, and in 1 patient (no. 9), the markers were not informative. In patient 4, the paternal origin of the mutated allele was identified (Fig. 1).

4. Discussion

Four mutations were initially detected by abnormal SSCP patterns in gels containing glycerol, while a fifth mutation was evident in gels without this additive, indicating that both types of gels are necessary for screening *RB1* mutations. The apparently low sensitivity of SSCP might be due to the fact that detection was restricted to PCR products consisting of exons and their short flanking regions, thus excluding mutations at introns or promoter regions. SSCP has been used frequently for mutation screening of *RB1* mutations [4,6–10], and its sensitivity can range from 58% [10] to 36% [4,15], depending on the polyacrylamide quality, presence of glycerol, running temperature, and buffer concentration. SSCP and denaturing high-performance liquid chromatography are mutation detection methods developed for point mutations, while quantitative multiplex PCR of short fluorescent fragments detects large rearrangements. Houdauer et al. [20] suggest that these techniques should be used in conjunction with SSCP.

Three mutations occurred in bilaterally affected patients and two in unilaterally affected patients; four mutations were constitutional and apparently de novo because none of them was present in the patients' progenitors. Two mutations were C→T transitions occurring at CpG dinucleotides and affecting an arginine codon (CGA). These are the most common *RB1* point mutations [9], presumably because of the high instability of 5-methylated cytosines. Another mutation (a G→A substitution) occurred at the first 5′ position of intron 12, affecting a site whose invariance is required for normal splicing.

The majority of germline mutations described in hereditary retinoblastoma are nonsense or frameshift mutations, while missense and in-frame mutations are associated with incomplete penetrance or milder expression [21]. All five mutations herein identified were nonsense mutations that generated internal premature stop codons.

One of them was a splice site mutation whose effect must be carefully evaluated because mutations affecting invariable splice sites result in premature stop codons or out-of-frame exon skipping [21,22], while other intronic mutations not affecting invariable splice sites might be associated to a less severe clinical phenotype.

The finding of a constitutional mutation in a unilaterally affected child (patient 4) placed this patient in a higher risk group for developing secondary tumors than most

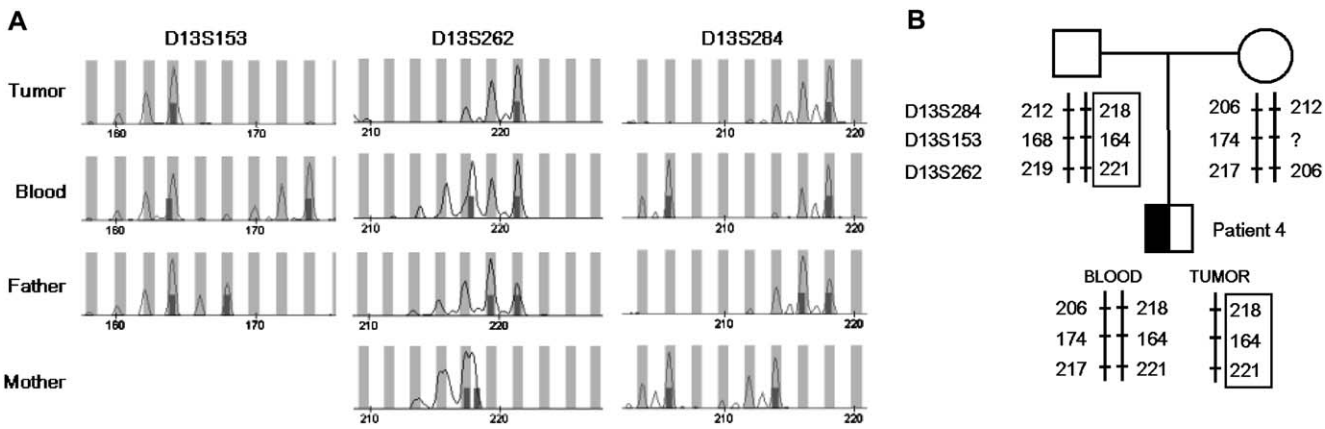

Fig. 1. (A) LOH analysis of microsatellite markers D13S153, D13S262, and D13S284 in the family of patient 4. Allelic peaks are shown as bars, and numbers indicate fragment sizes (in base pairs). Note LOH in the tumor sample, where one paternal allele is retained. (B) Pedigree of the same family showing haplotypes. The paternal haplotype (inside boxes) is both present in the tumor sample and in the patient's father (half-shaded square), unilateral retinoblastoma; question mark, unidentified allele.

unilaterally affected patients, who have a lower probability of carrying constitutional mutations and, consequently, belong to a lower risk group. Furthermore, the presence of constitutional mutations in four patients indicated a likely transmission to their offspring as well as a need to identify other at-risk individuals in their families.

In retinoblastoma, LOH is present in about 60% of cases [12–14], representing the most frequent M2 event. In this study, LOH was detected in 47.05% of the studied patients. In patient 4, who was a carrier of a constitutional mutation, analyses of his parents showed that the paternal allele was the mutated one while the maternal allele had been deleted in the tumor sample. This was coincident with the preferential retention of mutated alleles of paternal origin in bilaterally affected patients [23,24] who carry de novo germline mutations. This might result from a higher occurrence of mutations during spermatogenesis than oogenesis, higher mutation rates in males than females, or lack of repair mechanisms at initial development phases. Conversely, in patients with two somatic mutational hits, mutated alleles show no difference in parental origin [19].

This work helps to characterize the spectrum of mutations present in the Brazilian population, provide further information about the origin of the mutated allele, and prove that FFP is useful in genetic counseling studies by providing valuable information about *RB1* status in retinoblastoma patients.

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