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UNCOMMON *RB1* SOMATIC MUTATIONS IN A UNILATERAL RETINOBLASTOMA PATIENT

DANIELA OTTAVIANI[1], CRISTINA ALONSO[2], IRENE SZIJAN[1]

[1]Genética y Biología Molecular, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, [2]Departamento de Hemato-Oncología, Hospital Prof. Dr. Juan P. Garrahan, Buenos Aires, Argentina

Abstract Retinoblastoma (RB) is the most common primary intraocular malignancy in children. Somatic inactivation of both alleles of the *RB1* tumor suppressor gene in a developing retina is a crucial event in the initiation of tumorigenesis in most cases of isolated unilateral retinoblastoma. We analyzed the DNA from tumor tissue and peripheral blood of a unilateral retinoblastoma patient to determine the *RB1* mutation status and to provide an accurate genetic counseling. A comprehensive approach, based on our previous experience, was used to identify the causative *RB1* mutations. Screening for *RB1* mutations was performed by PCR direct sequencing, multiplex ligation-dependent probe amplification (MLPA) and Real Time-PCR analyses. Three different mutations were identified in the tumor DNA, which were absent in blood DNA. The somatic origin of these mutations was vital to rule out the heritable condition in this patient.

Key words: retinoblastoma, *RB1* tumor suppressor gene, mutations

Resumen **Mutaciones somáticas raras en un paciente con retinoblastoma unilateral.** El retinoblastoma (RB) es el cáncer ocular más común de la niñez. La inactivación somática de ambos alelos del gen supresor de tumores *RB1* en la retina en desarrollo es un evento crucial en la iniciación de la tumorigénesis en la mayoría de los casos de retinoblastoma unilateral. Nosotros analizamos el ADN de tumor y de sangre periférica de un paciente con retinoblastoma unilateral para identificar las mutaciones y así proveer un asesoramiento genético a la familia. Para ello utilizamos un protocolo basado en nuestra previa experiencia para identificar todas las mutaciones en el gen *RB1* que causaron el RB. El rastreo de mutaciones se realizó por medio de los siguientes análisis: PCR-secuenciación, amplificación multiplex de sondas ligadas (MLPA) y PCR-Tiempo Real. Se encontraron tres mutaciones diferentes en el ADN del tumor, las cuales estaban ausentes en el ADN de la sangre. El origen somático de estas mutaciones es importante para indicar que la enfermedad no es hereditaria.

Palabras clave: retinoblastoma, gen supresor tumoral *RB1*, mutaciones

Though the incidence of retinoblastoma (RB) is low (15.3-42.5 per million children aged 0-4) it appears as a hereditary cancer in approximately 50% of cases, thus, it is crucial to be diagnosed for its hereditary predisposition[1]. RB may be either bilateral (approximately 40%) or unilateral (60%), the former is caused by a germ-line mutation in one *RB1* allele, which may be inherited (10%) or arisen "de novo", followed by a somatic mutation in the other allele. Unilateral RB is caused in most cases (approximately 80%) by two somatic mutations[2]. Mutations in both *RB1* alleles result in a loss of function of a retinoblastoma protein, leading to deregulation of cell proliferation and tumor development. RB may be diagnosed by several presenting signs such as leukocoria or strabismus. An early diagnosis is critical for survival and eye preservation in children who carry the mutation. The most common treatment for RB in developing countries is enucleation, with or without adjuvant chemotherapy according to tumor presentation[3].

Screening for *RB1* mutations is challenging because of their heterogeneity and their distribution along the *RB1* exons, the promoter region and the flanking intronic sequences[4]. Moreover, only few mutations on specific sites are recurrent, such as the C > T transitions at the CpG dinucleotides. Most of mutations are unique or rarely reported (Retinoblastoma Mutation Data Base)[5]. Approaches using several techniques for mutation analysis may detect alterations in the *RB1* gene in about 80 to 90% of patients with germ-line mutations[6-8]. However, half of the RB patients do not carry mutations in constitutional DNA but they do only in tumor tissue, which is not readily available.

Most of the *RB1* mutations are nonsense or frame-shift including nonsense substitutions, large deletions, small deletions or insertions and splice site mutations. All these mutations result in an absence of retinoblastoma protein. Some *RB1* mutations such as missense, in frame deletions or promoter mutations are less common. This type of mutations is associated with low penetrance, consist-

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Postal address: Dra. Irene Szijan, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina
e-mail: iszijan@ffyb.uba.ar

ing in fewer tumors (unilateral RB). In this regard, it is noteworthy that some unilateral RB patients carry a low penetrant germ-line mutation[7, 9].

The unilateral retinoblastoma patient was diagnosed at 16 months and treated by enucleation and adjuvant chemotherapy. After signing the informed consent, blood and tumor biopsy samples were processed for deoxyribonucleic acid (DNA) extraction using the cetyltrimethylammonium bromide (CTAB) method (blood) or the proteinase K, phenol/chloroform purification and ethanol precipitation method (tumor).

Screening for *RB1* mutations was performed as follows:

1) PCR-direct sequencing of the 27 exons and the promoter region of *RB1* gene using an ABI 3130XL genetic analyzer[9].

2) Multiplex Ligation-dependent Probe Amplification assay (MLPA) using the Salsa MLPA kit PO47-B1 RB1 (MRC Holland).

3) Cloning of PCR products in pGEM-T vector: the PCR products were ligated to the vector pGEM-T and the mixture was transformed into DH5α competent bacteria growing in a media with an inducer of ß galactosidase (IPTG) and a chromogenic substrate 5-bromo-4chloro-3-indolyl-ß-galactoside (X-Gal). Recombinant vectors produced white colonies, while vectors without an insert originated blue colonies. The recombinant vector was extracted from white colonies and analyzed by sequencing.

4) Real-Time PCR: quantitative values were obtained from a threshold cycle number (Ct) at which an increase in the signal, associated with the exponential growth of PCR product, begins to be detected. The ΔCt value was

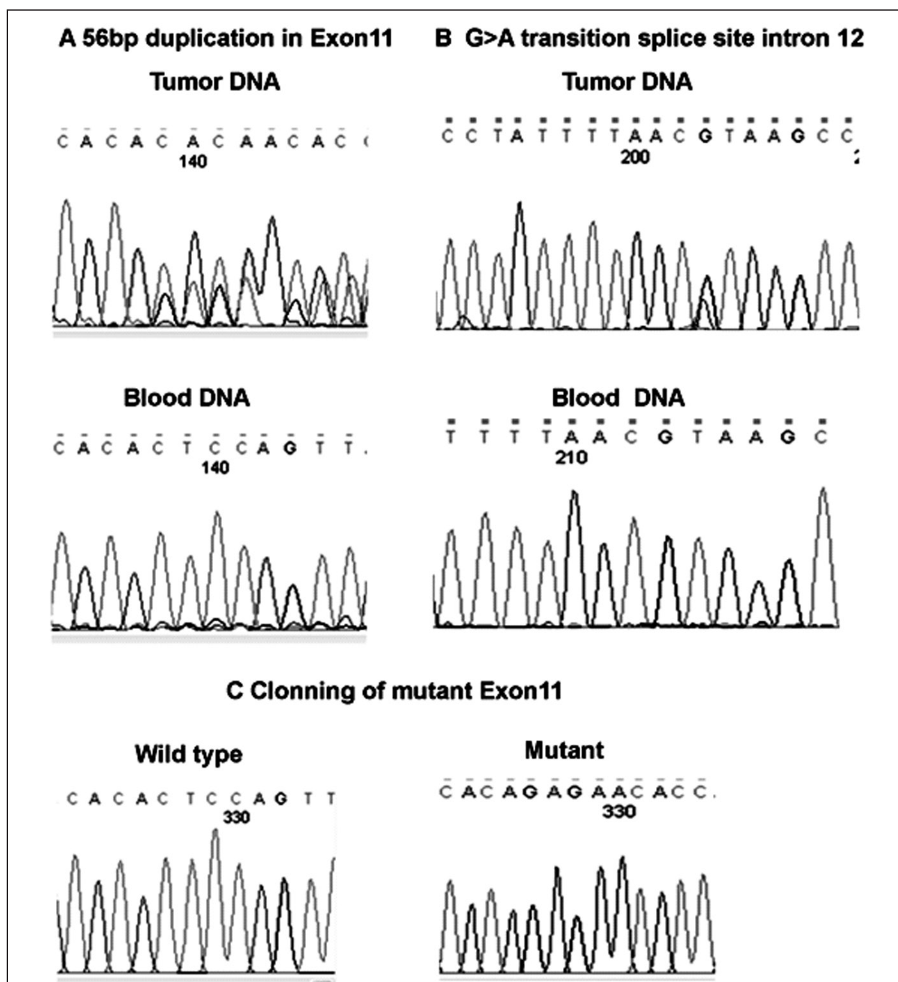

Fig. 1.– Results of sequence analysis of blood and tumor DNA. **A**. 56bp duplication in tandem in the Exon 11 in tumor DNA and a wild type sequence in the Exon 11 in blood DNA. **B**. G to A transition at the splice donor site of Intron12 in tumor DNA and a wild type sequence at the same site in blood DNA. **C**. Cloning of the PCR product of the tumor Exon 11 reveals two different clones, one with a wild type sequence and the other with a mutant sequence, confirming thus, the heterozygous duplication g.65432-65487dup in the tumor DNA.

calculated by normalizing the *RB1* exons to the Albumin gene, a single copy reference gene. For determination of the *RB1* gene copy number two exons were selected: the mutant exon 11 and the wild type exon 24, using the mutated sequence

5´-TAATTCCTCCACACAGAGA-3´ as a forward primer for exon 11 and the primer sequences described in Ottaviani et al. (2013)[9] as a reverse primer for exon 11 and the forward and reverse primers for exon 24. The primer sequences for Albumin gene were as follows:

Fw 5'-TGAAACATACGTTCCCAAAGAGTTT-3' and

Rev 5'-CTCTCCTTCTCAGAAAGTGTGCATAT-3'. Tumor and constitutional DNA of the patient were assayed using as a normal reference the DNA from a pool of 6 normal individuals. The quantitative PCR was performed in a total volume of 25 µl containing 12.5 µl of SYBR Premix (Bio-Rad), 2µl of genomic DNA from each of the four serial dilutions containing 60 ng, 30 ng, 15 ng and 7.5 ng of DNA, and 5 µl of primers (5 µM each), with two replicates per sample. The serial dilutions were performed to test if the efficiency of all reactions were comparable in order to use the quantitative method.

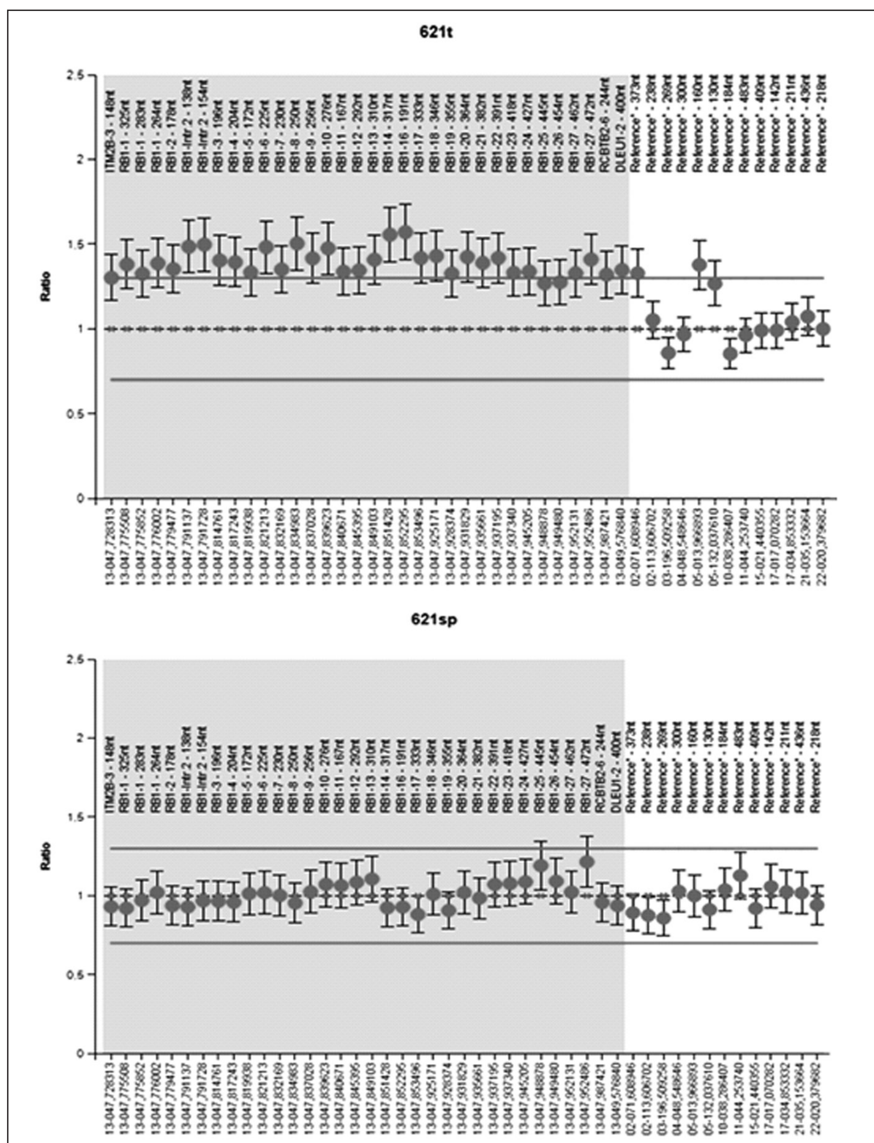

Fig. 2.– Multiplex ligation-dependent probe amplification (MLPA) analysis of tumor and blood DNA. Ratio: sample to reference ratio data. The data above the line of ratio 1.3 are considered as a duplication of the sequences of centromeric gene (*ITM2B*), the *RB1* Exons1 to 27 and the proximal and distal telomeric genes (*RCBTB2* and *DLEU*). 621t: Patient´s tumor DNA; 621sp: Patient´s blood DNA; Reference samples are the probes for DNA sequences from different human chromosomes. Two of these sequences show duplication in patient´s tumor.

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Mutations were described according to the nomenclature[10],using the *RB1* sequence from the GenBank, accession No L11910.

Analysis of tumor DNA showed three different mutations in the *RB1* gene: 1) in tandem duplication of a 56bp sequence in exon 11, g.65432-65487dup (Fig. 1A); 2) a base pair substitution at the conserved splice donor site of intron 12, c.1215+1G > A (Fig. 1B); 3) a duplication of the whole *RB1* gene plus the flanking centromeric and telomeric genes, g.*ITM2B*-?_*RB1*_*DLEU*+?dup (Fig. 2). All these mutations were heterozygous and were absent in constitutional DNA. The duplication of 56 bp sequence in exon 11 was validated by cloning of exon 11 PCR product in pGEM-T vector. Four white colonies were obtained, two of them contained the wild type exon 11 and the other two the mutant exon 11 (Fig. 1C). These data confirm the presence of a heterozygous 56bp duplication in exon 11. Duplication of the whole *RB1* gene was validated by Real-Time PCR analysis. The copy number of exons 11 (mutant) and 24 (wild type) compared with the reference gene were determined as follows:

I. $\Delta\Delta Ct$ of $RB1e24_B = (CtRB1e24_B\text{-}CtAlbumin_B)\text{-}(CtRB1e24_N\text{-}CtAlbumin_N)$

Where B indicates blood sample of the patient and N denotes a pool of normal blood samples. Using the ratio equation $2^{\Delta\Delta Ct}$ the relative copy numbers were calculated. The expected values were about 1 for a normal dose of DNA and 1.5 for heterozygous duplication.

$2^{\Delta\Delta Ct} = [\Delta CT\ E\text{-}24_B\,(1.21) - \Delta CT\ E\text{-}24_{N}\,(1.18) = \Delta\Delta Ct\ 0.03] = 1.02$

The number of *RB1* E-24 copies in blood DNA is similar to that of normal individuals

II. $\Delta\Delta Ct$ of $RB1e24_T= (CtRB1e24_T\text{-}CtAlbumin_T)\text{-}(CtRB1e24_N\text{-}CtAlbumin_N)$

Where T indicates tumor sample:

$2^{\Delta\Delta Ct} = [\Delta CT\ E\text{-}24_T\,(1.63) - \Delta CT\ E\text{-}24_N\,(1.18) = \Delta\Delta Ct\ 0.45] = 1.37$

The number of *RB1* E-24 copies in tumor is 1.37 times that of blood

There are 3 copies of *RB1* E-24 in tumor and 2 copies in blood

III. $\Delta\Delta Ct$ of $RB1e11m_T= (CtRB1e24_T\text{-}CtAlbumin_T)\text{-}(CtRB1e24_T\text{-}CtRB1e11_{mT})$

Where E-11mT indicates a mutant Exon 11 in tumor DNA:

$2^{\Delta\Delta Ct} = [\Delta CT\ E\text{-}24_T\,(1.63) - \Delta CT\ E\text{-}11_{mT}\,(1.08) = \Delta\Delta Ct\ 0.55] = 1.46$

The number of mutant *RB1* E-11 copies in DNA is 1/3 lower than that of *RB1* E-24. There were two copies of mutant *RB1* E-11 and three copies of *RB1* E-24 in tumor DNA. This analysis revealed that there were three copies of *RB1* gene in the tumor, two with a duplication of 56bp in exon 11 and one copy with a wild type exon 11, but presumably carrying the splice site mutation in intron 12.

Molecular genetic testing of RB patients identifies children with a heritable condition (approximately 50%), who have a genetic predisposition for second tumors[11]. Moreover, children with unilateral hereditary RB are at risk of bilateralization (metachronous bilateral RB)[12]. Inactivation of both alleles of the *RB1* tumor suppressor gene requires two mutations, being the first of them nonsense or frame shifting mutation and the second a chromosomal mutation in most of the cases. The results presented here revealed three different mutations: 1) frame-shifting insertion in exon 11 (novel mutation); 2) donor splice-site mutation in intron 12, which is the most common splice-site mutation in RB patients, leading to exon 12 skipping and generation of a stop codon and 3) duplication of a whole *RB1* gene (rarely reported). The presence of three *RB1* gene mutations in the tumor of the unilateral patient allows us to hypothesize that the *RB1* gene was inactivated by two mutations, an insertion of 56bp in exon 11 of one copy and a splice site mutation in intron 12 of the other copy, which led to development of the tumor. Later, during the continuous proliferation of retinoblasts, probably occurs another genetic alteration: duplication of the *RB1* copy with the mutant exon 11, since there were two copies of the mutant exon 11 and three copies of an *RB1* exon without mutation (exon 24).

This study allowed us to identify two uncommon and one recurrent *RB1* mutations in the same patient, being rare the occurrence of more than two mutations in the same tumor. The overall mutation rate of RB is very low, *RB1* is the only known cancer gene mutated in retinoblastoma[14].More than two *RB1* mutations have been reported in several RB patients, however most of them had undergone neoadjuvant chemotherapy, thus, the additional mutations may indicate a chemical damage of DNA[15]. The patient of our study had no chemotherapeutic treatment before enucleation (when tumor sample was isolated) thus, the finding of more than two *RB1* mutations in the same patient is a rare event and may be relevant to RB biology. The identification of only somatic mutations, which were absent in constitutional tissue of a unilateral patient, was useful to rule out the hereditary RB predisposition. The data obtained are essential for genetic counseling and support the relevance of performing complete genetic screening for *RB1* mutations in both tumor and constitutional tissues.

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Conflicts of interest: None to declare

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