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Genetics

Mitotic recombination map of 13cen–13q14 derived from an investigation of loss of heterozygosity in retinoblastomas

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ABSTRACT **Loss of heterozygosity at tumor-suppressor loci is an important oncogenic mechanism first discovered in retinoblastomas. We explored this phenomenon by examining a set of matched retinoblastoma and leukocyte DNA samples from 158 patients informative for DNA polymorphisms. Loss of heterozygosity at the retinoblastoma locus (13q14) was observed in 101 cases, comprising 7 cases with a somatic deletion causing hemizygosity and 94 with homozygosity (isodisomy). Homozygosity was approximately equally frequent in tumors from male and female patients, among patients with a germ-line vs. somatic initial mutation, and among patients in whom the initial mutation occurred on the maternal vs. paternal allele. A set of 75 tumors exhibiting homozygosity was investigated with markers distributed in the interval 13cen–13q14. Forty-one tumors developed homozygosity at all informative marker loci, suggesting that homozygosity occurred through chromosomal nondisjunction. The remaining cases exhibited mitotic recombination. There was no statistically significant bias in apparent nondisjunction vs. mitotic recombination among male vs. female patients or among patients with germ-line vs. somatic initial mutations. We compared the positions of somatic recombination events in the analyzed interval with a previously reported meiotic recombination map. Although mitotic crossovers occurred throughout the assayed interval, they were more likely to occur proximally than a comparable number of meiotic crossovers. Finally, we observed four triple-crossover cases, suggesting negative interference for mitotic recombination, the opposite of what is usually observed for meiotic recombination.**

Loss of heterozygosity of the long arm of chromosome 13 as a somatic event in mammalian cells was first documented through studies of retinoblastomas (1–3). It is the most common mechanism by which the remaining wild-type allele at the retinoblastoma tumor-suppressor locus (*RB1*) is lost in a retinal cell that is heterozygous for a null mutation. A retinoblastoma can arise from the resulting daughter cell that is either homozygous or hemizygous for the mutant allele. Many other human cancers arise after loss of heterozygosity at tumor-suppressor loci on chromosome 13 or other chromosomes (4).

The rate at which allele loss occurs during mitosis has been measured in few cell types *in vitro* and only for a small number of chromosome arms. The measured rates are low; e.g., loss of heterozygosity involving chromosome 6p in lymphocytes approximates 10^{-6} (5). Different chromosomal mechanisms, such as chromosomal nondisjunction, chromosomal nondisjunction followed by duplication, gene conversion, mitotic recombination, and deletion can be responsible for the loss of heterozygosity (2), but the relative frequencies of these different mechanisms are not established. In the case of mitotic recombination, very little is known about the relative frequencies of mitotic crossovers occurring along defined intervals of a chromosome arm and how they compare with the frequencies of meiotic recombination along the same intervals (5, 6).

To investigate this phenomenon, we collected matched sets of leukocyte and tumor DNA samples from patients with retinoblastoma. By using polymorphic markers within the *RB1* gene and elsewhere on 13q, we measured the proportion of cases exhibiting homozygosity at the *RB1* locus and determined the fraction of cases with homozygosity occurring through apparent nondisjunction or mitotic recombination. We also determined whether these proportions correlated with patients being male vs. female, with initial mutations being germ line vs. somatic in origin, and with the initial mutations arising on the maternal vs. paternal allele. Finally, we mapped the chromosomal regions where somatic recombination events responsible for homozygosity had occurred.

MATERIALS AND METHODS

Ascertainment of Cases. Our survey of the files of the Retinoblastoma Diagnostic Service at the Massachusetts Eye and Ear Infirmary revealed 395 families with at least one affected member who underwent DNA analysis of the *RB1* gene. More than 90% of the families were from the United States and Canada. Of these families, there were 162 in which both leukocyte DNA and tumor DNA were available from the index case. In cases from which more than one retinoblastoma was received, only one of the tumors was included in this analysis. Clinical records were reviewed, and, in some cases, the patients or referring health care providers were contacted to obtain demographic information. Patients were categorized as having a germ-line initial mutation if they had an affected relative, if they had bilateral disease, or if a mutation of the *RB1* gene had been previously identified and found to be present in their leukocyte DNA. Unilaterally affected patients without a family history of retinoblastoma and without a mutation identified in leukocyte DNA were categorized as having an initial somatic mutation.

Evaluation of Intragenic DNA Polymorphisms. Five polymorphisms within the *RB1* gene were analyzed in the leukocyte and tumor DNA samples from each case. Three of the polymorphisms were restriction fragment length polymorphisms revealed with the restriction endonucleases *Bam*HI, *Xba*I, and *Tth*111I at positions 2,300, 99,426, and 171,933, respectively (GenBank accession no. L11910) (7). One was a minisatellite repeat polymorphism of the variable number of tandem repeats type in intron 17 commencing at position 123,912 and one was a tetranucleotide repeat polymorphism commencing at position 156,895 (7). A polymorphism was defined as "informative" in a particular case if the patient's leukocyte DNA was heterozygous. A tumor was categorized as exhibiting allele loss at an informative marker if one of the two alleles present in leukocyte DNA was absent in the corresponding tumor DNA (1–3). In many of the cases in which

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blood samples were available from one or two parents, an analysis of the same polymorphisms allowed the determination of the parental origin of the allele with the initial mutation (8).

Southern Blot and Densitometric Analysis. Leukocyte and tumor DNA samples were analyzed with genomic and cDNA probes derived from the *RB1* gene to determine which cases had deletions accounting for the loss of alleles. Matched DNA samples were digested with restriction endonucleases according to the manufacturer's instructions (Boehringer Mannheim). Digested DNA was separated by electrophoresis through 0.8% agarose gels and transferred to nitrocellulose by standard methods. Blots were probed separately with two genomic DNA fragments, p128M1.8 (a 1.8-kb fragment containing exon 1 of the *RB1* gene) (9) and p68RS2.0 (bases 123,853–126,036) (7, 10), and two cDNA fragments, p2R*Hpa*I0.7 (a cDNA fragment extending from the *Hpa*I site at codons 75–77 to the *Eco*RI site at codons 300–302) and p2R3.8 (a cDNA fragment extending from the *Eco*RI site at codons 300–302 through the polyadenylation signal sequence). Probe p128M1.8 was hybridized to genomic DNA samples digested with *Hin*dIII and separately with the combination of *Sac*I and *Sac*II to check for hypermethylation of the promoter region. Probes p2R*Hpa*I0.7 and p2R3.8 were hybridized separately to genomic DNA samples digested separately with *Hin*dIII, *Xba*I, and *Taq*I. Probes were radiolabeled with ^{32}P by using a random-primer method. Hybridization, washing, and autoradiography were performed according to standard methods. Not every case was analyzed with all probe and enzyme combinations. In a few cases without an informative polymorphism, additional intragenic DNA polymorphisms were analyzed, such as RBi2 (AFM058xd6), RBi4, and RBi17 (7).

To confirm the absence of a deletion of *RB1* in the 75 cases that were analyzed with microsatellite markers between 13cen and 13q14 (see below), *Taq*I-digested DNA was probed simultaneously with genomic *RB1* fragment pH3–8 (bases 40,781–42,292) (7, 11, 12) and a cDNA fragment named pHRDS7 from the human *RDS* gene on chromosome 6p that overlaps with the published fragment pHRDS8 (13). Densitometric analysis was used to quantitate the relative hybridization intensity obtained with pH3–8 and pHRDS7 probes. Resulting intensities were analyzed with IMAGEQUANT software (Molecular Dynamics).

Mutation Screen. In many cases, an exon-by-exon search for mutations in the *RB1* gene was performed with single-strand conformation and direct-sequencing methods (14–17). Whenever a mutation was found in a tumor DNA sample, the corresponding leukocyte DNA sample, if available, was assayed for the same mutation. The results were used in the categorization of patients as described above.

Microsatellite Analysis. Dinucleotide repeat polymorphisms analyzed were obtained from the Généthon human linkage map (18). The Généthon marker AFM058xd6 is known to be within the *RB1* gene and served to place *RB1* on this map (7). Relevant regions were amplified with PCR and the published primers for each microsatellite marker (18). PCR was performed in 20 μl of a buffer containing 1 ng DNA/μl, 0.5–1.5 mM $MgCl_2$, pH 8.4–8.6, and 0% or 10% dimethyl sulfoxide. The $MgCl_2$ concentration, pH, presence or absence of dimethyl sulfoxide, and the annealing temperature were optimized for each primer pair. In each case, the forward primer was labeled with ^{32}P by using polynucleotide kinase before PCR. Radiolabeled fragments were separated by electrophoresis through 6% polyacrylamide gels with urea as a denaturant. Gels were dried onto Whatman 3MM paper, and results were visualized by autoradiography.

Statistical Analysis. The likelihood of the nonrandom assortment of sets of cases was calculated with the Fisher exact test and the χ^2 test by using the web sites http://www.matforsk.no/ola/fisher.htm and http://home.clara.net/sisa/twoby2.htm. In all cases, probabilities did not substantially vary according to the statistical test; in this paper, the *P* values generated by the Fisher exact test are provided unless otherwise specified. Some calculations were confirmed by directly calculating the χ^2 values applying the method described by Rosner (19).

RESULTS

All but four of the 162 patients included in this study had leukocyte DNA that was hemizygous or heterozygous at one or more polymorphic sites in the *RB1* gene, allowing us to determine whether their tumors had allele loss. Of the 158 tumors from informative patients, 101 (64%) exhibited loss of heterozygosity at *RB1*. Of these, seven were hemizygous (i.e., a deletion was present in tumor but not leukocyte DNA), and 94 were homozygous. The remaining 57 tumors (36%) retained heterozygosity; of these, 12 were hemizygous for a deletion of *RB1* in both leukocyte and tumor DNA.

Tumors from female patients were approximately equally likely to exhibit homozygosity (47 of 73, 64%) as tumors from male patients (47 of 85, 55%) ($P = 0.26$). Tumors in which the initial mutation was in the germ line were also approximately equally likely to exhibit homozygosity (32 of 60 germ-line mutations, 53%) as tumors in which the initial mutation arose somatically (62 of 98 somatic mutations, 63%) ($P = 0.24$). In 95 cases, it was possible to determine the parental origin of the allele with the initial mutation. There was no apparent correlation between the parental original of the initial mutation and the occurrence of homozygosity: 33 of 36 cases (92%) with the initial mutation on the maternal allele exhibited homozygosity, whereas 46 of 59 cases (78%) with an initial mutation on the paternal allele exhibited homozygosity ($P = 0.10$).

Of the 94 cases that exhibited homozygosity for markers inside the *RB1* gene, there was sufficient leukocyte and tumor DNA from 75 for further analysis of the mechanisms leading to homozygosity. The absence of a deletion of *RB1* in these tumors was confirmed through Southern blot studies with probes pH3–8 and pHRDS7. In all of these cases, we determined the alleles at 17 microsatellite polymorphisms distributed at approximately equal genetic intervals along 13cen–q14, an interval of approximately 47.5 cM. At each marker locus, alleles found in the leukocyte DNA were compared with alleles found in the tumor DNA. Of the 75 tumors studied in this way, 41 (55%) had developed homozygosity at all informative marker loci including the most centromeric informative markers (Fig. 1), indicating that the homozygosity occurred because of either chromosomal nondisjunction or a crossover event very close to the centromere. For simplicity, these cases are henceforth referred to as "nondisjunction" cases. The remaining 34 tumors retained heterozygosity for one or more proximal markers, indicating that homozygosity at the *RB1* locus had occurred through somatic recombination distal to the most centromeric marker. These cases will be referred to as "recombination" cases. Six microsatellite markers distal to the *RB1* locus (AFM190ya3, AFM259zg1, AFM269yb1, AFM284za9, AFM037xa1, and AFM240xg5) were analyzed in all 75 cases. In every case, the region of homozygosity extended past *RB1* to the most distal informative marker (up to 10.8 cM beyond *RB1*).

There was a higher proportion of recombination cases in females vs. males [21/37 (57%) vs. 13/38 (34%)], but the difference was of borderline statistical significance ($P = 0.06$ by Fisher exact test and $P = 0.05$ by the χ^2 test). We did not detect a statistically significant association between the proportions of nondisjunction vs. recombination cases among patients with a somatic vs. germ-line initial mutation ($P = 0.60$) or among patients with a paternally derived vs. maternally derived initial mutation ($P = 0.22$).

Somatic Recombination Analysis. The locations of the crossovers in the 34 recombination cases were more precisely mapped through the analysis of 35 additional microsatellite

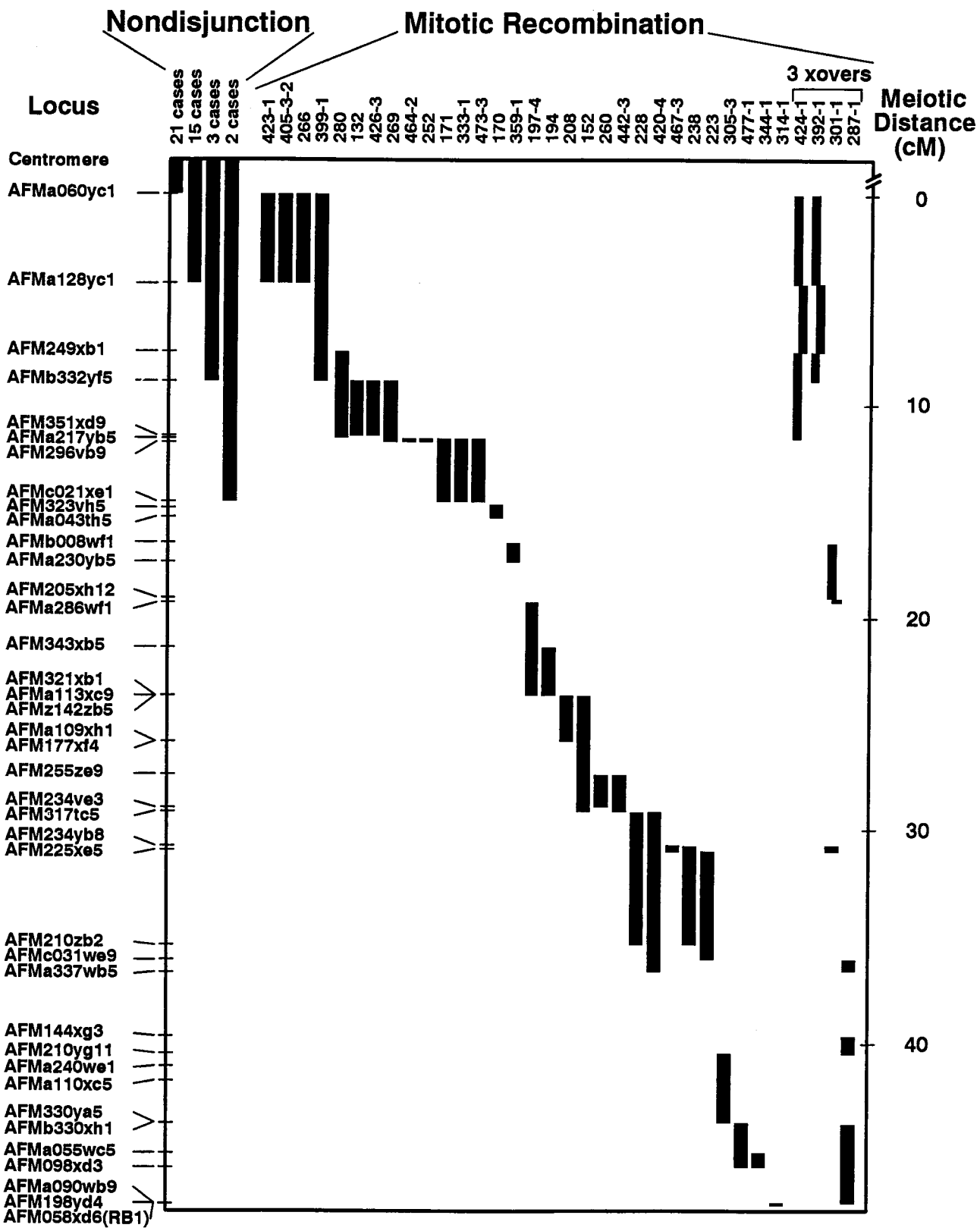

FIG. 1. Summary of the data derived from the analysis, by using markers in the interval 13cen–q14, of 75 retinoblastomas that exhibited isodisomy at *RB1*. In the recombination cases, the ends of each black box denote the locations of the informative markers that serve to delimit the region within which a mitotic crossover occurred. In cases with a single crossover, informative markers proximal to the box maintained heterozygosity in the corresponding tumor, whereas informative markers distal to the box were homozygous in tumors. In the triple-crossover cases, the three boxes indicate the three intervals within which crossovers occurred. In the 41 nondisjunction cases, the proximal ends of the boxes extend to the centromere because the most proximal markers were uninformative. The nondisjunction cases are split into four separate groups of between two and 21 cases each (labeled at the top of the figure) defined by the location of the first informative marker at the distal ends of the boxes. For all cases, markers within the boxed intervals were tested and were uninformative. The laboratory identification numbers for the recombination cases are at the top. At the left are the marker names spaced according to the intermarker meiotic recombination distance (18).

polymorphisms. Each case was analyzed with only the markers necessary to refine the location of the crossover. Thirty tumors each showed evidence for a single crossover, whereas four tumors showed evidence for three crossovers each. No novel alleles at any polymorphic site were detected in any tumor DNA that were not present in the corresponding leukocyte DNA. Fig. 1 shows the intervals within which the crossover(s) occurred in each case.

We plotted the cumulative number of observed mitotic crossovers as a function of the meiotic recombination distance (Fig. 2). If the relative propensities of defined chromosomal regions for mitotic recombination match those for meiotic recombination, one would expect the cumulative numbers of observed mitotic recombinations to approximate the diagonal line in Fig. 2. Instead, over most of the proximal region of 13q, the observed cumulative number is greater than that expected if mitotic and meiotic crossovers were distributed comparably. This indicates that there is a relatively high frequency of mitotic crossovers in the proximal region of 13q. This abundance of proximal crossovers would be even more striking if some of the cases categorized as nondisjunction were actually proximal crossovers. For example, the first informative marker in two of the nondisjunction cases (Fig. 1) was AFMc021xe1, which is at least 14.5 cM distal to the centromere; one or both of these two cases might actually have proximal crossovers that were not detected.

There appears to be a cluster of mitotic crossovers in the region around marker AFMa217yb5 (Fig. 2). In particular,

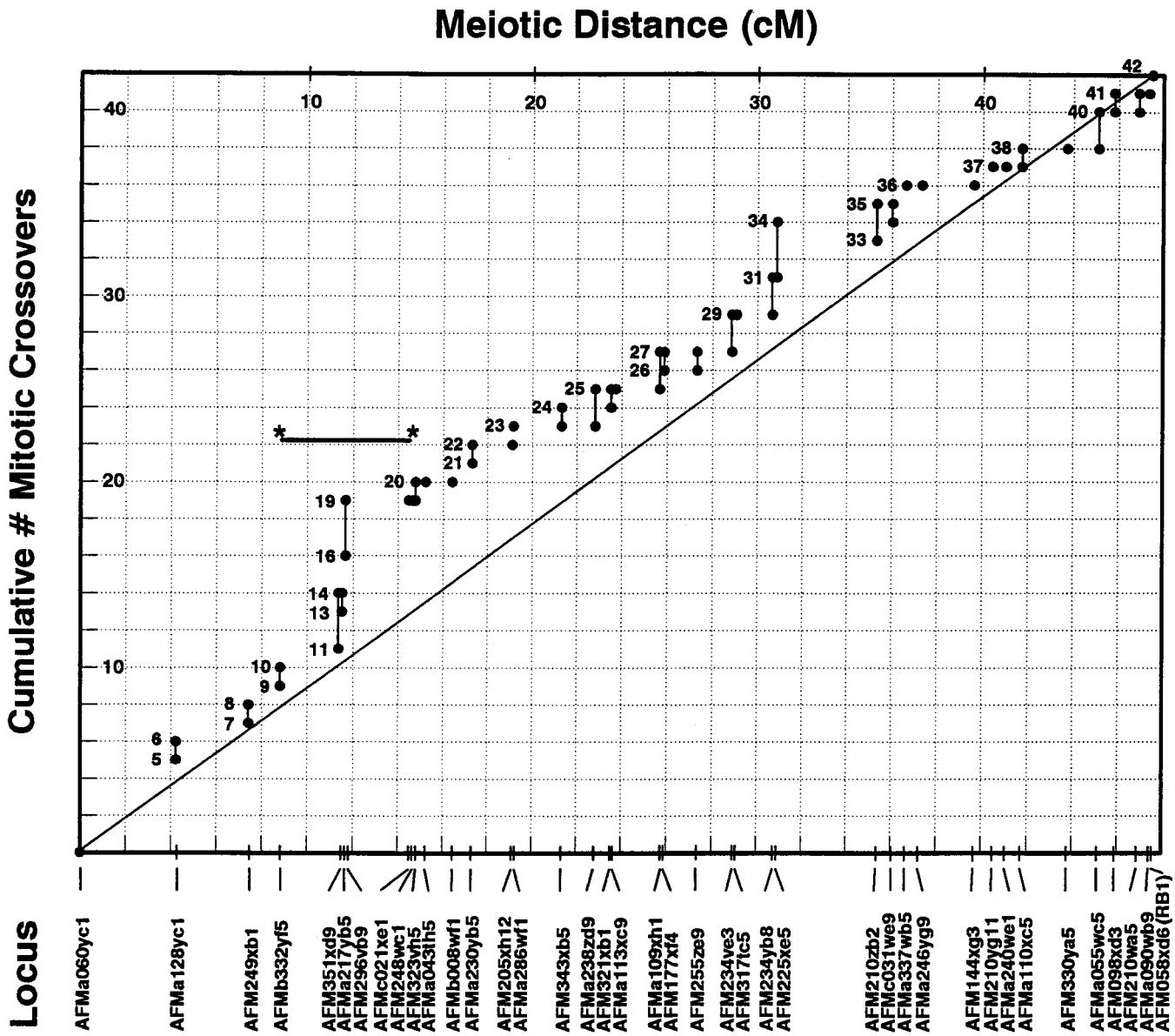

FIG. 2. Cumulative number of mitotic crossovers detected in the recombination cases in the interval AFMa060yc1 to *RB1* as a function of meiotic recombination distance. The data are derived from 42 recombination events detected in the 34 recombination cases (30 with a single detected crossover event and four with three crossovers each). Marker names are spaced along the *x* axis according to published meiotic recombination distances. The solid diagonal line indicates the expected results if the relative propensities of defined chromosomal regions for mitotic recombination match those for meiotic recombination. Data for each marker are displayed as a range because of uncertainty arising from uninformative markers. The lower boundary of each range is the minimum number of crossovers that definitely occurred between the most proximal marker (AFMz060yc1) and that locus, and the upper boundary is the maximum cumulative number of crossovers that might have occurred. The region exhibiting a cluster of crossovers discussed in *Results* (between markers AFMb332yf5 and AFMc021xe1) is denoted with a horizontal line with asterisks. Two pairs of markers (AFM205xh12 and AFMa286wf1, and AFM234yb8 and AFM225xe5) that previously were assigned to the same genetic location were ordered in this analysis by observing an informative mitotic recombination event.

there are between two and eight mitotic crossovers in the interval between markers AFM351xd9 and AFM296vb9, a meiotic distance reported to be only 0.2 cM (18). This region accounts for only 0.4% (0.2/47.5 cM) of the sex-averaged meiotic recombination distance between the most proximal marker and *RB1* and, therefore, should account for only 0.4% of the 42 mitotic crossovers observed, or about 0.18 crossovers. The observation of between two and eight crossovers in this region is statistically significant ($P = 0.027$ if there were two mitotic crossovers in this region; $P < 10^{-10}$ if there were eight, applying the one-sample binomial test, exact method) (19). However, the significance of this apparent cluster of crossovers is tempered because the measured meiotic recombination distance between these markers is very approximate. The meiotic recombination map is derived from the analysis of only a few hundred meioses (18), and an interval of 0.2 cM would, therefore, be based on detecting only one or two recombinants. The apparent cluster of mitotic crossovers might simply be an indication of a larger meiotic (and physical) distance between markers AFM351xd9 and AFM296vb9. An analysis of a larger, more precisely measured interval that encompasses this region might better test the significance of this cluster of crossovers. The 5.7-cM interval between markers AFMb332yf5 and AFMc021xe1 has either nine or 10 mitotic crossovers. This is greater than the five recombinants expected based on the meiotic recombination frequency, but it is of borderline or no statistical significance ($P = 0.05$ if there were 10 mitotic crossovers in this region; $P = 0.12$ if there were nine, applying the one-sample binomial test, exact method) (19).

DISCUSSION

Early evidence of the existence and importance of tumor-suppressor genes in human cancer was obtained through studies of retinoblastomas. These retinal cancers arise in cells that have sequentially lost the function of both wild-type copies of the tumor-suppressor locus called *RB1* in chromosome band 13q14. The first mutant *RB1* allele (the initial mutation) is either inherited from a carrier parent or created through somatic mutation. The loss of the second wild-type allele in an embryonic retinal cell or its precursors creates a progenitor without a functional *RB1* allele, and only such cells or their descendants give rise to retinoblastomas. Some tumors arise from cells that have lost the second allele through mutation and others through chromosomal mechanisms that produce homozygosity at loci through most or all of chromosome 13. Although these mechanisms have been documented in retinoblastomas since 1983 (1–3), the proportion of tumors showing homozygosity for the initial mutation has been measured in only a few studies and these involved far fewer cases than the present study (3, 6, 20). There has been even less attention paid to the mechanisms generating homozygosity and, in particular, to the mitotic recombination events that produce homozygosity in many cases.

Data from this study of 158 informative cases provide a greatly improved measure of the relative proportions of tumors arising from the different mechanisms for losing the second wild-type *RB1* allele: approximately 4% lose the second allele through deletion, 59% through chromosomal mechanisms leading to homozygosity (isodisomy) for the initial mutation, and 36% through mechanisms that retain heterozygosity at the *RB1* locus such as a second mutation other than a deletion. These proportions are similar to those found in a previous study involving 13 informative tumors (6). Furthermore, our data allowed us to explore whether there is any relationship between the various mechanisms and certain clinical parameters. No evident correlation exists between the occurrence of isodisomy and the sex of the patient, whether the initial mutation is in the germ line or arose somatically or whether the allele with the initial mutation is derived from the patient's mother or father. The overall frequency of tumors with loss of heterozygosity (i.e., cases with isodisomy or hemizygosity) observed in this series is comparable to that found in previous studies of smaller numbers of cases (2, 3, 6), including a study of 43 informative tumors from Japan (20). However, we failed to confirm the higher proportion of loss of heterozygosity among patients with an initial somatic mutation compared with an initial germ-line mutation found in the Japanese study (90% vs. 54%) (20). The reason for the discrepancy is obscure, but it should be noted that the Japanese study included only 20 informative tumors with an initial somatic mutation.

A more intensive analysis of 75 tumors with isodisomy at the *RB1* locus provided a mechanistic categorization of such cases. Slightly more than half of the isodisomic tumors were isodisomic for all informative markers tested on chromosome 13q. Such cases are usually assumed to be the product of nondisjunction with reduplication (i.e., isodisomy for the entire chromosome 13) (2, 6, 21). However, although the physical distance between the centromere and the most centromeric marker we tested is presumably very small, there is nevertheless the possibility that a hot spot for mitotic recombination could exist within this interval. If this were the case, the short arm of chromosome 13 and a small portion of the centromeric long arm might retain heterozygosity. Unfortunately, this possibility cannot currently be tested because there are no available polymorphic DNA markers from 13p or the most proximal region of 13q. In this regard, it is noteworthy that a study of isodisomy for 6p spontaneously generated in lymphocytes *in vitro* found no evidence for nondisjunction, because markers from 6q invariably retained heterozygosity (5).

Mitotic recombination is the likely mechanism for isodisomy in almost half of the tumors, because proximal markers retained heterozygosity. The 34 tumors in this category provided an opportunity to determine how the distribution of mitotic crossovers relates to the meiotic recombination distance in proximal 13q. We explored this phenomenon by comparing the positions of somatic recombination with the previously reported meiotic recombination map (Fig. 1). Our interpretation of the data produced the following observations. First, recombination in mitosis, as in meiosis, does not occur at only a few positions but, instead, appears to occur anywhere along the assayed interval. Second, through most of the assayed interval, the cumulative number of mitotic crossovers is greater than that expected if they were distributed similarly to an equal number of meiotic crossovers (i.e., the cumulative number is usually above the diagonal in Fig. 2). This is explained either through a relative propensity for mitotic crossovers or a relative deficit of meiotic crossovers in the proximal part of the assayed region. The frequency of meiotic crossovers of human chromosomes varies widely among chromosome regions of equal physical size, and it generally decreases with proximity to the centromere. Our findings suggest that the likelihood for a mitotic crossover between two loci may correlate more closely with the physical distance between them.

The third observation relates to the detection of four cases with triple crossovers. Detecting triple-crossover events in the assayed interval would not be highly unlikely among a set of 34 meioses because crossovers are so frequent during meiosis and they usually involve every chromosome. However, the rate of recombination per mitotic cell division is very low, probably on the order of 10^{-6} for any particular chromosome arm (5). The corresponding likelihood of double- or triple-crossover events involving a particular chromosome arm during mitosis would be vanishingly small (10^{-12} and 10^{-18}, respectively). Our detection of four examples of triple crossovers out of a set of 34 mitotic recombination cases strongly suggests negative interference; i.e., a mitotic recombination event greatly increases the likelihood of a similar event in its vicinity. This is the opposite of the positive interference usually found in

meiotic recombination. It may reflect a fundamental difference in the mechanisms leading to recombination in the two settings. For example, mitotic recombination might occur during the repair of aberrant chromosome breaks that might arise from insults that are likely to produce multiple breaks simultaneously. Negative interference could also explain the one retinoblastoma with a multiple-crossover event observed among six cases analyzed by Zhu *et al.* (6). The lack of detected double crossovers in our study is presumably methodological: a double crossover proximal to *RB1* would not produce isodisomy at *RB1*, and, thus, would not produce a tumor.

A final observation regards an apparent excess of crossovers occurring between markers AFMb332yf5 and AFMc021xe1, although of only borderline statistical significance. Whether the apparent cluster of crossovers reflects a mitotic recombination hot spot will require either the analysis of additional tumors or a better measure of the meiotic recombination distance between the markers in this region.

Through a study of a series of retinoblastomas, we have obtained a refined measure of the frequencies of various mechanisms for allele loss at a tumor-suppressor locus. The mitotic recombination map afforded by the data yields a distinctive, although rudimentary, picture of infrequent but clinically important chromosomal interactions in somatic cells. We have related the mitotic recombination events to the known meiotic recombination map of the region 13cen–q14. When the genomic sequence of the region is established, a reevaluation of the data should permit a comparison of mitotic recombination events and physical distance. Because human tumor-suppressor loci on other chromosomes exist, similar studies of mitotic recombination involving other chromosome arms are possible. Mitotic recombination maps could also be constructed through *in vitro* studies of cell lines with selectable markers on specific chromosome arms. Such studies would serve to determine whether the patterns of mitotic recombination observed on proximal 13q are pan-chromosomal and whether they vary according to the somatic cell type in which they occur.

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