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Hereditary Retinoblastoma Transmitted by Maternal Germline Mosaicism

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Background. Investigating transmission of a constitutive, g78238C > T (R552X), *RB1* mutation in four affected children descended from three different unaffected fathers and an unaffected mother. ***Procedures.*** Sequence data analyses and allele-specific PCR assays were used to investigate the presence of the mutation in four affected children, five unaffected sibs (or half-sibs), and the unaffected mother. Haplotyping was carried out for confirming that the children descended from different fathers. ***Results.*** Haplotyping excluded the possibility of paternal transmission of a de novo mutation and provided evidence of maternal germline mosaicism. The mutation was apparently absent in blood- and buccal cell-DNA of the mother who also showed a normal fundoscopy. ***Conclusions.*** Our findings indicated that mosaicism was restricted to the maternal germline. The mutational event must have occurred at least 4 weeks post-conception, unlike the early mutational events of most mosaics, occurring between fertilization and the 8th day of conception. The implications of these findings are discussed in view that genetic counselling should discriminate between germline mosaicism and de novo events in pseudo-low-penetrant hereditary retinoblastoma. Pediatr Blood Cancer 2008;51:598–602.

Key words: genetic counselling; hereditary retinoblastoma; maternal germline mosaicism; *RB1* mutations

INTRODUCTION

Retinoblastoma is a malignant tumour originated in developing retinoblasts, with an incidence of 1/15,000–1/20,000 live births and accounting for 3% of childhood neoplasms in the Western world [1]. This condition might be familial-hereditary (5–10%), de novo-hereditary (20–30%) or sporadic (60–70%), and in hereditary cases, mutations are constitutional, mostly associated with bilateral presentation [2]. *RB1* mutations are inherited as dominant traits from affected parents or may originate at pre-zygotic or post-zygotic events. Pre-zygotic mutational events may occur in healthy progenitors, mainly in males and during spermatogenesis, when DNA repair mechanisms are apparently inoperative. Post-zygotic events frequently occur in paternally derived chromosomes, probably as a consequence of the residual impairment of the male germline genome in efficiently repairing DNA [3].

Post-zygotic events, occurring at different stages of embryonic development, frequently result in mosaicism that may be unevenly present in different organs, tissues and cell types. This may account for gonadal, or germline, mosaicism by which an *RB1* mutation may be exclusively transmitted by gametes derived from a single germ cell progenitor, masking the pattern of dominant inheritance and resulting in pseudo-low-penetrant hereditary retinoblastoma [3].

Transmission of *RB1* mutations by germline mosaics has been more frequently reported in males consequently to the possibility of analysing sperm DNA. Conversely, in females, in which analyses of mature gametes might require more complex and invasive procedures, germline mosaicism has been demonstrated by pedigree analysis [3,4]. Both in males and females, germline mosaicism frequently coexists with somatic mosaicism, sometimes with mutated *RB1* alleles at levels below the expected 50% for heterozygote carriers. Thus, the demonstration of somatic mosaicism represents a valuable indicator of germline mosaicism, especially in healthy progenitors of affected offspring.

Here we report transmission of a constitutional *RB1* mutation by an unaffected female progenitor to four of her nine offspring. The fact that all carriers of the same mutation descended from three different unaffected fathers ruled out a pre-zygotic, de novo mutational event. Haplotypic analyses indicated that maternal germline mosaicism accounted for the transmission of this mutation. Interestingly, somatic mosaicism was apparently absent.

PATIENTS AND METHODS

We studied a family with four affected and five healthy offspring (Fig. 1) referred to genetic counselling at the Instituto Nacional de Câncer (Rio de Janeiro, Brazil). All participating families provided an informed consent for the research team to perform molecular tests and to use samples for retinoblastoma research in an anonymous manner. This study followed the tenents and guidelines of the Declaration of Helsinski.

According to clinical data, affected children were born at different intervals (13 years between II-1 and II-8; 7 years between II-8 and II-9, and 2 years between II-9 and II-10). All children and their mother were subjected to ophthalmologic examinations, including fundoscopy, to search for retinoma or other retinal lesions.

As a constitutional, g78238C > T mutation in *RB1* exon 17, resulting in a truncated RB protein (R552X), had been previously found in individuals II-1 and II-8 of this family [5], we further searched for this mutation in peripheral blood DNA of the seven other sibs (or half-sibs), and in two separate blood DNA samples of their mother, and in a DNA sample from buccal swab epithelial cells.

Additional Supporting Information may be found in the online version of this article.

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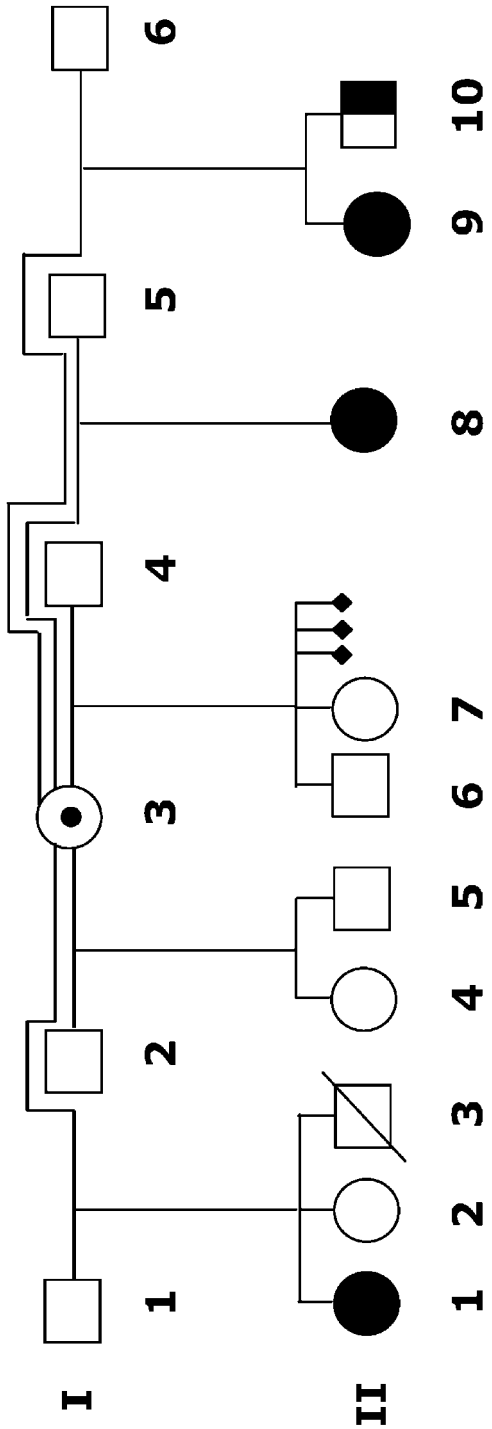

Fig. 1. Heredogram showing transmission of retinoblastoma. Black circle: bilaterally affected female; half-black square: unilaterally affected male; white circle and square: unaffected individual; circle with dot: unaffected female with germline mosaicism. Death of II-3 was unrelated to retinoblastoma. Three miscarriages occurred between II-7 and II-8. The four affected children descended from three different unaffected fathers (see Supplemental Table for genotypic analyses of this family).

Amplification of exon 17 of the *RB1* gene was performed as previously reported [5] in 50 µl reaction mixes containing 1X Mg-free buffer (Promega Corporation, Madison, Wisconsin, USA), 3 mM $MgCl_2$, 0.15 mM of each dNTP, 0.4 pm of each primer (forward and reverse) and 1 U of *Taq* DNA polymerase (Promega Corporation). PCR was performed with 40 cycles at 94°C (1 min), 54°C (40 sec) and 72°C (35 sec) in a programmable thermocycler (PT-100; MJ Research, Inc., Waltham, Massachussets, USA).

An allele-specific, sense primer (5′-AAATGATAAAACATT-TAGAAT-3′), designed for PCR amplification of a 116 bp fragment containing the R552X mutation and a reverse primer (5′-AATT-TGTTAGCCATATGCACATG-3′) were used for testing somatic mosaicism in the mother. The sense primer was specific for either the wild-type or mutant genomic DNA templates at the ultimate 3′ base position. Reaction mixes were prepared as previously described, with *Taq* DNA Polymerase, Recombinant® (Invitrogen, Carlsbad, California, USA). PCR conditions [40 cycles at 94°C (1 min), 49°C (40 sec) and 72°C (35 sec), in a programmable thermocycler (PT-100; MJ Research, Inc.)] were optimised for strongly amplifying samples with the mutation and only in the presence of the mutant allele. PCR products of two positive control samples (two affected children), a DNA blood sample of the mother, and of five negative non-carriers of the mutation, were resolved by acrylamide gel electrophoresis.

Direct sequencing was performed with an ABI PRISM 377 automatic DNA sequencer (Applied Biosystems, Foster City, California, USA) and sequences were edited using Sequence Navigator software (Applied Biosystems 1994).

We also analysed three 13q microsatellite markers, one located within *RB1* (D13S153, in intron 2) and two downstream, tightly linked *RB1* flanker markers (D13S262 and D13S284), for genotyping the mother and all children, and for deducing their presumptive haplotypes. Amplifications were carried out with labelled primers as previously described [6]; amplified products were diluted, denatured and subsequently analysed in a MegaBace™ 1000 DNA automatic sequencer (Amersham Biosciences, Piscataway, New Jersey, USA). Data were analysed with MegaBace™ Genetic Profiler (Amersham Biosciences).

Further genotyping was carried out with PowerPlex™ 16 System (Promega Corporation), following the recommendations of the manufacturer, for unambiguously corroborating maternity, sibship and half-sibship, and descendence from different fathers as indicated in the heredogram. This comprised multiplex PCR amplifications of 15 STR loci, including D13S317 (a microsatellite marker located at 13q22-q31). Furthermore, in order prove that the children descended from five different fathers we analysed the HUMANDREC region of the X-linked androgen receptor (*AR*) gene in all female children as previously reported [7].

RESULTS

The family heredogram shows the offspring of an unaffected mother and five different unaffected fathers, in which affected children descended from three different unaffected fathers (Fig. 1). Genotyping with PowerPlex™ 16 System confirmed maternal descendence, sibships and half-sibships as indicated in the heredogram, and descendence from five different male progenitors. This latter finding was also confirmed by analysis of the HUMANDREC region in the female offspring of each male progenitor (see Supplemental Table).

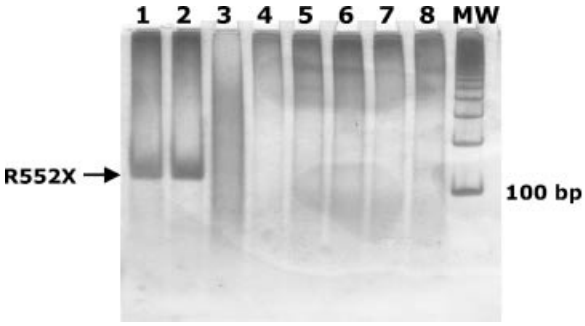

Fig. 2. Two positive controls (lines 1 and 2), the mother (line 3) and negative controls for the R552X mutation (lines 4–8) were tested with allele-specific PCR amplification of blood DNA followed by electrophoresis of amplified products. A strong 116 bp fragment, indicating the presence of the mutated allele, was only amplified in the positive controls, indicating that the mutated allele was apparently absent in the mother's leukocytes.

All unaffected children and their mother were found to be normal by fundoscopy. Direct DNA sequencing of exon 17 showed that all affected children carried the same, constitutive g78238C > T mutation that was absent in all unaffected sibs (or half-sibs). Similarly, we did not detect this mutation in the mother's blood DNA and its absence was twice confirmed in a second blood sample and in buccal epithelial cells. Furthermore, allele-specific PCR assays, while confirming the constitutive mutation in two affected children (used as controls for the presence of the mutation), did not produce detectable amplifications with the mother's blood DNA or with samples of five different non-carriers of the mutation (Fig. 2).

Analyses of D13S153, D13S262, D13S284 and D13S317 allowed for the unequivocal identification of five different paternal haplotypes and four different maternal haplotypes in five children (II-1, II-5, II-7, II-8 and II-10). The presumed haplotypes of the mother were therefore deduced based on the 4:1 proportion of the presumably parental to recombinant haplotypes in her children (Table I).

In these five children, the maternal haplotypes comprised: a parental haplotype 166-219-205-11 (in II-1 and II-10) and a recombinant haplotype 166-219-205-12 (in II-8), both carrying the g78238C > T mutation, and two other parental haplotypes carrying a wild-type *RB1* allele: 166-219-205-11 (in II-5) and 164-207-205-12 (in II-7). In II-1, II-8 and II-10, as in another affected child (II-9) whose haplotype could not be deduced, the 166 allele of the D13S153 intragenic polymorphic marker co-segregated with g78238C > T. However, a 166 allele of unequivocal maternal origin also coexisted with a wild-type *RB1* gene in the case of II-5 and in the somatic cells of the mother, both in blood and buccal epithelial cells. In individual II-6, three amplified signals were evident following amplification of D13S153, with two peaks corresponding to the maternal alleles (164 and 166), and a third peak to a paternal 161 allele.

DISCUSSION

The four affected children, carrying the same constitutional *RB1* mutation, were born at different intervals, and all descended from the same unaffected mother. Analysis of direct sequence data of blood- and buccal swab DNA and allele-specific PCR assays in the mother did not provide evidence of somatic mosaicism, indicating that mosaicism was apparently restricted to the maternal germline. Fundoscopy showed a normal, ectodermally derived retinal tissue in the mother and in all unaffected children.

Microsatellite analysis and genotyping confirmed the heredogram, and clearly demonstrated that the affected children descended from three different unaffected fathers, indicated by different paternal 13q-haplotypes in affected descendents of I-1, I-5 and I-6 (Table I). Altogether, these findings made it highly unlikely that a same mutation could have thrice occurred, independently, as a de novo event.

Genotypic and haplotypic analyses and exclusion of paternal transmission indicated that the g78238C > T mutation was transmitted by the unaffected mother, by co-segregation with the 166 allele in II-1 and II-10 (with the parental haplotype 166-219-205-11), in II-8 (with the recombinant haplotype 166-219-205-12), and probably in II-9, where only the maternal 219 and 205 alleles could be unquestionably identified. Conversely, in II-5, where the maternal haplotype 166-219-205-11 could be unequivocally identified, the 166 allele co-segregated with a wild-type *RB1* allele. The 166 allele was also found in three other unaffected children (II-2, II-4 and II-6), although its maternal origin could not be unequivocally determined. Finally, a third maternal haplotype (164-207-205-12) was also transmitted to another unaffected offspring (II-7). These findings provided good evidence of a maternal germline mosaicism, and that the 166 intragenic *RB1* allele was present in two different germ cell lines, one containing the g78238C > T mutation and another with the wild-type *RB1* allele (Fig. 3).

The recombination event accounting for the maternal haplotype found in II-7 could not be precisely delimited because the mother was homozygous at the D13S284 locus. However, this locus, located in 13q14.3, is strongly linked to D13S153 and D13S262 (in 13q14.2-q14.3) while D13S317 is more distally located (at 13q22-q31), a fact that makes the recombinant event more likely to have occurred between D13S284 and D13S317. In individual II-6,

TABLE I. Genotype of the Mother (I-3) and Her Nine Children Shown in Figure 1

	I-3	II-1	II-2	II-4	II-5	II-6	II-7	II-8	II-9	II-10
D13S153	164/166	166/**166**	164/166	164/166	**159**/166	**161**/164/166	**161**/164	166/**168**	164/166	166/**172**
D13S262	207/219	219/**223**	219/**221**	**215**/219	**217**/219	207/219	207/**211**	**217**/219	**217**/219	219/**227**
D13S284	205/205	205/**207**	205/**207**	205/**211**	205/**223**	205/**205**	**193**/205	205/**209**	205/**213**	205/**211**
D13S317	11/12	11/**11**	11/12	11/**13**	**8**/11	11/12	12/**13**	**9**/12	11/12	**9**/11

Alleles are named according to their fragment size (in number of base pairs) following PCR amplification. Paternal alleles are shown in bold case. Maternal and paternal haplotypes were identified in individuals II-1, II-5, II-7, II-8 and II-10. Grey columns show data of affected children.

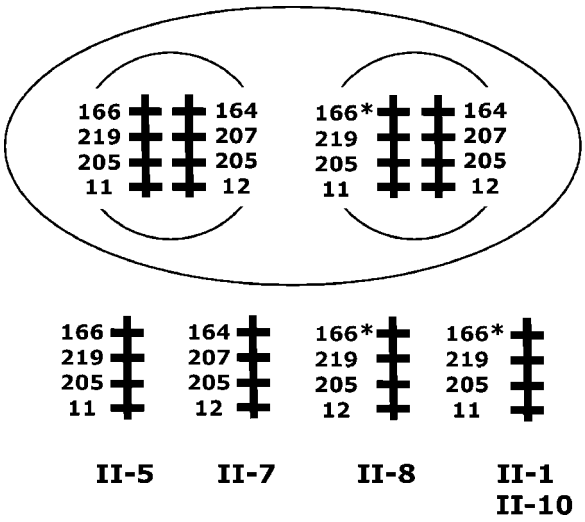

Fig. 3. Maternal germ cells and maternal haplotypes (top). In one germ cell line, the 166* allele is linked to the R552X mutation (top right). Four maternal haplotypes were transmitted (bottom): (1) a parental haplotype with the 166 allele with wild-type *RB1* to II-5, (2) a parental haplotype to II-7, (3) a recombinant haplotype with the 166* allele and the mutation to II-8, and (4) a third parental haplotype with the 166* allele with the mutation to II-1 and II-10.

the three D13S153 signals might have resulted from a triplication as previously reported [8] or from a recombination event between *RB1* intron 2 regions upstream and downstream of D13S153 (data not shown).

In mosaics, post-zygotic mutational events have been postulated to occur at early stages, that is in a mutation window encompassed between fertilization and the eighth day of embryonic development, and more likely, 2 days after conception consequently to the residual methylation status of the paternal haploid genome [3]. This is because the spermatozoal haploid genome has been found to be strongly methylated and lacking efficient DNA repair mechanisms with respect to the undermethylated oocyte genome. This explains why mutations in mosaics may occur in the paternally derived chromosome of the zygote genome. Moreover, the early occurrence of these mutational events also explains the co-existence of germline and somatic mosaicism.

Conversely, the occurrence of an exclusive, germline-restricted mosaicism in the mother of the affected children indicated that the g78238C > T mutation must have occurred at a later stage of development because gonadal development starts with migration of primordial germ cells from the yolk sac to the gonadal primordium 4 weeks post-conception [9]. This explains why this mutation was absent in two maternal cell types of earlier embryonic origin (endoderm-derived lymphocytes and mesoderm-derived buccal epithelial cells), a finding that was confirmed by allelic specific PCR assays. And it also rules out that this mutation might have occurred by defective DNA repair mechanisms associated to residual methylation.

Transmission of hereditary retinoblastoma associated to maternal germline mosaicism has been reported, although in few families [4]. In one of them, an affected mother with bilateral retinoblastoma passed the same haplotype to three children, one of which was bilaterally affected and heterozygous carrier of a nonsense mutation. In another family, an unaffected mother passed a mutation to a bilaterally affected child. In both families, somatic mosaicism was also evident because the mutations were detected in maternal blood DNA, although below the 50% level expected for heterozygotes. Conversely, other cases of documented mosaicism were found in affected children with mutations apparently absent in blood DNA. Although these findings were not confirmed by allele-specific PCR assays, these affected children might be eventual transmitters of these mutations. These cases would, however, differ from the family herein described in which a mutation was transmitted by an unaffected mosaic parent.

With respect to genetic counselling, it is important to discriminate between de novo, pre-zygotic or post-zygotic mutations from those present in a precursor germ cell and transmitted by germline mosaics. The former mutations might be sporadic while the latter might be responsible for familial retinoblastoma with a pseudo-low-penetrant hereditary pattern of transmission [10]. The demonstration of somatic mosaicism in an unaffected parent might provide a useful indicator of germline mosaicism. This, however, might not occur either because mosaicism might be restricted to the gonad or because the proportion of somatic cells carrying the mutation might be very small. This latter possibility would actually account for cryptic mosaicisms that could only be demonstrated by more refined procedures, like allele-specific PCR assays and, in male mosaics, by analysis of sperm DNA. It should also be advisable to haplotype the affected offspring and their unaffected parents to identify the parental origin of the mutated allele by pedigree analysis. However, this approach might not be satisfactory for demonstrating germline mosaicism in small sized families which might result from family planning following the occurrence of one affected child. The unfortunate situation of this family, with four affected children, showed the importance of diagnosing germline mosaicism as the causal event responsible for hereditary retinoblastoma, especially in the case of an unaffected mother without evidence of somatic mosaicism.

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