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Somatic Mosaicism in a Patient with Bilateral Retinoblastoma

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Summary

We describe two cell lines with different deletions of the retinoblastoma gene in a patient with bilateral retinoblastoma. This patient has transmitted the mutation less frequent in his lymphocytes to two affected children. We cloned, mapped, and sequenced the junction fragments of the two deletions and found that they share one breakpoint but extend into opposite directions. An insertion of 4 bp of unknown origin is present between the breakpoints in one of the deletions. The second deletion shows a more complex rearrangement, including an inversion at the 5′ end. Short regions of homology were found at the breakpoints and flanking the inversion. These results support the notion that bilateral retinoblastoma may not only be due to a germ-line mutation but also to a postzygotic mutation leading to somatic mosaicism.

Introduction

The retinoblastoma gene (RB-1) is the prototype of a recessive oncogene. Tumor development is initiated by the loss of function of both alleles of the gene (Knudson 1971). In about 30% of patients the predisposition to retinoblastoma is present as an autosomal dominant trait, either by transmission from an affected parent or by a new germinal mutation (for review, see Vogel 1979). In these patients, all cells carry one mutation and about 90% of them develop bilateral or multifocal unilateral retinoblastoma.

RB-1, which is located on the long arm of chromosome 13, band 14, has recently been cloned (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987) and shown to be the target of mutations in retinoblastoma and other tumors (Dryja et al. 1986; Friend et al. 1987; Bookstein et al. 1988; Goddard et al. 1988; Harbour et al. 1988; Lee et al. 1988; Horowitz et al. 1989). Point mutations and very small deletions have been investigated by the RNase protection method (Dunn et al. 1988, 1989) and by direct sequencing of enzymatically amplified axons (Yandell et al. 1989). About 20% of mutations are deletions detectable by Southern blot hybridization (Horsthemke et al. 1987*b*; Goddard et al. 1988; Canning et al. 1989). We have cloned and sequenced the junction fragments of two intragenic deletions observed in a family with bilateral retinoblastoma.

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Methods

Patients

The family HÖ-0027 with hereditary retinoblastoma was ascertained through the Retinoblastoma Clinic of the Department of Ophthalmology, University of Essen. The diagnosis of retinoblastoma had been established by current ophthalmological criteria.

DNA Analysis

Genomic DNA was purified from whole blood according to standard methods (Kunkel et al. 1977). Aliquots of DNA (2 μg each) were digested with the restriction enzymes (Boehringer Mannheim) indicated below. The fragments were separated by gel electrophoresis and transferred to Hybond N membranes (Amersham). Blots were hybridized according to a method described elsewhere (Horsthemke et al. 1987*b*). The polymorphic probes p68RS2.0 (Wiggs et al. 1988) and p2R0.9 (Goddard et al. 1990) were gifts from Dr. T. P. Dryja (Boston). Probe R0.6 is an *Hpa*I-*Eco*RI fragment of RB-1 cDNA subclone p2R0.9. Probe H3-8 is a genomic 1.5-kb *Hin*dIII fragment containing exon 4

of RB-1 (Lalande et al. 1984) and was provided by the late Dr. S. A. Latt (Boston).

Library Construction

We constructed phage libraries from lymphocyte DNA of the father and his affected son. DNA (50 μg each) was digested to completion with *Bcl*I in a final volume of 200 μl. Fragments of 4–7-kb size were isolated on a 10%–30% sucrose gradient according to a method described by Maniatis et al. (1982, pp. 270–294). The ends of these fragments were partially filled in with dATP and dGTP by using the Klenow fragment of the *Escherichia coli* DNA polymerase. Lambda L47.1 was digested with *Xho*I, and the ends were partially filled in with dTTP and dCTP. Aliquots of these preparations (100-ng fragments and 900-ng vector) were ligated overnight with T4 ligase. Ligations were packaged in vitro (Amersham kit) to yield a total of 2.5×10^6 (father's library) and 9×10^5 (son's library) plaque-forming units on host WL66. About 200,000 plaques from each library were screened with the probe R0.6. Positive clones were plaque-purified. The insert DNA plus adjacent vector sequences were cut out with *Bam*HI and *Sal*I and subcloned into puc 19.

Mapping and Sequencing

A restriction map (McGee et al. 1989) and a set of overlapping genomic phage clones (Wiggs et al. 1988) of RB-1 was provided by Dr. T. P. Dryja (Boston). From these phages, we subcloned the sequences corresponding to the 5′ and 3′ ends of the breakpoints. We constructed restriction maps of the cloned junction fragments. Correct alignment to the normal gene was confirmed by hybridizing *Hin*dIII fragments to a panel of *Hin*dIII-digested DNA of the normal gene. The junction fragments and corresponding normal fragments were sequenced using the dideoxy chain-termination method and sequenase (United States Biochemical Corporation). At least 60 bp surrounding the breakpoint were sequenced in both directions.

Polymerase Chain Reaction (PCR)

PCR was carried out in a total volume of 100 μl with 2 μg genomic DNA by using a Perkin Elmer-Cetus kit and the conditions recommended by the manufacturer. After 35 cycles of amplification (DNA thermal cycler; Perkin Elmer-Cetus), the fragments were separated by electrophoresis through a 1.4% agarose gel. Primers for the PCR and for sequencing were synthesized on an Applied Biosystems DNA synthesizer (PCR mate 391). Primer sequences are as follows: oVG1, ATATAATTGATATTTCAAAA; oVG2, AAAGTAGAGAAAAAACTATC (for map location of primers, see fig. 4).

Results

Using Southern blot analysis we screened patients with bilateral retinoblastoma for deletions and gross structural rearrangements of RB-1. In one family, HÖ-0027, we noticed an aberrant *Bcl*I fragment of 5.2 kb in the affected members. Figure 1 shows the pedigree of this family and a marker analysis with two intragenic RFLPs. The patient described in the present study (individual I-1) is bilaterally affected. His parents (not shown) are free of retinoblastoma. He has two unaffected children and two children with bilateral retinoblastoma. The RFLP analysis shows that affected and unaffected children inherited different paternal haplotypes. We did not observe additional *Bcl*I bands either in the unaffected children or in the father's parents.

The additional *Bcl*I band in this family is detected with the cDNA subclone R0.6 (fig. 2*a*) and represents the junction fragment of a small intragenic deletion. To our surprise, the abnormal fragment of the father—but not those of the two affected children—hybridized to the genomic subclone H3-8 (fig. 2*b*). Further analysis with the restriction enzyme *Kpn*I and with the probe R0.6 revealed abnormal bands of different size in the DNA of the father and his affected children (fig. 2*c*).

To define the genetic defect at the molecular level, we cloned the abnormal 5.2-kb *Bcl*I fragments of both the father and the affected son. We prepared partial genomic libraries in lambda L47.1 enriched for the abnormal *Bcl*I fragments. These libraries were screened with R0.6. We isolated 11 identical recombinants (represented by p41A) from the library constructed from the son's DNA. From the library constructed from the father's DNA we obtained two classes of positive clones containing an 5.2-kb insert: three clones, represented by p62, hybridize to H3-8 and R0.6, as expected from

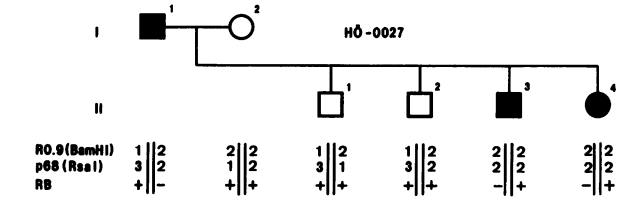

Figure 1 Marker analysis of family HÖ-0027. The family is informative for two RFLPs at the 5′ and 3′ ends of RB-1 (R0.9 and p68, respectively). RB + = wild-type allele; RB − = mutant allele.

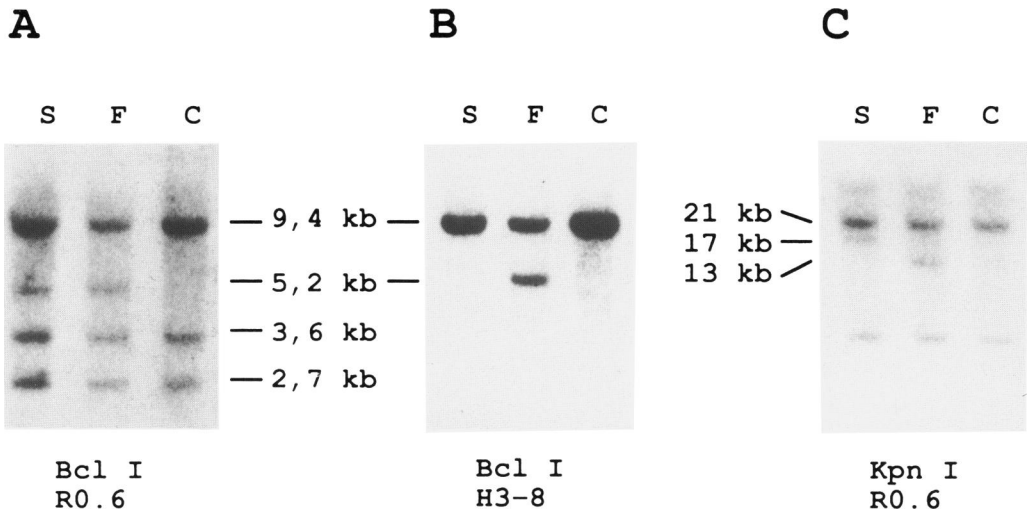

Figure 2 Southern blot analysis of DNA from the father (F), the affected son (S), and a normal control (C). The enzymes and probes are given beneath the blots. The cDNA subclone R0.6 identifies an additional 5.2-kb *Bcl*I fragment in both patients (*A*), but only the father's additional *Bcl*I fragment hybridizes to H3-8 (*B*). R0.6 identifies abnormal *Kpn*I bands of different sizes (13 kb and 17 kb, respectively) in the father and the affected son (C). The affected daughter has the same abnormal bands as her affected brother (data not shown).

the Southern blot data; clone p618 hybridizes only to R0.6. DNA from the recombinant clones was digested with the restriction enzyme *Hin*dIII to construct the restriction maps shown in figure 3. The restriction maps of p618 and of p41A are identical.

Alignment of the junction fragments and of the normal gene revealed two different deletions. Both deletions share one breakpoint. From this point they extend to opposite directions. The deletion in p62 spans about 7.5 kb and includes exons 5 and 6. One *Bcl*I site is deleted. The size of the resulting *Bcl*I junction fragment is 5.2 kb. In p41A, approximately 4 kb including exons 3 and 4, as well as the sequence of H3-8, are missing. In both cases, the loss of these exons disrupts the normal reading frame and results in several premature stop codons.

We sequenced the DNA surrounding the deletion breakpoints in both clones and the corresponding normal fragments (fig. 4). In p41A 4bp of unknown origin are inserted between the breakpoints. We did not obtain any evidence for the involvement of Alu-repetitive sequences or other repeats.

p62 shows a more complex rearrangement. At the 5′ breakpoint we found an inverson of 205 bp. Twelve basepairs of the normal sequence are missing 5′ of this inversion. Two different short regions of homology are

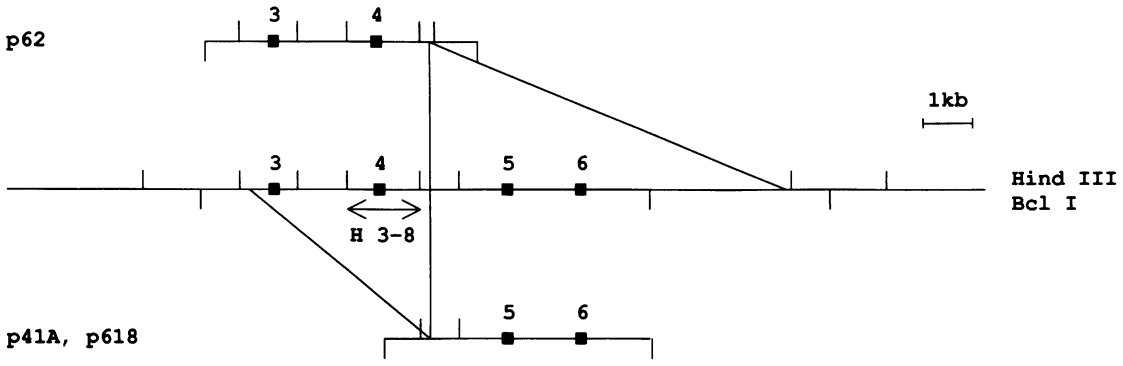

Figure 3 Restriction maps of the clones *Bcl*I deletion junction fragments (p62, p618 for father; p41A for son) and of the corresponding section of RB-1. Numbered boxes represent exons.

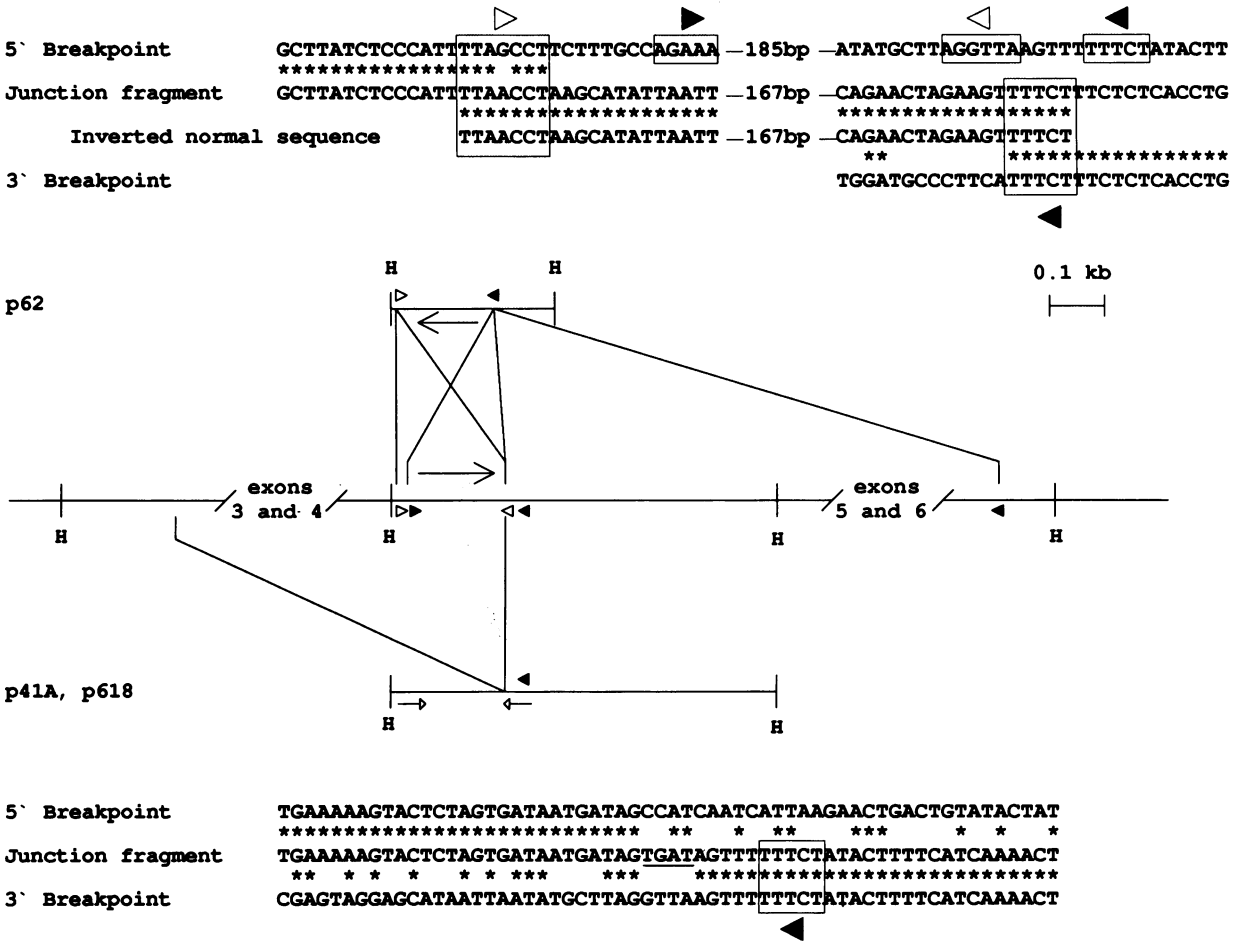

Figure 4 Partial restriction map of RB-1 and the *Hin*dIII junction fragments p62 (affected father) and p41A (affected son). H = *Hin*dIII restriction sites. The sequences of the 5′ breakpoint region and the p62 junction fragment (upper part of the figure) start at the *Hin*dIII site downstream of exon 4. The sequence of the 5′ breakpoint region spans the 12-bp deletion and the 205-bp inversion of p62. For clarity, only relevant parts of the sequences are shown. To demonstrate the 205-bp inversion, the corresponding normal sequence has been inverted and is shown in the third line. The 3′ breakpoint of the 7.5-kb deletion is downstream of exon 6. Two different kinds of inverted repeats in the sequence at the inversion breakpoints and at the 3′ breakpoint are boxed. Triangles indicate the position and orientation of these repeats in the map. Δ = TTA(G/A)CCT; ▲ = AGAAA. In p41A (lower part of the figure), an insertion of 4 bp between the breakpoints is underlined. Small arrows indicate the location of primers used in the PCR analysis (fig. 5).

present in the sequence across the inversion and the breakpoints. We found a pair of imperfect 7-bp inverted repeats (TTAPCCT; P = purine) flanking the region inverted in the mutant allele. Only one copy of the repeat is found in the mutant allele. Another pair of inverted repeats (AGAAA) is located next to this sequences. A third (inverted) copy of this 5-bp sequence lies at the 3′ breakpoint. Only one inverted copy of this repeat is present in the mutant allele.

Since we isolated two classes of clones from the library of the father, we suspected that he has two cell lines with different deletions. Southern blot analysis is obviously not sensitive enough to detect both aberrant *Kpn*I fragments. Thus, we used the PCR method to detect the junction fragment. We synthesized primers from the 5′ side and the 3′ side of the breakpoint in p41A. As shown in figure 5, we were able to amplify the expected 264-bp fragment from the DNA of father and son, whereas it was not seen in the DNA of a normal control.

Discussion

We have described a patient with bilateral retinoblastoma who has two cell lines with different intragenic deletions. The deletions share one breakpoint and

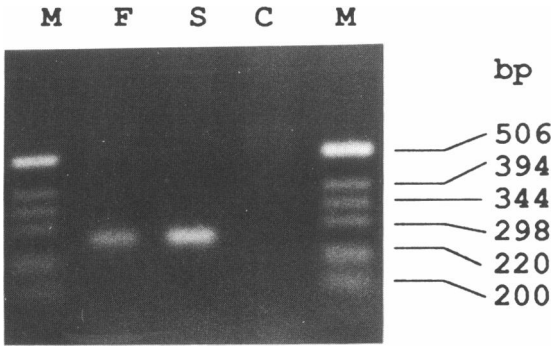

Figure 5 PCR analysis of the deletion junction corresponding to clone p41A. DNA of the father (F), the affected son (S), and a normal control (C) was amplified as described in Methods. M = marker. The expected 264-bp fragment is seen in F and S but not in C.

extend to opposite directions. Since this finding cannot easily be explained by successive mutations, we assume that both mutations originate from a single postzygotic genetic event. Unless this event occurred during the first division of the zygote, the patient should have a third cell line with two normal alleles. Southern blot analysis of lymphocyte DNA shows no striking difference between the intensity of the signals of the abnormal bands from the mosaic father and the intensity of the abnormal bands from the heterogyzous children, indicating that the majority of these cells seems to carry one of the mutant alleles. In fact, the data would suggest that all the father's cells have a mutant allele. Up to now, it has not been possible to prove the absence or presence of a normal cell line. Since the patient transmitted one mutation to two of his children, the mutation must have arisen before the germ line was partitioned off from other cell lines. Work in mice indicates that partitioning occurs at a very early stage (Soriano and Jaenisch 1986), but the situation in humans is less clear (Luckett 1978; Gardner 1983). It is interesting that the mutation that appears to be less frequent in lymphocytes was transmitted to both of the affected children. Since a sperm sample was not available, we do not known whether mosaicism is also present in the germ line.

Our findings have implications for predictive DNA diagnosis in further children of the patient. If there is a normal cell line in the father's germ line, the chromosome with the haplotype found in the two affected children will not necessarily carry a deletion in all germ cells—and a diagnosis based solely on RFLP analysis could lead to a wrong prediction. A valid diagnosis must demonstrate the presence or absence of the deletion junction fragment.

At the 5′ end of the breakpoint shared by the deletions 205 bp of the sequence are inverted. This inversion is located in an intron and possibly represents a neutral variant. We speculated that the patient might have inherited this inversion and that the deletions arose as a result of mispairing of the normal and the inverted sequence during a mitotic recombination event. In this case, the normal cell line, if present at all, should carry the inverted allele. We tested this hypothesis by using PCR (data not shown) and did not find an inversion without a deletion in either the patient's or his parents' lymphocyte DNA. This indicates that the inversion results from the mutation event.

To understand better the possible mechanisms for the formation of the deletions, we sequenced the breakpoints. Alu-repetitive elements, which are known to be involved in the formation of deletions in other human genes—e.g., the LDL receptor gene (Lehrman et al. 1985, 1986; Horsthemke et al. 1987*a*)—obviously play no role in this case. In p62 we found short repeats at the ends of the breakpoint and of the inversion. This corresponds to the results of Canning and Dryja (1989). These authors reported short regions of homology of 4–7 bp at six of eight sequenced deletion termini. They conclude from their data that the "slipped mispairing" mechanism (Efstratiadis et al. 1980; Albertini et al. 1982; Brunier et al. 1988) may predominate in the generation of mutations at this locus. Our data support this conclusion, although such a mechanism alone does not explain the mutations described here.

The occurrence of bilateral retinoblastoma in an individual without family history is generally attributed to a germ-line mutation in one of the parents. A recent report and review of the literature (Ribeiro et al. 1988), however, pointed out that bilateral retinoblastoma can also occur as a result of somatic 13q− mosaicism. In the majority of cases the mutation is too small to be detectable by cytogenetic methods, and it may be impossible to distinguish an early postzygotic mutation from a de novo germ-line mutation. Thus, the frequency of somatic mosaicism in karyotypically normal patients with isolated bilateral retinoblastoma may be higher than generally assumed. The present study emphasizes the importance of somatic mosaicism in genetic diseases (Hall 1988).

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