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Mutations in the *RB*1 Gene in Argentine Retinoblastoma Patients and Uncommon Clinical Presentations

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Abstract

Background: Retinoblastoma, the most common ocular cancer of childhood, is caused by inactivation of the RB1 tumor suppressor gene in the developing retina. It may occur as unilateral, bilateral or rarely as multicentric retinoblastoma, including pineal or suprasellar tumors. Being the retinoblastoma a hereditary cancer, identification of the causative mutation is important for risk prediction in the family members. An early detection of tumor is critical for survival and eye preservation. Screening for *RB*1 mutations is important for early tumor detection, critical for survival and eye preservation.

Purpose: To identify causative *RB*1 mutations in retinoblastoma patients with different clinical presentations, some of them with a rare multicentric retinoblastoma or with a second non ocular malignancy, as well as the rare association with down syndrome. A comprehensive approach was used to identify the mutations and to detect children with a hereditary condition.

Methods: A cohort of 20 patients with unilateral, bilateral and multicentric retinoblastoma was studied. Blood and tumor DNA was analyzed by sequencing, segregation of polymorphisms and MLPA analyses. Some of the rare mutations were validated by cloning or by Real-Time PCR

Results: Six germline and seven somatic mutations were identified; they include nonsense, frameshift, splice mutations and gross rearrangements, four of them novel. Three out of four nonsense/frameshift germline mutations were associated with severe phenotype: bilateral and multicentric retinoblastomas. The at-risk-haplotype was identified in a familial case including one patient with osteosarcoma; it was useful for detection of mutation carriers.

Conclusions: This study allowed us to identify causative *RB*1 mutations, including several novels. Some patients showed uncommon clinical presentations of retinoblastoma. These data are significant for genetic counseling. Our results support the relevance of carrying out complete genetic screening for RB1 mutations in both constitutional and tumor tissues

Keywords

Retinoblastoma; Hereditary and non-hereditary; Clinical presentation; *RB*1-tumor suppressor gene; *RB*1-mutations; At-risk-haplotype

Introduction

Retinoblastoma (*RB*) is the most common ocular cancer in children. Though the incidence of *RB* is low (15.3-42.5 per million children aged 0-4) it presents as a hereditary cancer in approximately 50% of cases, thus, it is crucial to be diagnosed for its hereditary predisposition [1]. The development of *RB* is due to inactivation of the RB1 tumor suppressor gene in the developing retina (GenBank accession No L11910, MIM 180200). *RB* may be either bilateral (approximately 40%) or unilateral (60%) and in some rare instances it may present as a trilateral or quadrilateral tumor, including an intracranial primitive neuroectodermal tumor in the pineal or less frequently suprasellar region (5% of children with constitutional mutations) [2,3]. Hereditary *RB* is often associated with second, even multiple primary nonocular malignancies, with a cumulative incidence throughout the lifespan [4]. Sometimes, *RB* may occur in association with other syndromes, such as the 21 trisomy (Down) [5].

Bilateral *RB* is caused by a germline 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 by two somatic mutations (approximately 80%) [6]. Mutations in both RB1 alleles result in a loss of function of the retinoblastoma protein, leading to deregulation of cell proliferation and tumor development.

Individuals with germline mutations have hereditary predisposition to retinoblastoma, thus, the identification of the causative mutation is important to predict the risk for tumor development in family members. Retinoblastoma is a potentially curable cancer and may be diagnosed by several presenting signs such as leukocoria and strabismus, which correlate with a high survival rate of the patient. An early diagnosis is critical for survival and eye preservation in children who carry the mutation, which could be detected even by prenatal analysis. Moreover, a preimplantation test to select embryonic cells free of mutation, followed by *in vitro* fertilization may be employed to prevent the birth of children at risk. On the other hand, children not carrying the mutation can be excluded from the invasive ophthalmologic procedure to detect the tumor [7,8]. The most common treatment for *RB* in developing countries is enucleation, with or without an adjuvant chemotherapy according to tumor presentation [9,10,11].

Screening for *RB*1 mutations is challenging because of their heterogeneity, with over 900 mutations reported to date [12] and their distribution along the RB1 exons, the promoter region and the flanking intronic sequences. Moreover, only few mutations on specific sites are recurrent, such as the C>T transitions at the CpG dinucleotides, especially those affecting the arginine codon. Most of the mutations are unique or rarely reported [13,14]. Approaches using several techniques for mutation analysis may detect alterations in the RB1 gene in about 80 to 90% of patients [13,15,16]. The main barriers for achieving an efficient detection of RB1 mutations is the presence of mosaicism [17] and the location of mutation within non coding regions, far from the exon-intron junctions [18]. In addition, about half of the *RB* patients do not carry mutations in constitutional DNA but only in tumor DNA. Tumor tissue is not readily available; either because the patient was not enucleated, or was enucleated many years ago or otherwise the tumor sample is reserved in the bank

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for other analyses. In the absence of tumor DNA the failure to detect mutations in constitutional DNA does not provide certainty about the non-heritable, somatic nature of the causative mutation, since there is still a little chance that the constitutional heritable mutation is located outside the analyzed regions.

Most of the RB1 mutations are nonsense or frameshift including whole *RB*1 gene deletions, interexonic deletions, small intraexonic frameshift deletions or insertions and splice site mutations. All these mutations result in the absence of retinoblastoma protein. Some of the *RB*1 mutations such as missense, in frame deletions or those located in the promoter region are less common. This type of mutations is associated with low penetrance, consisting in fewer tumors (unilateral *RB*) or even an absence of tumors. In this regard, it is noteworthy that some unilateral *RB* patients carry a low penetrant germline mutation [13,15,19].

In a previous study we reported the results of our experience in retinoblastoma testing throughout 20 years, using a methodology that has evolved over the study period [19]. Here we present the results of RB1 mutations in retinoblastoma patients with different clinical presentations, some of them with a rare multicentric retinoblastoma or with a second nonocular malignancy, as well as the rare association of *RB* with Down syndrome. A comprehensive approach, based on our previous experience, was used to identify the causative RB1 mutations, including the novel ones, and to detect children with hereditary condition. These data are crucial to provide genetic counseling to the family.

Materials and Methods

Patients

Retinoblastoma patients were referred from the "Hospital de Pediatría JP Garrahan" (Buenos Aires, Argentina) as well as other pediatric health care centers in Argentina. The *RB* diagnosis was established by current ophthalmologic/histological criteria. A total of twenty retinoblastoma cases were studied, including fourteen sporadic unilateral, one of them associated with Down syndrome, three sporadic bilateral, one familial with two bilateral patients, one of them with a second tumor, and two sporadic multicentric. Informed consent for genetic analyses was signed by parents of the affected children.

DNA isolation and genotyping of polymorphic loci

Deoxyribonucleic acid (DNA) was obtained from peripheral blood leukocytes using the cetyltrimethylammonium bromide (CTAB) method and from frozen tumors by treatment with proteinase K, phenol/chloroform purification and ethanol precipitation. Segregation analysis of five polymorphic loci within the RB1 gene included two restriction fragment length polymorphisms (RFLPs: BamHI and XbaI, introns 1 and 17), and three microsatellites (Rbi2, Rbi4 and Rb1.20 introns 2,4 and 20), it was performed as previously described [19].

Mutation analysis

The mutation screening was performed in blood DNA samples from all patients and in tumor DNA samples from the six patients with an available tumor biopsy. PCR-amplification and sequencing of the 27 exons and the promoter region of RB1 gene was performed as already described using an ABI 3130XL genetic analyzer [19]. All the mutations were confirmed by both direction sequencing from separate PCR reactions and by their absence in normal control individuals. Mutations were described according to the nomenclature [20] using the RB1 sequence from the GenBank, accession No L11910. The identified mutations were submitted to the Retinoblastoma Database 2011 [14].

Multiplex Ligation-dependent Probe Amplification assay (MLPA) was performed using the Salsa MLPA kit PO47-B1 RB1 (MRC Holland). Fluorescent in situ hybridization analysis (FISH) on metaphase spreads was performed using 13q14 (RB1) specific green probe (Qbiogene Molecular Cytogenetics, Irvine, CA, USA). Quantitative multiplex-PCR analysis of the 27 RB1 exons and the main body of the promoter was performed as described [13].

Cloning of PCR products in pGEM-T vector

The vector contains thymidine residue (T) in the 3´ends for its pairing with the (A) residue incorporated by Taq polymerase in the PCR products. This vector also includes a multiple cloning site in the region encoding for α peptide of ß galactosidase, inactivation of this gene by insertion of a PCR product allows the identification of the recombinant clones. Cloning was performed as described [21]. In brief, the PCR products are 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 the chromogenic substrate 5-bromo-4chloro-3-indolyl-ß-galactoside (X-Gal). Recombinant vectors produced white colonies, while vectors without the insert originated blue colonies. The recombinant vector was extracted from white colonies and analyzed by digestion, electrophoresis and sequencing.

Real-time quantitative PCR

Quantitative values were obtained from the threshold cycle number (Ct) at which the increase in the signal, associated with the exponential growth of PCR product, begins to be detected. The results, presented as N fold differences in target gene relative to Albumin gene (one copy gene) and termed N target, were determined as follows: N target=2ΔCt, where the ΔCt value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the Albumin gene. For determination of the copy number of RB1 gene two exons were selected: the Exon 11, which is mutated in one tumor DNA, and the wild type Exon 24, using a forward primer for Exon 11 that only recognized the mutated sequence, 5´ TAATTCCTCCACACAGAGS 3´.

The reverse primer for Exon 11 and the primers for Exon 24 were the same as those used for sequencing, the primers for albumin gene were: Fw 5' TGAAACATACGTTCCCAAAGAGTTT 3' Rev 5' CTCTCCTTCTCAGAAAGTGTGCATAT 3'. Tumor and constitutional DNA (leukocyte DNA) of the patient were assayed using the DNA from a pool of 6 normal individuals as normal reference. The quantitative PCR was performed in a total volume of 25 µl containing 12.5 µl of SYBR Premix (Bio-Rad), 2 µl 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. Reaction run with the following conditions in an ABI 7900HT (Applied Biosystems, Foster City, CA, USA): 95°C for 10 min and 40 cycles of 95°C 10 s/60°C 15 s/72°C 20 s.

RNA analysis

Total RNA was extracted from blood leukocytes of the patient and a control individual using the TRIzol reagent (Invitrogen) and following the manufacturer´s protocol. Prior to RNA extraction, leukocytes were treated with 200 µg/ml of puromycin (Sigma-Aldrich, Inc. Saint Louis, USA) for 4hr at 37 ºC in order to prevent the degradation of mutant transcripts [18]. First strand of cDNA was synthesized with a mixture of random hexamers and the reverse transcriptase Superscript II (Invitrogen). A total of 2 µl of the cDNA reaction mixture where amplified in four overlapping fragments using PCR primers designed to obtain full coverage of the RB1 cDNA coding region [18]. PCR products were purified and subjected to sequencing.

Results

Clinical data, treatment and outcome

Most of the patients presented a unilateral *RB* (70%), and the tumor biopsy was available from six of them. The remaining showed different clinical presentations, bilateral, trilateral and quadrilateral *RB*, one *RB* patient with a second tumor and one *RB* associated with Down syndrome. The patient with a trilateral retinoblastoma included a neuroectodermal pineal tumor while another patient developed a suprasellar and also a small pineal tumor in addition to *RB*, thus he was considered as a quadrilateral retinoblastoma [22].

This patient died at seven months as a result of multiple brain metastases, despite an aggressive clinical treatment. The patient with a rare presentation of two syndromes, 21 trisomy (Down) and unilateral retinoblastoma had poor health and suffered a relapse. Thirteen of the *RB* patients were enucleated and eight of these received additional chemotherapy. The remaining, including those with multicentric retinoblastoma and the younger sibling of the familial case, were not enucleated and received chemotherapy and/or radiotherapy. The eye outcome of the patients and the causative mutations are displayed in Table 1.

RB1 mutations

Six germline and seven somatic mutations were found in eleven patients, these included three nonsense, three frameshift, two splice site mutations and five gross RB1 rearrangements.

Germline mutations were heterozygous (mutations that are present at a level of approximately 50% of the leukocyte DNA), while somatic mutations, identified in tumor DNA, were both homozygous or hemizygous (three) and heterozygous (four).

Nonsense and frameshift mutations

Nonsense germline mutations were identified in two sporadic patients: one bilateral (#565) - diagnosed at seven months - and the other unilateral (#600) - diagnosed at 30 months. Both were treated by enucleation followed by chemotherapy. A nonsense somatic mutation was identified in a tumor of one sporadic unilateral patient (#656), who was diagnosed and enucleated at 25 months without any other treatment. The second mutation in this tumor was the loss of heterozygosity (LOH). These three nonsense mutations were the well-known recurrent C>T transitions, one at CGA codon of exon 10, the other at CAA codon of exon 18 and the third at CGA codon of exon 23 respectively.

Two frame shift germline mutations-CC and TA-2bp deletions-were identified in exon 18 and exon 22 from a trilateral and a quadrilateral patient respectively. Both were considered novel mutations as they had not been reported in the RB1 gene mutation database [11]. The trilateral patient (#582), presenting bilateral *RB* and a pineal tumor, was diagnosed at 10 days and received chemotherapy according to the COG scheme (children oncology group). At present she is 3 years old and clinically stable. The quadrilateral patient (#568) had a rare presentation with a huge suprasellar mass, massive bone marrow and skull metastases and, in addition, a small pineal tumor. This patient was diagnosed at one month and received chemotherapy according to the COG scheme; however, he died at seven months.

Splice mutations

The same recurrent splice mutation-a G to A transition at the conserved donor site of intron 12-was identified in tumor DNA of two unilateral patients. One of them diagnosed at 17 months and enucleated without any other treatment (#551), and the other diagnosed at 16 months and treated by enucleation and chemotherapy (#621). This mutation was absent from constitutional DNA of both patients The second mutation identified in these tumors was a LOH in one of the patients (#551) and an insertion in exon 11 in the other patient (#621), which is described in the next section.

Gross rearrangements

The gross rearrangements were identified in five patients and comprised either deletions or duplications, ranging from two exons to a whole RB1 gene, including neighboring centromeric and telomeric genes. A deletion of two exons (22 and 23) was identified in the constitutional DNA of a bilateral patient (#570), diagnosed at 6 months and treated by irradiation. Her current age is 17 years and she preserved her vision. A second grade *RB* cousin of this patient did not carry this mutation and no other mutations were detected in his blood DNA. As this was a unilateral patient the causative mutation probably occurred in the tumor. Thus, both cousins developed *RB* as a result of different mutations.

Whole RB1 gene deletions were found in two patients, one in the constitutional DNA from a child who presented tumors in both eyes at the early age of 15 days, and the second, in the tumor DNA from a patient with retinoblastoma associated with Down syndrome. This deletion was heterozygous in tumor DNA (the second mutation was not identified) and it was absent from constitutional DNA. The patient with the two syndromes was diagnosed for *RB* at 20 months and enucleated, however, he suffered a relapse and received chemotherapeutic treatment, finally, and he died due to poor health.

Two different duplications were identified in tumor DNA from one patient (#621): 1) in tandem duplication of a 56 bp sequence in exon 11; and 2) duplication of the whole RB1 gene plus the flanking centromeric and telomeric genes. The 56 bp duplication was validated by cloning of the 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. These data confirmed the presence of a heterozygous 56bp insertion in exon 11. The duplication of the whole gene was validated by Real-Time PCR analysis of two RB1 exons, one carrying the mutation (exon 11) and the other with a wild type sequence (exon 24).

This analysis revealed the presence of three alleles of exon 24 and two alleles of the mutant exon 11 as compared with the albumin gene (two alleles). Therefore, there were three copies of RB1 gene in the tumor, two with an insertion of 56 bp in exon 11 and one with a wild

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Table 1: Description of retinoblastoma patients with RB1 gene mutations **(**Mutation description according to den Dunen and Antonarakis nomenclature using the genomic sequence of GenBank (L11910.1); **BiRB** Bilateral retinoblastoma; **del**: large deletion; **ITM2B**: centromeric gene; **DLEU1**: distal telomeric gene; **Centrom**: centromeric; **Telom**: telomiric; **dup:** large duplication; **ch**: chromosome.).

Patient ID	Phenotype	Age at diagnosis (months)/ Treatment/2nd tumour	Tissue Analyzed	Mutation Description	Location relative to exon/intron	Expected consequence	Recurrence	Comments
551	Sporadic Unilateral	17/Enucleation/No other tumours	Tumour Blood	1:g.70330G>A 2: LOH Absence of Mutation	IVS12+1G>A	Exon 12 skipped/ Frameshift/ Stop codon p380	Very recurrent	Nonhereditary
554	Sporadic Unilateral	29/Enucleation and Chemotherapy/ No other tumours	Tumour Blood	1:c.264?_501+?dup 2: LOH Absence of Mutation	Insertion in Intron 4	Duplication of exons 3 and 4/ Frameshift/ Stop codon p188	One recurrence as a germline mutation	Nonhereditary
565	Sporadic Bilateral	7/Enucleation, Chemotherapy, Radiotherapy	Blood	g.64348C>T	Exon 10	Premature stop codon: p.R320X	Very recurrent	Hereditary
568	Sporadic Quadrilateral	Chemotherapy/ Died at 7 months	Blood	g.162045-46delTA	Exon 22	Frameshift Stop codon pV754fsX756	Novel	Hereditary
570	Sporadic Bilateral Second cousin Unilateral Rb	6/Radiotherapy 27/Enucleation	Blood Blood	c.2211-?_2490+? del No mutation detected	Deletion exons 22/23 No deletion	Exons 22/23 missing. Frameshift, stop codon: p744 ND	One recurrence ND	Hereditary Nonhereditary
582	Sporadic Trilateral	10 days/chemotherapy current age: 28 months	Blood	g.150057-58delCC	Exon 18	Frameshift, stop codon p.H585fsX586	Novel	Hereditary
583	Sporadic Bilateral	15 days	Blood	g. ITM2B-?_DLEU1+? del c.-138_2784+? del	*RB1*+Centrom+Telom genes	Deletion of whole *RB1* and other genes on ch13	Recurrent	Herditary
600	Sporadic Unilateral	30/Enucleation	Blood	g.150025C>T	Exon 18	Premature stop codon p.Q575X	Recurrent (9 times reported)	Hereditary
621	Sporadic Unilateral	16/Enucleation & Chemotherapy	Tumor Blood	1:g.65431-65432 ins56bp 2:g.70330G>A 3:g.ITM2B-?_DLEU1+? dup Absence of mutations	Exon 11 IVS12+1G>A *RB1*+Centrom+Telom genes	1.Frameshift/Stop codon p.363 2.Exon12 skipping/ Frameshift/ Stop codon p.380 3.Duplication of a mutant *RB1* gene	Novel Recurrent Recurrent	Non hereditary
636	Sporadic Unilateral *RB* +Down	20/Enucleation & Chemotherapy	Tumor Blood	g. ITM2B-?_DLEU1+? del Heterozygous 1:Absence of *RB1*Del 2:Trisomy 47XY+21	RB1+Centrom+Telom genes Ch.21	Deletion of RB1 and other Ch. 13 genes Down syndrome	Recurrent	Non hereditary
656	Sporadic Unilateral	25/Enucleation	Tumor Blood	1:g.162237C>T 2: LOH Absence of mutation	Exon 23	Premature stop codon p.R787X	Very recurrent	Non hereditary

type exon 11. In addition to these duplications a third mutation was identified in the same tumor, the splice site G to A substitution in intron 12, aforementioned in the section "Splice Mutations". Thus, the third copy of RB1 gene, with a wild type exon 11 presumably carried the splice site mutation in intron 12. All three mutations were heterozygous and were absent from constitutional DNA.

Duplication of two exons, 3 and 4, was identified in the tumor DNA of a unilateral patient (#554) diagnosed at 29 months and treated by enucleation and chemotherapy. This was a somatic mutation as it was absent from constitutional DNA, the second mutation in this tumor was a LOH.

Familial *RB* case

One family included two bilateral *RB* siblings, one asymptomatic sibling and asymptomatic parents. The older *RB* patient was diagnosed at 18 months of age and was treated by enucleation, chemotherapy and radiotherapy. At the age of 18 years he also developed an osteosarcoma and received chemotherapeutic treatment.

His younger sister was diagnosed at an earlier stage and treated solely with focal therapy (laser coagulation). Molecular testing by sequencing, MLPA, FISH and QM-PCR analyses at the DNA level was uninformative for the causative mutation. Analysis at RNA level, in conditions that inhibit the surveillance pathway of nonsense mediated decay, showed a double peak chromatogram with superimposed sequences of exons 8 and 9. These results indicate an absence of exon 8 in one RB1 copy, which would result in frameshifting and generation of a stop codon.

However, the control DNA (normal individual) treated with puromycin also showed a heterozygous absence of exon 8 in the blood sample, therefore this anomaly would not be the causative mutation and the lack of exon 8 might result from an alternatively spliced transcript [23]. Segregation analysis of five polymorphic loci within the RB1 gene revealed the haplotype-at-risk, shared by the two *RB* siblings, the asymptomatic sibling and the asymptomatic father (Figure 1).

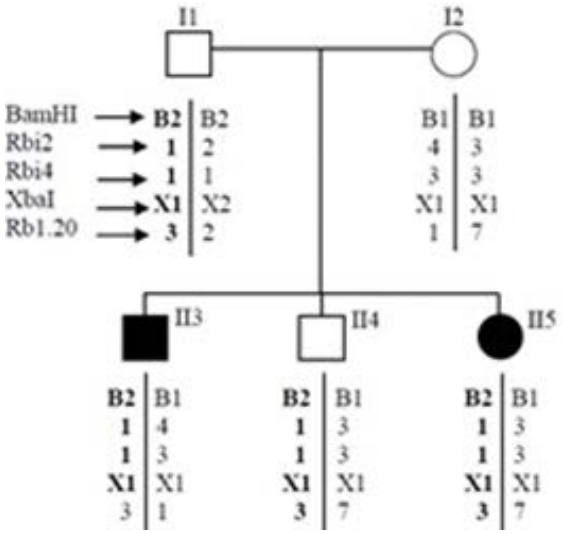

Figure 1: Pedigree and haplotype analysis of one family with two *RB* siblings. The polymorphisms analyzed are indicated at the left of the pedigree. The haplotype at risk is indicated in bold.

Discussion

Different *RB* presentations and outcome

Molecular genetic testing of *RB* patients identifies children with a heritable condition (approximately 50%) who have a genetic predisposition for second tumors. Moreover, the children with unilateral hereditary *RB* are at risk of bilateralization (metachronous bilateral *RB*) [24]. Molecular analysis of patient`s relatives detects presymptomatic *RB* children, allowing early diagnosis and treatment. For these reasons, the identification of mutations is essential for planning treatment strategies and a long-term follow up, in order to improve survival rates and the life quality of patients and their relatives [8]. On the other hand, the search for mutations among the family members detects the non-carriers of *RB* mutations, reducing in this way the clinical screening procedures, which have potential morbidity for these children.

The high survival rate (90-95%) in developed countries contrasts with poor prognosis for *RB* in developing countries, where the vast majority of *RB* patients live [25]. In our country a mean of 44 new *RB* patients are treated yearly at the Garrahan Hospital (reference *RB* center), but probably there are still more children with *RB*, living in poverty and without receiving medical attention. At present, the survival rate of the ascertained patients is about 80%, with the follow-up continuing into adulthood. The average age at diagnosis, according to uni, bi or trilaterality of the tumor, is similar to that reported in other countries [26].

Trilateral patients represent 3.7% of the children with germline RB1 mutations in our cohort of *RB* patients (three in a total of 81 patients with bilateral/familial *RB*), in full agreement with previously reported data [2]. Two of these *RB* patients had had a pineal tumor and the third had developed a suprasellar tumor and later a small pineal one. The suprasellar malignancy was presented as very aggressive and at an early age, causing a prompt death in spite of intensive treatment. The two patients with pineal tumor were less severely affected, one of them, reported previously [19] died at the age of four years and the other is still alive at the age of three years. A patient, who had had a bilateral/familial *RB* (#559), later developed a second primary tumor, an osteosarcoma. This fact along with our previous results on second malignancies in another patient with familial *RB* (#112) [19] support the findings that patients with familial *RB* have a slightly higher risk of developing second tumors [27]. One patient was a rare case with two different syndromes, Down and retinoblastoma. Previous reports have suggested an increased incidence of leukemia and some solid tumors like retinoblastoma in Down patients [28,29]. A possible mechanism of tumor development may be related to gene dosage on chromosome 21 [28].

Genotype and Phenotype

Germline mutations were identified in five of the six patients with bilateral/familial *RB* and in one of the fourteen unilateral patients. Additionally, somatic mutations were found in tumor DNA from five unilateral patients. All functional classes of mutations were identified and they were distributed throughout the RB1 gene, but predominantly in the 3´ region. Nonsense/frameshift mutations, which have been reported to be the most common RB1 mutations, were found in six patients (46%), gross rearrangements were identified in five patients (39%) and the splice site mutation was observed in two patients (15%). Three out of the four nonsense/frameshift mutations of germline origin were associated with severe phenotype: a bilateral

RB (nonsense mutation), a trilateral *RB* and a quadrilateral *RB* (frameshift 2bp deletions in each one). Of note, one trilateral case, previously reported, was also caused by a frameshift 1bp deletion [19]. Thus, the multicentric *RB* cases were associated with frameshift intraexonic deletions in our group of *RB* patients. Unexpectedly, one nonsense germline mutation was associated with a unilateral *RB* of late diagnosis and an advanced development of tumor that had to be enucleated. These data will be significant for genetic counseling.

One of the gross rearrangements, the duplication of exons 3 and 4 found in tumor DNA, is similar to that previously reported in constitutional DNA of an *RB* patient, duplication of exons 3 and 4 in tandem orientation, resulting in frameshifting and generation of a stop codon [18]. The appearance of the same uncommon mutation in different tissues, germ cells and retinoblasts, suggest that it may occur by a similar mechanism in both tissues, namely duplication in tandem orientation, leading to premature termination of translation. The other gross rearrangement, a germline deletion of exons 22-23, presumably had the same consequence as the above mentioned, frameshifting and a premature stop codon. It is remarkable that one germinal whole RB1 gene deletion was detected in a bilateral *RB* patient soon after birth. Our previous results as well as other reports show that the whole RB1 gene deletions and cytogenetic deletions occur mostly in unilateral patients [19,30]. The donor splice-site mutation in intron 12, identified in two of our patients, is the most common splice-site mutation in *RB* patients, which leads to exon 12 skipping generating a stop codon [18]. The presence of three mutations in the RB1 gene of one tumor DNA is an uncommon event. In addition, two of them were rare mutations, the 56bp duplication in exon 11 was novel, and the duplication of the whole gene was reported in tumor DNA of two patients, but probably without functional inactivation of the RB1 gene [31]. On the contrary, the presence of three mutations in one patient from our study allows us to hypothesize that the RB1 gene was inactivated by two mutations, an insertion of 56 bp 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 occurred another genetic alteration, a duplication of the RB1 copy with the mutated exon 11, since there were two copies of the mutant exon 11 and three copies of an RB1 exon without mutation (exon 24).

Indirect analysis in familial *RB*

The polymorphic loci within the RB1 gene were useful in identifying the at-risk-haplotype in one familial *RB* case, where the direct DNA analysis was uninformative. These data allowed us to detect individuals likely of carrying the mutant RB1 allele but who do not show signs or symptoms of *RB*. In order to clarify what type of RB1 mutation caused tumor development in the two affected siblings we analyzed the genetic and clinical data in this family. Previous findings show that *RB* patients with nonsense mutations are more susceptible to develop second non ocular tumor than *RB* patients with low penetrant mutations [32]. Thus, the fact that both affected siblings had had a bilateral *RB* and that the older one, in addition, developed an osteosarcoma, suggest that the causative RB1 mutation should be highly penetrant, leading to a functionally disabled protein. However, this assumption contrasts with the presence of at-risk-haplotype, and hence a mutant RB1 allele, in two unaffected members of the family, the father of *RB* siblings and their healthy brother. This discrepancy may be explained by the presence of mosaicism in the unaffected father, who could have transmitted the mutant chromosome to the affected offspring and the wild type chromosome, with the same haplotype, to the healthy son. Another possibility is the influence of genetic factors that can modify the expressivity of *RB* and therefore the genotype-phenotype outcome [33].

Mutations at RNA level

The absence of mutations in the DNA coding regions may indicate that the alteration occurred in other DNA locations, such as the deep intronic sequences, not reached in the amplified products of exons plus flanking intronic sequences [18,34]. Analysis at RNA level of the familial *RB* case showed an absence of exon 8 in the patient and also in a normal individual. These results are in line with previous findings in normal breast and prostate tissue that show a skipping of exon 8 originating an alternative transcript in small amounts ~3% [23]. This transcript would be subjected to nonsense-mediated decay (NMD) and can only be detected by pretreatment of cells with puromycin. No other alterations at RNA level were identified until now. Since deep intronic mutations are a rare event, found in a small percentage of patients with undetectable DNA mutations [18], other genetic alterations should be investigated. The search for a causative RB1 mutation in this family is being pursued at molecular and cellular levels.

Conclusions

This study allowed us to identify interesting and rare RB1 mutations, as those found in one *RB* tumor with three mutations in the RB1 gene; this finding may be relevant to *RB* biology. Furthermore, several rare clinical presentations were observed: trilateral and even quadrilateral tumors, developed from precursor cells similar to retinoblasts in the pineal and suprasellar regions and retinoblastoma associated with Down syndrome. The identification of somatic mutations in five unilateral patients was useful to rule out hereditary *RB* predisposition. Segregation analysis of polymorphic loci enabled the identification of mutation carriers in one familial case without evidence of RB1 mutations. The data obtained in this study are crucial for genetic counseling of the affected families and support the relevance of performing complete genetic screening for RB1 mutations in both tumor and constitutional tissues.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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