THE RELEVANCE OF MOLECULAR BIOLOGY STUDIES IN THE GENETIC COUNSELLING OF ARGENTINE RETINOBLASTOMA FAMILIES

IMPORTANCIA DE LOS ESTUDIOS DE BIOLOGÍA MOLECULAR EN EL ASESORAMIENTO GENÉTICO DE FAMILIAS ARGENTINAS CON RETINOBLASTOMA

PARMA DL¹, DALAMON VK¹, FERNÁNDEZ C², SZIJAN I¹, DAMEL A³

ABSTRACT

Objective: Evaluate the relevance of RB1 mutations detection in the genetic counselling of Argentine retinoblastoma families.

Methods: We included in this study 34 Argentine families with bilateral and unilateral Retinoblastoma (Rb). 130 DNA samples from leukocytes, tumors and chorionic villus were analyzed by indirect and direct molecular biology assays like Southern blot, segregation of polymorphisms BamHI, Rbi4, XbaI and Rb 1.20 (PCR-RFLP, PCR-STR), PCR-heteroduplex and sequencing of RB1 gene.

Results: Molecular biology analysis was informative in 18 out of 34 families studied (53%), 56% with bilateral and 44% with unilateral Rb. DNA tumor samples of 11 patients were available and could be studied by loss of heterozygosity (LOH) detection, that allowed us to identify the mutated RB1 allele in 9 (82%) patients. When tumor samples were not analized, the studies were informative only in 9 out of 23 patients (39%); we used direct mutation

RESUMEN

Objetivo: Evaluar la importancia de la detección de mutaciones del gen RB1 en el asesoramiento genético de las familias argentinas con retinoblastoma.

Métodos: Se incluyeron en este estudio 34 familias argentinas con Retinoblastoma (Rb) bilateral y unilateral. Se analizaron 130 muestras de ADN de leucocitos, tumores y vellosidades coriónicas, por ensayos de Biología Molecular indirectos y directos, como Southern blot, segregación de los polimorfismos BamHI, Rbi4, XbaI y Rb 1.20 (PCR-RFLP, PCR-STR), PCR-heteroduplex y secuenciación del gen RB1.

Resultados: El análisis molecular fue informativo en 18 familias de las 34 incluidas en el estudio (53%), el 56% con Rb bilateral y el 44% con Rb unilateral. Se contó con muestras de ADN tumoral de 11 pacientes que se estudiaron para detectar pérdida de heterocigosidad (LOH), que posibilitó identificar el alelo RB1 mutado en 9 pacientes (82%). Cuando no se analizaron las muestras tumorales,
INTRODUCTION

Retinoblastoma (Rb) is a malign tumor of childhood that begins with the concurrence of 2 mutational events in locus 13q14 which deactivates both alleles of the retinoblastoma gene (RB1) (1). RB1 is part of the tumor-suppressing gene family, that normally inhibit cellular growth and negatively regulate proliferation (2). This gene has 200 kb and 27 exons and encodes for a cellular cycle-regulating protein called pRb or p110 RB (100KDa) (3).

Alfred Knudson proposed that the deactivation of RB1 occurs through the «double hit» mechanism. The first mutation may appear in the germinal line (hereditary Rb, 40%) or in somatic cells (non-hereditary Rb, 60%) (4). The second mutation is somatic in all cases (it occurs only in retinoblasts) and arises with frequency. In the hereditary form, which expresses as a multifocal or bilateral Rb, one of the mutations is present in all the cells of the individual, including somatic (retinal) cells and germinal cells. In the non-hereditary form of the disease, which expresses as a unifocal unilateral Rb, both mutations must occur in the same retinoblast (5).

In 70% of tumors, the second mutation involves the somatic loss of the normal allele. These allele loss mechanisms can be manifested by molecular biology tools such as the loss of heterozygocity (LOH) of polymorphic loci in RB1, comparing the patient constitutional genotype and the tumor genotype (6,7).

Therefore, due to the high frequency of occurrence of the second mutation in RB1, the Rb is transmitted as a dominant autosomic disease with a penetration of 90% (8), i.e., only 10% of patients exhibiting a mutation in said gene would remain as healthy carriers. In the hereditary form, only 25% of cases exhibit familial history (hereditary transmitted Rb), the remaining 75% is due to new mutations in the germinal line or during gestation, which will be transmitted by the patient to his/her descendants (hereditary de novo). In non-hereditary cases, both mutations are present only in the tumor tissue and therefore the mutation is not transmissible.

The types of mutations described in RB1 are: microscopic deletions (5%), sub-microscopic deletions (15%) and small mutations (80%) (9). Molecular biology strategies for studying these mutations can be direct or indirect depending on whether the involved mutation is detected.

The study of mutations is fundamental for early diagnosis, detection to a predisposition to Rb and other cancers and genetic counseling for the families. The diagnostic time is important for eyesight prognosis and survival.

SUBJECTS, MATERIAL AND METHODS

This article comprised 34 families with bilateral and unilateral retinoblastoma (Rb), of different ethnic origins and all geographical regions of Argentina (table I). The patients were referred from the ophthalmology service of the Children’s Hospital of Buenos Aires city, and the diagnostic was establis-
Molecular diagnosis of retinoblastoma

The molecular analysis was informative in 18 families of the 34 included in the study (53%) (table I), 56% with bilateral Rb and 44% with unilateral Rb (10-14). In 15 families it was possible to identify whether the mutated gene was paternal (in 6) or maternal (in 9).

RESULTS

The molecular analysis was informative in 18 families of the 34 included in the study (53%) (table I), 56% with bilateral Rb and 44% with unilateral Rb (10-14). In 15 families it was possible to identify whether the mutated gene was paternal (in 6) or maternal (in 9).

Tabla I.

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m: male; f: female; ND: not determined.
Tumor DNA samples of 11 patients were available and studied to detect loss of heterozygocity (LOH), which made it possible to identify mutated RB1 allele in 9 patients (82%). When tumoral samples were not available, the studies were informative in only 8 of the patients (39%). Direct detection was utilized in 17 patients (41% was informative) and indirect in 20 (60% was informative).

Below the analyses made in four families of this group are shown. Individual 60 (bilateral tumor, no history) carried mutation g.156820delA, in exon 20 of gene RB1, evidenced by heteroduplex and posterior sequentiation (14). The peripheral blood DNA was analyzed for two brothers in the risk age group to establish the probability of developing Rb. In both cases, the sequence was comparable to the sequence obtained for a normal control. Accordingly, both brothers were excluded from the risk of developing or transmitting Rb to their descendants (fig. 1).

Individuals 148 and 291 (without history, unilateral tumor of early appearance, suggesting hereditary de novo characteristic), exhibited LOH in the tumor, which allowed us to determine the parental inheritance of the affected allele in the family (figs. 2 and 3). For the RB1.20. polymorphism, patient 148 maintained allele 4 in the tumor and patient 291 allele 2. As the normal allele is lost, it is assumed that the allele remaining in the tumor (maternal) is the one linked to the mutation. Assuming that the Rb was hereditary, the identification of the mutated chromosome in the patient would allow us to predict susceptibility to Rb, only through exclusion, in siblings or descendants, thus becoming a part of the genetic counseling. The sister of patient 291 inherited paternal (NC/158/NC/4) and maternal alleles (C/162/C/3) different to those of the patient. Thus, she was excluded from risk because she did not inherit the maternal allele linked to the mutation.

Individual 194 (without history, bilateral tumor) evidenced a deletion in region 3’ of gene RB1 in constitutional DNA, when segregating alleles for the 4 intragene polymorphisms. The segregation of alleles for the polymorphisms located in gene region 5’ and central, revealed the presence of both the paternal and maternal alleles. On the contrary, the analysis of polymorphism RB1.20 evidenced the constitutional deletion by the absence of the maternal allele variation for said locus (fig. 4). Extreme 5’ of the deletion should be found in intron 17, downstream from the polymorphic site for the Xbal restriction enzymes. In this case, the study of

Fig. 1: Normal sequences (left, centre) obtained for both siblings of the Rb patient. Punctual deletion in exon 20, g.156820delA, identification in the patient (red arrow), generates displacement of the reading framework (right).

Fig. 2: Segregation of polymorphisms for patient 148 and his family. A. Polyacrylamide gel for RB1.20. The tumor sample of the patient (TU) shows the absence of paternal allele 3. B. Genealogy with haplotypes determined.
intra-gene polymorphisms becomes a direct study
which evidenced the deactivating mutation of gene
RB1. By means of this same study it was possible
to exclude the sister of patient 194 as carrier of the
mutation which predisposes to the pathology becau-
ses she inherited the other maternal allele and the
absence of allele RB1.20 was not detected.

**DISCUSSION**

The results demonstrate the need of including the
tumor DNA when the patient was enucleated, and
emphasize the importance of direct detection of the
mutation in families with early sporadic Rb without
tumor sample. However, as a consequence of a
substantial improvement in detection and treatment
methods, the number of enucleations is gradually
diminishing, making it increasingly difficult to
obtain tumor tissue for this type of study.

The absence of an anomaly which can be detec-
ted in the DNA of a constitutional tissue (such as
the peripheral blood leucocytes), suggests a non-
hereditary form of the disease. In any case there
could be small structural alterations which are not
detectable with the methods being used. Therefore
there cannot be 100% probability of a sporadic
case, without risk for the future descendants of the
patient. However, the majority of unilateral Rb (60-
75%) are not hereditary, whereas 25-40% are.

Having identified the mutated RB1 allele it is
possible to determine the risk for the patient’s rela-
tives. Accordingly, the results obtained allow for
the establishment of several aspects of genetic
counseling for each family (19-21). If it is a germi-
nal mutation, it’s a hereditary case. The descendan-
ts have 50% probability of inheriting a copy of the
mutated RB1 gene and, with these studies, the pre-
symptomatic test can be determined for the future
progeny. For the children that don’t have a mutated
allele, it is not necessary to carry out the frequent
and uncomfortable ophthalmological explorations
because they have a very low risk of developing Rb,
while monitoring can be focused on the carriers to
increase the probability of early detection and ther-
fore of preserving their eyesight. It is important to

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Fig. 3: Segregation of polymorphisms for patient 291
and his family. A. Polyacrylamide gel for RBI.20. In the
tumor (Tu) paternal allele 1 is absent. B. Genealogy with
haplotypes determined. The sister inherited alleles which
are different to those of the patient.

Fig. 4: Segregation of polymorphisms for patient 194
and his family. A. Polyacrylamide gel for RBI.20. The
patient does not have maternal allele 2. The sister inhe-
rited paternal allele 3 and maternal 2. B. Genealogy with
haplotypes determined.
determine the status of the parents as being carriers or not because this allows for assessing the risk of transmitting to their children the predisposition to developing the tumor. If the parents are not carriers of the mutated allele, their future children will not be at risk of developing Rb or other related tumors. However, if either of the parents is a carrier, their children will have 50% probability of inheriting the same mutation, with the risk of developing the disease. On the other hand, if the mutation is not detected in the constitutional DNA of the patient with unilateral Rb, it probably is a sporadic, non-heritable case and the risk for the future descendants is low (22).

In this group of families, the molecular biology studies contributed to adequate genetic counseling and an appropriate design of early treatment.

REFERENCES