Letters to the Editor

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Evidence of Somatic and Germinal Mosaicism in Pseudo–Low-Penetrant Hereditary Retinoblastoma, by Constitutional and Single-Sperm Mutation Analysis

To the Editor:

Retinoblastoma is a pediatric cancer of the retina, initiated by two consecutive inactivating mutations at the retinoblastoma locus (RB1; Friend et al. 1986; Fung et al. 1987; Lee et al. 1987). Tumorigenesis may occur by two distinct pathways: in nonhereditary retinoblastoma (60% of patients), unifocal clonal expansion occurs after two somatic RB1 mutations in a single retinal precursor cell; however, in hereditary retinoblastoma (40% of patients), the first RB1 mutation is inherited classically and is present in both retinas, where uni- or multifocal tumorigenesis can be initiated in any cell by the somatic mutation of the remaining allele.

Typically, germ-line mutation may be passed dominantly by an affected genitor, or it can be transmitted as a prezygotic, mostly paternal neomutation from a healthy parent. However, postzygotic mutagenesis has long been suspected to contribute to the pool of transmittable mutations of RB1. Two main predictions characterize retinoblastoma patients with mutational mosaicism: (1) somatic mosaicism may significantly reduce tumor susceptibility, and (2) gonadal mosaicism may cause linkage-based analysis of inheritance to be biased toward apparent low penetrance, as a result of a non-Mendelian output of mutant versus wild-type gametes.

Several cases of retinoblastoma with mosaicism are mentioned in the literature (Greger et al. 1990; Huang et al. 1992; Shimizu et al. 1994; Thonney et al. 1996; Lohmann et al. 1997; Sippel et al. 1998). The incidence of mosaicism as well as its phenotypic influence on hereditary retinoblastoma remain to be determined, to improve genetic counseling and to shed light on the mechanisms underlying expression and penetrance of retinoblastoma.

To detect the presence of mosaicism in hereditary retinoblastoma, we selected pedigrees found through either healthy carriers or affected individuals in whom linkage analysis concomitantly documented an apparent low penetrance, from a series of 210 consecutive index patients referred for genetic counseling to the Retinoblastoma Clinics at the Jules Gonin Hospital during the period 1986–96. Among these patients, 147 (70%) had bilateral disease, and 34 (16.2%) had a familial history of retinoblastoma. All patients with familial retinoblastoma were investigated genetically by linkage analysis using intragenic DNA-sequence polymorphisms, and all were informative (Munier et al. 1992, 1996). Apparent low penetrance was present in eight pedigrees. After the exclusion of two families with fortuitous familial aggregation of independent retinoblastoma cases (Munier et al. 1993), the six remaining pedigrees were documented for reduced penetrance, as defined by the presence of healthy individuals >3 years of age in linkage phase with affected family members. A systematic search for the RB1 mutation in these six families was then performed in order to describe the molecular basis of the presumed low penetrance.

Among the six families (A–F) with apparent low penetrance, linkage analysis detected 21 unaffected carriers, of whom 6 were obligate retinoblastoma transmitters; 2 of the 6 obligate transmitters were founders (in families A and B; data not shown). The other four pedigrees were founded either by unilaterally (families C, D, and F) or bilaterally (family E) affected males, and attenuated expressivity or retinoma was present in three of these males: two in family E (the affected father had one eye enucleated for unilateral retinoblastoma and a unifocal flat chorioretinal scar, reminiscent of type IV regression, in his untreated eye; the affected son had bilateral multifocal retinomas) and one in family F (the affected grandfather had a unifocal staphylomic macular scar).

Two abnormal conformers were identified when SSCP analysis of exons 8 and 23 was performed for the two index patients of families D and E, respectively (fig. 1). Sequence analysis of these DNA fragments revealed C→T transitions at CpG dinucleotides of two arginine codons, at positions 251 and 787, in families D and E, respectively. These changes also abolished two *Taq*I restriction sites. When segregation of the mutations was analyzed in both families, the *Taq*I restriction digest showed a faint undigested band in affected fathers, in addition to the expected digested products, suggesting the presence of somatic mosaicism. Confirmation of mosaicism was

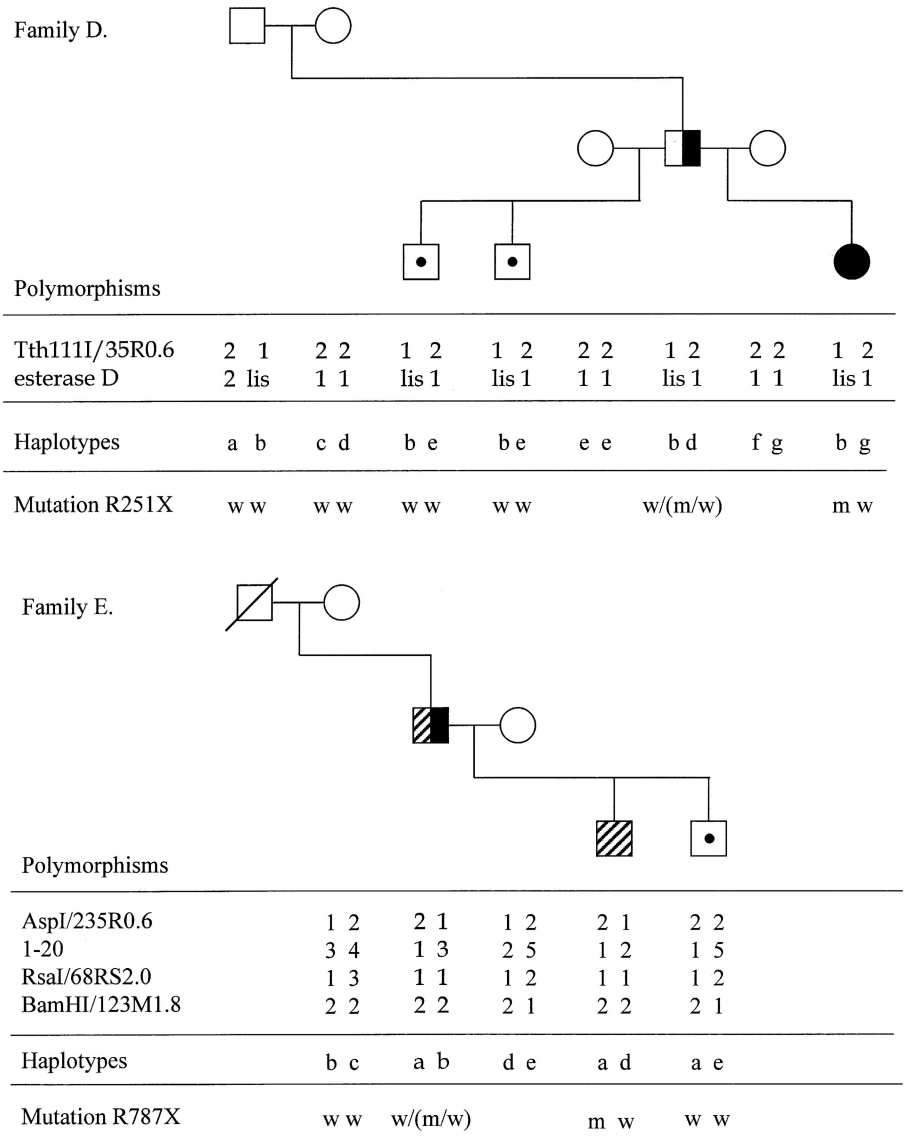

Figure 1 Pedigrees of two low-penetrant retinoblastoma families (D and E), and genotypes after linkage and/or mutation analysis at the RB1 locus. mw = heterozygote for the RB1 mutation, ww = homozygote for the RB1 wild type, w/(m/w) = mosaic, and lis = esterase D lisbon isoenzyme. The blackened circle represents a bilaterally affected female patient; half-blackened squares represent unilaterally affected males; hatched and half-hatched squares represent patients with bilateral and unilateral retinomas, respectively; and squares containing a black dot represent unaffected male carriers of the apparently disease-linked haplotype.

obtained following analysis of the *Taq*I site in cloned PCR amplicons of exons 8 and 23. The mutant digestion pattern was recovered in only 8 (10.7%) of 75 inserts from family D and in 4 (12.1%) of 33 inserts from family E. In contrast with the results of the linkage analysis, nonpenetrance failed to be validated by the presence of mutations in the three unaffected sibs (individuals III-1 and III-2 in family D and individual III-2 in family E) of the probands, strongly suggesting germinal mosaicism in the founders.

Direct quantitative analysis of gonadal mosaicism was performed on semen from only the family E founder (fig. 2), by means of a *Taq*I restriction assay in amplicons of exon 23. Single sperms were isolated by fluorescence-activated cell sorting into 96-well microtiter plates (Cui et al. 1989; Li et al. 1991). We performed amplification reactions in 576 wells and obtained an amplification signal for 415 wells. No amplification was obtained for the 40 negative controls (wells without cells), which is consistent with absence of contamination. All 18 positive controls (10 spermatozoa/well) gave a signal on an agarose gel; two alleles were observed in 3 wells, indi-

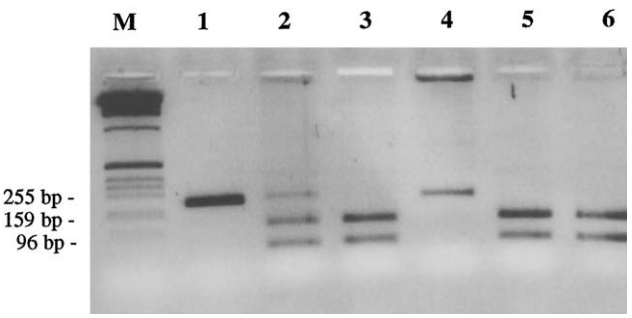

Figure 2 Determination of genotypes in single sperm from patient II-1 from family E, as detected by ethidium-bromide staining of *Taq*I-digested PCR products. Lane 1, Undigested PCR product. Lane 2, Digested PCR product from 10 spermatozoa (presence of the two alleles). Lanes 3–6, PCR product from single sperm with a mutant allele (lane 4) or a normal allele (lanes 3, 5, and 6). Lane M, Molecular-weight marker (1-kb ladder; Gibco BRL).

cating the presence of more than one spermatocyte. The remaining 394 wells had only one allele. The mutated and wild-type alleles were easily distinguished, after *Taq*I digestion, as one 255-bp amplicon or as products of 159 + 96 bp, respectively (fig. 2). Of 394 unicellular digested products, 365 (92.64%) revealed the presence of a normal allele, and 29 (7.36%) revealed the presence of a mutant allele.

Intragenic linkage analysis of 34 familial retinoblastoma cases revealed a low-penetrance pattern of inheritance in eight families, of which four followed pseudo–low-penetrant mechanisms, including independent occurrence of RB1 mutations in two different sets of cousins (Munier et al. 1993) and, as shown in this study, germ-line mosaicism in two affected founders. For the four remaining families, the molecular basis of the apparent low penetrance could not be determined and will await identification of the disease-causing mutation(s). Given the reported sensitivity (26%–83%) of the various RB1 mutation-scanning methods for hereditary retinoblastoma (Blanquet et al. 1995; Liu et al. 1995; Lohmann et al. 1996), the observation of mutations in 33% of the cases was not surprising. One possible explanation is that several mutations can be missed by SSCP screening or may lie outside the scanned RB1 exons (exons 2–26). Analysis of incomplete penetrance in retinoblastoma has previously led to the identification of two major types of gene alterations, resulting in either transcription reduction via a promoter mutation (Sakai et al. 1991; Cowell et al. 1996) or partial protein inactivation via missense mutations (Kratzke et al. 1994; Lohmann et al. 1994; Ahmad et al. 1997) and in-frame deletions (Lohmann et al. 1992; Dryja et al. 1993; Bremner et al. 1997; Schubert et al. 1997).

We report two affected patients with somatic and gonadal mosaicism for two RB1 nonsense mutations (R251X and R787X). The pathogenicity of these two mutations has been well established (Yandell et al. 1989; Cowell et al. 1994). For both affected patients, somatic mosaicism has been documented in peripheral lymphocytes and possibly involves the retina, as suggested by the nonpenetrance of retinoblastoma in one eye of the founder of family D. Mosaicism further extends to include the germ line, as proved by the segregation of three different chromosomes 13 in both families (fig. 1). In family D, indirect evidence of gonadal mosaicism in the father was provided by the analysis of three informative meiotic events in his progeny, after exclusion of nonpaternity, since his affected girl and two unaffected sons inherited the same paternal haplotype, including a rare esterase D polymorphism, ESD*Lis (Munier et al. 1988). In family E, direct evidence of germ-line mosaicism in the father's semen is based on the study of 394 meiotic events, from which 7.3% of the spermatozoa are mutant, which is not very different from the 12.1% observed in the peripheral leukocyte DNA.

In contrast to the ectodermal lineage of the retina, leukocytes and primordial germ cells have an extraembryonic origin and derive from the blastocyst endoderm. Since the didermic stage starts at ~8 d after conception, it is tempting to adopt this age as the upper limit for the occurrence of the postzygotic mutations R251X and R787X. On the other hand, the earliest mutational event leading to mosaicism can already have taken place in the postmeiotic gametes, as "half-chromatid mutations" (Carlson and Desnick 1979). The fact that the mutations in both mosaics occurred on the paternally derived chromosomes suggests that the well-known preferential prezygotic paternal mutagenesis (Dryja et al. 1989; Zhu et al. 1989) lasted in the zygote until the 8th d of development. Interestingly, these two mutations are C→T transitions at CpG dinucleotides, most likely occurring by spontaneous 5-methylcytosine deamination. In support of this mutational mechanism, the genome of haploid spermatozoa is known to have a higher methylation content than the undermethylated DNA of the oocyte and to be completely devoid of repair capabilities (Monk 1995). Hypermutability of the male-derived conceptus genome may be momentarily repressed following the massive demethylation that takes place at ~2 d after conception. De novo methylation of the unmethylated blastocyst genome occurs again, at ~6 d after conception, at the time of implantation (Dost and Lee 1995; Razin and Shemer 1995). The CpG of codon 251 in RB1 was recently proved to be constitutively methylated, whereas no information is yet available with regard to the methylation status of the CpG of codon 787 (Mancini et al. 1997). In summary, we tentatively can assign a mutational window spanning from shortly before fertilization to the 8th d of development. Since both ob-

served mutations in the two mosaics occurred on the paternally derived chromosomes, most likely following a cytosine methylation–mediated mechanism, the mutational events involved likely happened no later than 2 d after conception, on the methylated paternal genome, before massive demethylation took place. Furthermore, we postulate that the different methylation status between sperm and oocyte genomes may temporarily persist in the zygote and, hence, may cause a preferential paternal origin of mosaicism.

This study indicates that hereditary retinoblastoma does not originate exclusively from gametic neomutations but also may result from embryonic mutagenesis. The relative contribution of gametic and embryonic neomutations in hereditary retinoblastoma remains unknown but may be determined by systematic screening for both somatic mosaicism in patients with presumed de novo mutations and cryptic mosaicism in their parents. The time interval between the end of meiosis and the differentiation of soma from the germ line is viewed by some as a significant source of transmittable dominant neomutations, with estimates ranging from 5% to >15% (Dost and Lee 1995).

The mosaic nature of the two mutations described in this study could not be detected in any of the other 10 germ-line mutations identified by us (data not shown). A search was performed in index patients and their healthy parents. On the basis of this small series, we estimated a mosaic prevalence of 16.6% (2/12). Interestingly, Lohmann et al. (1997) estimated a similar prevalence, with 1 (16.6%) in 6 mutations that cause isolated unilateral hereditary retinoblastoma found to be mosaic in nature. Such a frequent occurrence of mosaicism was also highlighted recently by Sippel et al. (1998), whose data indicate a 10% rate of mosaicism in a population of 156 retinoblastoma patients. Finally, in a review of 140 retinoblastoma cases associated with constitutional 13q14 chromosomal rearrangements, 25 (18%) had proved mosaicism (Munier et al. 1989).

Taken together, these data suggest that mosaicism may be a frequent phenomenon, often interfering with expression and transmission of retinoblastoma. Somatic mosaicism may cause attenuated expression, such as unilaterality or nonpenetrance of retinoblastoma. Likewise, germinal mosaicism may be associated with apparent reduced penetrance of retinoblastoma in linkage-based molecular assessments of inheritance. This fact should be included in the calculation of the recurrence risk of retinoblastoma, especially for families with unilaterally affected male founders.

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