Am. J. Hum. Genet. 45:252–260, 1989

Linkage Analysis of Families with Hereditary Retinoblastoma: Nonpenetrance of Mutation, Revealed by Combined Use of Markers Within and Flanking the RB1 Gene

Hans Scheffer,* Gerard J. te Meerman,* Yvonne C. M. Kruize,* Anke H. M. van den Berg,* Dirk P. Penninga,* Karel E. W. P. Tan,† Daan J. der Kinderen,‡ and Charles H. C. M. Buys*

*Department of Human Genetics, State University of Groningen, Groningen, The Netherlands; and Departments of †Ophthalmology and ‡Experimental Pathology, State University of Utrecht, Utrecht

Summary

Nonpenetrance of the inherited mutation responsible for retinoblastoma has been reported. By DNA analysis in families with hereditary retinoblastoma, it is possible to identify healthy individuals in whom the mutation is nonpenetrant. This requires the use of DNA markers both within and flanking the retinoblastoma gene. We have analyzed the segregation of several markers in 19 families (69 meioses) with hereditary retinoblastoma. In two families a carrier was identified who showed nonpenetrance of the mutation predisposing to retinoblastoma. The intragenic markers were informative in 15 pedigrees. The use of flanking markers from the same chromosomal region caused an increase of the number of informative families to 18. No crossing-over within the gene was observed. In one family an inherited deletion involving one of the RB1 alleles was detected. Our findings emphasize the use of a combination of both intragenic and flanking markers to obtain both the highest reliability of carrier detection in families with hereditary retinoblastoma and an accurate estimate of the frequency of nonpenetrance.

Introduction

Retinoblastoma is a childhood cancer that is hereditary in 40% of cases. A total of 10% of cases are due to the transmission of a germ-line mutation from an affected parent, and 30% of cases are due to new germ-cell mutations. There is a dominant pattern of inheritance with a penetrance of about 90% (Vogel 1979). Knudson (1971) hypothesizes that retinoblastoma is a cancer caused by two mutational events. In the inherited form the first mutation is inherited via a germ cell and therefore is present in all somatic cells, and the second mutation occurs in a somatic cell. In the nonhereditary form both mutations occur in one and the same somatic cell.

Received September 16, 1988; final revision received March 30, 1989.

Address for correspondence and reprints: Dr. C. H. C. M. Buys, Department of Human Genetics, State University of Groningen, Antonius Deusinglaan 4, NL-9713 AW Groningen, The Netherlands.

© 1989 by The American Society of Human Genetics. All rights reserved.

0002-9297/89/4502-0007$02.00

Deletion of band 13q14 in one of the chromosomes 13 of normal cells occurs in a proportion of hereditary cases. Cytogenetic analysis of tumor cells from some patients with a normal constitutive karyotype also revealed chromosome rearrangements involving this chromosome band (Chaum et al. 1984). The retinoblastoma locus (RB1) has accordingly been assigned to 13q14 (Sparkes et al. 1980).

Cavenee et al. (1983), using chromosome 13–specific DNA markers, found evidence for Knudson's hypothesis in an analysis of rearrangements of chromosome 13 in retinoblastoma tumors. Although in children at risk almost all tumors are diagnosed by the age of 1 year, they should be ophthalmologically examined until the age of 4 or 5 years and, less frequently, even after that (Ellsworth 1969). Some mutation carriers never become affected. Although all their retinal cells have an RB1 allele carrying a mutation, in none of them does a second somatic mutation involving the normal allele occur. Here we present an approach to determine what fraction of unaffected offspring carries a predisposing mutation.

Until a few years ago, the only genetic marker available for segregation analysis of the chromosomes 13 in pedigrees with hereditary retinoblastoma was the polymorphic enzyme marker esterase D (ESD), also coded for by a gene located at 13q14 (Sparkes et al. 1980). Several groups then started to isolate polymorphic chromosome 13–specific DNA markers (Cavenee et al. 1984; Lalande et al. 1984; Scheffer et al. 1986*b*). Recently, the presumed retinoblastoma gene has been isolated and characterized by three groups (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987). Wiggs et al. (1988) described five RFLPs inside the RB1 gene. Thus, a whole collection of highly informative DNA markers, both intragenic and flanking, are now available for presymptomatic (including prenatal) diagnosis of hereditary retinoblastoma.

We have analyzed the segregation of RB1 gene markers in kindreds with hereditary retinoblastoma in order to (*a*) evaluate the occurrence of possible cross-overs between the variant site detected by the respective intragenic markers and the mutation itself predisposing to retinoblastoma, (*b*) estimate the occurrence of nonpenetrance in hereditary retinoblastoma by identification of asymptomatic carriers of a predisposing mutation, and (*c*) determine the frequency of detection of deletion mutations by the various intragenic markers (see Horsthemke et al. 1987; Wiggs et al. 1988). In asymptomatic carriers of a mutation predisposing to this disease, alleles of intragenic markers seem not to segregate with the retinoblastoma trait, thereby mimicking a situation of intragenic crossing-over. A combined application of intragenic and flanking markers, however, allowed in our families the exclusion of crossing-over within the RB1 gene. Thus, we could identify in different families two carriers who showed nonpenetrance of the mutation predisposing to retinoblastoma.

A summed maximum lod score of 16.567 (θ = .000) for linkage between the retinoblastoma trait and the intragenic RB1 markers is presented for both the kindreds we studied and the 18 kindreds reported by Wiggs et al. (1988) to have an established diagnosis of retinoblastoma. An inherited deletion involving one of the RB1 alleles was detected in only one of the families.

Subjects and Methods

Subjects

This paper presents some results obtained from a study of 19 families (18 from The Netherlands and one contributed by Dr. A. Petrakova from Czechoslovakia) having two or more members with retinoblastoma. The patients in these families had no microscopically detectable chromosome 13 abnormalities in their lymphocytes.

DNA Analysis

DNA was isolated from white blood cells that were recovered from whole blood after osmotic lysis of erythrocytes and platelets by NH_4Cl. Cells from 40 ml blood were resuspended in 20 ml SE buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0) and were incubated with proteinase K (100 μg/ml) and 1% SDS for 2 h at 37°C. The DNA was extracted three times with phenol/chloroform, precipitated by adding 1/30 vol 3 M sodium acetate and 1 vol isopropanol, was washed in 70% ethanol, and was dissolved in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4.

Five micrograms DNA per sample were digested with the appropriate restriction endonucleases: *Apa*I, *Dra*I, *Hin*dIII, *Kpn*I, *Msp*I, *Rsa*I, *Tth*111I, or *Xba*I. The resulting fragments were separated by electrophoresis in a 0.6%–1.5% agarose gel in TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA, pH 8.0) and were transferred onto Genescreen Plus™ (New England Nuclear) nylon filters by Southern blotting in 0.4 M NaOH, 0.6 M NaCl. Pulsed-field gel electrophoresis (PFGE) was carried out using the contour-clamped hexagonal field (CHEF) device described by Chu et al. (1986). High-molecular-weight DNA used in this technique was prepared according to a method described by Van Ommen et al. (1986). Gels were run for 48 h at 6 V/cm in TBE at 12°C, using switch times of 180 s. After irradiation of the gels by 302 nm UV light during 5 min, the DNA was transferred to Genescreen Plus as described above.

Filters were prehybridized for 15 min at 65°C in 10 ml 0.5 M $NaHPO_4$ pH 7.2, 10 mM EDTA, 1% BSA (fraction V; Boehringer Mannheim), 7% SDS (Church and Gilbert 1984). The probes used are listed in table 1. They were labeled by the primer extension method (Feinberg and Vogelstein 1983) to a specific activity of approximately 10^9 cpm/μg. The prehybridization solution was replaced by 10 ml of the same solution containing 25 ng labeled probe, and hybridization was carried out overnight at 65°C. After hybridization the filters were washed twice at 65°C in 2 × SSC (1 × SSC = 0.15 mol NaCl/liter, 0.015 mol trisodium citrate/liter), 0.1% SDS for 30 min, twice in 1 × SSC, 0.1% SDS, and once in 0.3 × SSC, 0.1% SDS. Filters were covered with plastic wrap and were exposed to Fuji RX film backed by an intensifying screen at −80°C for 18 h–3 d.

Table 1

Characteristics of Applied Chromosome 13–specific Probes

Clone		Localization	Restriction Enzyme	Minor-Allele Frequency	Total PIC Value	References
pG14E3.8	D13S22	13q14.1	*Hin*dIII	.50	.70 (from eight haplotypes)	Scheffer et al. 1987
pG14E1.9	D13S22		*Dra*II	.33		
pG14E0.9	D13S22		*Hin*dIII	.15		
pESD14.1.1	ESD	13q14.1	*Apa*I	.20		Squire et al. 1986
p95HS0.5	RB1	13q14.1	*Kpn*I	.05		Wiggs et al. 1988
p88PR0.6	RB1		*Xba*I	.45		Wiggs et al. 1988
p68RS2.0	RB1		*Rsa*I	. . .	.77 (eight-allele system)	Wiggs et al. 1988
p35R0.6	RB1		*Tth*111I	.20		Wiggs et al. 1988
pGH2	RB1	0.9 kb 5′cDNA	*Hin*dIII	Not polymorphic		Fung et al. 1987
pG3.8M	RB1	3.8 kb 3′cDNA	*Hin*dIII	Not polymorphic		Fung et al. 1987
pG18E2.1	D13S12	13q21	*Msp*I	. . .	.67 (four-allele system)	Scheffer et al. 1986*a*
WC5	D13S39	13q14-q22	*Msp*I	. . .	.37 (three-allele system)	Leppert et al. 1987
WC83	D13S41	13q14-q22	*Taq*I	.37		Leppert et al. 1987

Linkage Analysis

Linkage calculations were made manually by assuming a penetrance of 0.9 and were verified by using the computer program LINKAGE, which also allows the assumption of an incomplete penetrance (Lathrop and Lalouel 1984). Haplotypes were deduced under the assumption of a minimum number of cross-overs.

Results

Probe Informativity

The 19 families (69 meioses) with hereditary retinoblastoma that were analyzed by us are shown in figure 1. The intragenic probes appeared to be fully informative in 15 of the 19 families (48 meioses) and to be partly informative in family Rb-H (two meioses). The highly informative marker p68RS2.0, which detects a polymorphism caused by variation of the number of tandem repeats (VNTR) of a 50-bp sequence (Wiggs et al. 1988), accounted already for 13 families (43 meioses). The application of the D13S22 markers increased the number of informative families to 18 (54 meioses). Segregation analysis was consistent with the absence of intragenic crossing-over in these families. The large family Rb-L (10 meioses) and part of family Rb-H (5 meioses) remained uninformative.

In *Rsa*I-digested genomic DNA a new 1.55-kb allele was detected with the VNTR marker in one of 80 chromosomes from 40 unrelated individuals.

Molecular Detection of Inherited Deletions

The cloning of the RB1 cDNA sequence by several groups (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987) and of part of the genomic DNA sequence of the RB1 gene by Wiggs et al. (1988) has made possible the detection of inherited deletions inside the RB1 gene.

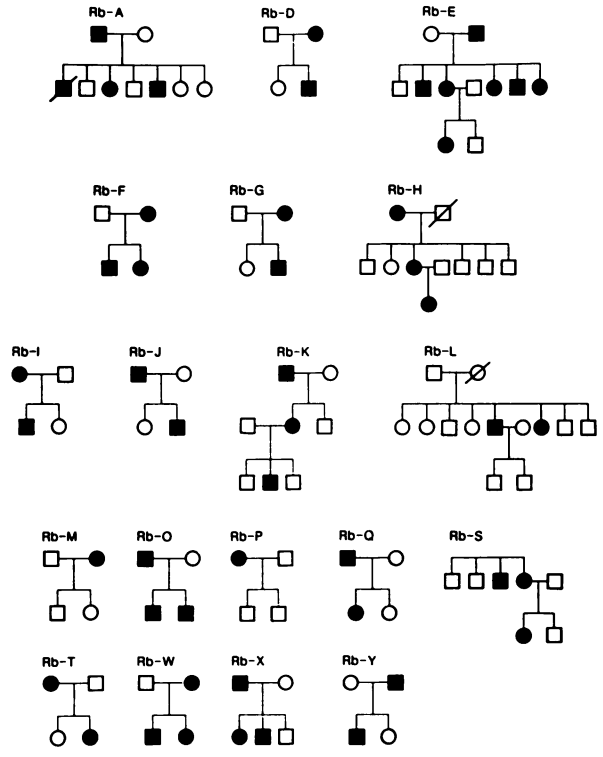

Figure 1 Pedigrees of families with hereditary retinoblastoma. Affected individuals are indicated by solid circles (females) or solid squares (males). Presence of a heritable RB1 allele segregating in families Rb-M and Rb-P is assumed because of the presence of other affected individuals in parts of the families not analyzed.

Thus, one type of mutation predisposing to retinoblastoma can be analyzed directly. Using the genomic intragenic markers summarized in table 1, we found by dosimetric analysis in family Rb-W a deletion that was transmitted from the affected parent to the affected offspring. The segregation pattern for the p35R0.6 marker is shown in figure 2*a*. The unaffected father I.1 is heterozygous for this *Tth*111I RFLP, and the affected mother I.2 is hemizygous for the 4.35-kb allele. Both affected children are hemizygous for the 4.95-kb allele inherited from I.1. Apparently they lack a maternal allele. Since all intragenic markers reveal the presence of just one allele (data not shown), the deletion must span the whole genomic DNA sequence coding for one of the RB1 alleles. PFGE data were consistent with the absence of a breakpoint within either of the RB1 alleles. Using the 3.8-kb 3′ RB1 cDNA sequence as a probe, we did not detect any aberrant *Sac*II restriction fragments along with the normal 750-kb fragment in DNA from the affected individuals I.2, II.1, and II.2 (see fig. 2*b*). Since the same *Sac*II fragment is detected with the 0.9-kb 5′ cDNA sequence, virtually all RB1 coding sequences must be contained within this fragment. By dosimetric analysis using the chromosome 3–specific probe pH3E4 (Carritt et al. 1986) for reference, we found that the intensity of the hybridization signal was reduced to 50% in I.2, II.1, and II.2, consistent with the presence of just one RB1 allele (see fig. 2*c*). The proximal marker ESD (13q14.1-2) as well as the distal marker D13S12 (13q21) revealed heterozygous phenotypes in II.1 and II.2, indicating a deletion size not extending as far as these two markers. Karyotypical analysis in this family did not reveal a microscopically detectable deletion involving 13q14.

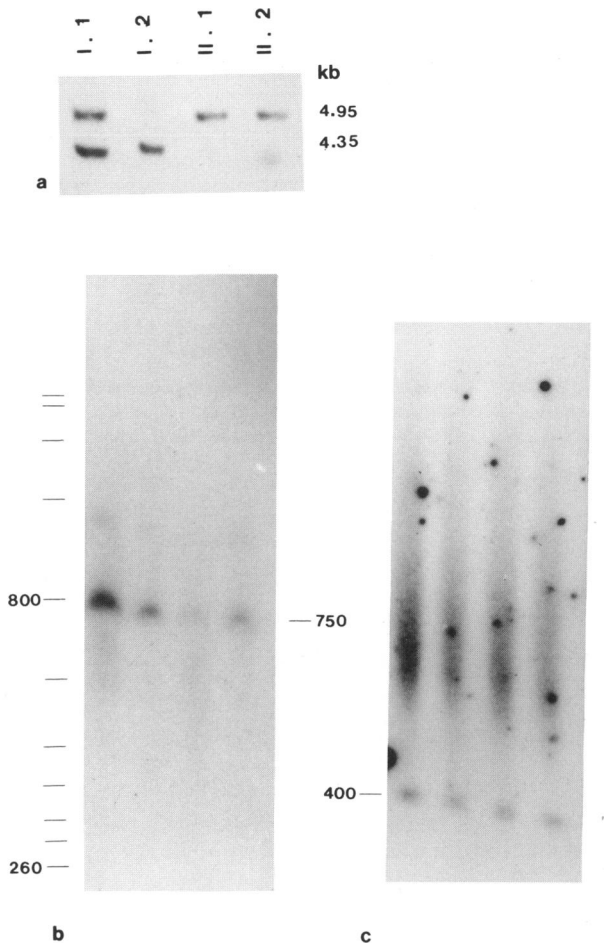

Figure 2 Detection of the deletion of one of the RB1 alleles segregating in family Rb-W. *a*, Segregation of the intragenic p35R0.6 marker. *b*, Detection of the presence of normal *Sac*II restriction fragments hybridizing to the 3′ RB1 cDNA probe pG3.8M in all members of family Rb-W. The same *Sac*II fragment is detected by the 5′ RB1 cDNA probe pGH2 (data not shown), which implies that virtually all RB1 coding sequences must be contained within this fragment. Yeast chromosomes (*Saccharomyces cerevisiae* XX-11) were run as molecular-weight standards. The positions of the chromosomes I–X a and b are indicated by bars. *c*, Rehybridization of the same filter by using probe pH3E4 (Carritt et al. 1986) for reference, followed by dosimetric analysis, revealed the presence of two RB1 copies in I.1 and one RB1 copy each in I.2, II.1, and II.2.

Linkage Analysis

The degree of linkage between the respective 13q14 markers (including the markers within the RB1 gene) and the retinoblastoma trait has been calculated from the data obtained for the 18 families in which one or more markers were informative. It was assumed that in 90% of the individuals in which a mutant retinoblastoma allele is present the disease will actually develop (90% penetrance). Table 2 shows the results. For the intragenic markers the lod score reached its maximum at a recombination fraction of zero. The summed lod score for the intragenic markers obtained from both Wiggs's study and our own was 16.567 (θ = .000). For the proximal marker D13S22 (pG14E3.8, pG14E1.9, and pG14E0.9) the lod score reached its maximum at a recombination frequency of approximately .07. For the ESD marker, which is also proximal to RB1 (Mitchell and Cowell 1988), the lod score reached its maximum at a recombination fraction of zero.

Retinoblastoma is characterized by a heterogeneous spectrum of mutations involving the RB1 gene. Since the precise sites of the predisposing mutation and the

Table 2

Lod Scores for Linkage of the 13q14 Markers to the Retinoblastoma Trait

	Recombination Fraction							
	.000	.001	.01	.05	.1	.2	.3	.4
D13S22/retinoblastoma (all families) . . .	.965	1.072	2.118	2.850	2.910	2.350	1.424	.345
ESD/retinoblastoma (all families)	2.812	2.797	2.745	2.543	2.232	1.582	.895	.304
RB1/retinoblastoma (all families)	6.300	6.265	6.155	5.681	4.986	3.502	1.973	.654
RB1/retinoblastoma (family Rb-E)	1.021	1.025	1.032	1.032	.987	.804	.530	.202
RB1/retinoblastoma (family Rb-K)	.080	.081	.082	.160	.195	.196	.150	.081
Summed lod scores from the present study and that of Wiggs et al. (1988)[a], RB1/retinoblastoma	16.567	16.465	16.155	14.108	13.132	9.290	5.495	1.880

Note.—The RB1 markers used are summarized in table 1. No haplotypes were constructed for these probes.
[a] Under the assumption of 90% penetrance and leaving out family Rb-Sa of Wiggs et al. (1988).

variant restriction site detected by a marker within the RB1 gene are not identical, they may segregate separately as a consequence of an intragenic crossover. Whether this is the case can be verified by analyzing the segregation of flanking markers. Occurrence of a crossover within the gene can be confirmed by deviating segregation of either proximal or distal marker alleles. No case of intragenic recombination was observed in the 47 meioses informative for one or more intragenic markers. Apparent separate segregation of the retinoblastoma trait and of a couple of intragenic markers was observed in the kindreds Rb-E and Rb-K. In these cases, however, this is due to the occurrence of an asymptomatic carrier of a mutation predisposing to retinoblastoma in either family (see below). Consequently, for the pedigrees Rb-E and Rb-K the maximum lod score for linkage between intragenic markers and the retinoblastoma trait was observed at a recombination fraction clearly different from zero (see table 2). A detailed description of the exclusion of the possible occurrence of intragenic recombination in these kindreds is given in the following section.

Asymptomatic Carriers of the Mutant Gene

Pedigrees of two families with potential crossovers for the VNTR marker p68RS2.0 are shown in figures 3 and 4. Of the intragenic probes described by Wiggs et al. (1988), p68RS2.0 and p88PR0.6 were informative in family Rb-K (fig. 3). The mutation predisposing to retinoblastoma is associated with the 1.65-kb p68-RS2.0 allele and the p88PR0.6 allele 1 in II.2, which alleles are also present in III.2. In the unaffected individual III.3, however, the 1.65-kb p68RS2.0 allele and the p88PR0.6 allele 1 are also present. We have used flanking markers, both proximal and distal (see fig. 3), to exclude the possibility of an intragenic crossover between both intragenic markers p68RS2.0 and p88PR0.6 and the mutation predisposing to retinoblastoma. The closest proximal marker is ESD at 4.8 cM (Bowcock et al. 1988), and the closest informative distal marker is D13S39 at approximately 15 cM (authors' unpublished results). No crossovers are detected between RB1 and the flanking markers. Between the distal markers D13S39 and D13S41, however, a maternal crossover was detected. To infer on the basis of the combined haplotypes, individual III.3 in family Rb-K must have inherited from his mother II.2 the part of the chromosome 13 homologue carrying the mutation. On scrupulous ophthalmological inspection no signs of retinoblastoma development were observed in this boy by the age of 21 mo. Most likely, this is an illustration of nonpenetrance of the mutation. The observed pattern of segregation could also result if predisposition to retinoblastoma were caused by mutations at more than one locus. However, both the fact that no crossovers between the intragenic markers and the retinoblastoma trait were observed in the remaining part of family Rb-K (see fig. 3) and the general lack of evidence for locus heterogeneity make nonpenetrance of the mutation predisposing to retinoblastoma the most plausible explanation of our results.

A comparable case was observed in family Rb-E (fig. 4). An analysis of the parents, brothers, and sisters (fig. 1) of I.1 in figure 4 made clear that the mutation predisposing to retinoblastoma was associated with the 1.50-kb allele of the VNTR marker p68RS2.0 and with

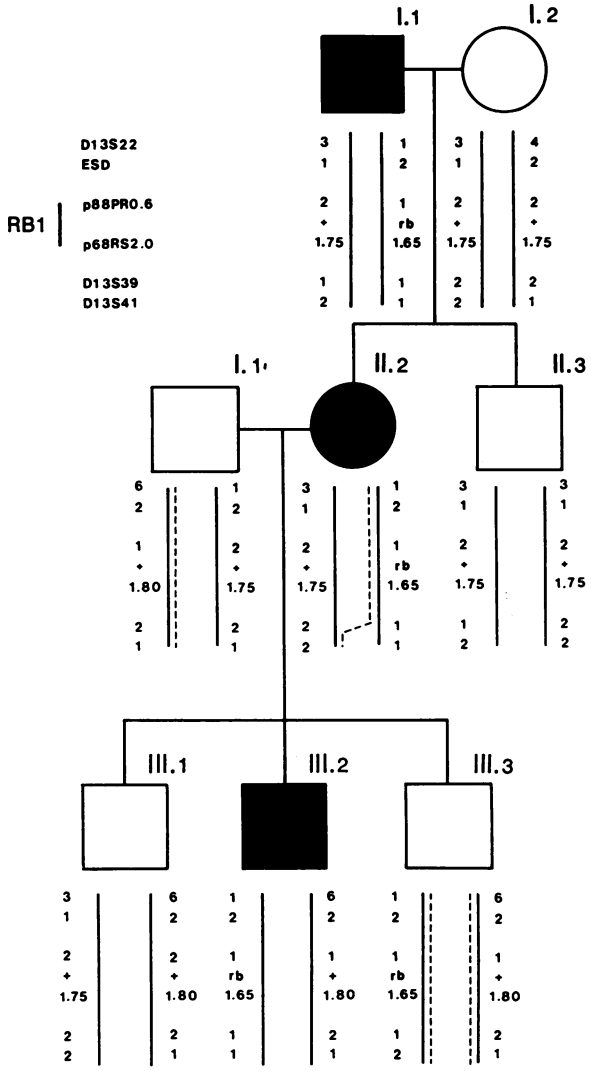

Figure 3 Pedigree of family Rb-K, showing the segregation of RFLPs within and flanking the RB1 gene. The haplotypes of the D13S22 locus are deduced from the alleles of the pG14E3.8/*Hin*dIII RFLP, the pG14E1.9/*Dra*I RFLP, and the pG14E0.9/*Hin*dIII RFLP, respectively. The numbers indicating different haplotypes re given according to the haplotype frequencies. The alleles of the proximal marker ESD, the distal markers D13S39 and D13S41, and the intragenic marker p88PR0.6 are indicated by 1 for the larger allele and by 2 for the smaller allele. The intragenic VNTR marker alleles of p68RS2.0 are indicated by the lengths (in kb) of their characteristic bands. The localization of ESD proximal to RB1 and the orientation of the RB gene have been determined by deletion analysis in a retinoblastoma cell line by Mitchell and Cowell (1988). The localization of D13S22 proximal to ESD is derived from linkage data obtained by Bowcock et al. (1988), and the order of the markers distal to RB1 is derived from linkage data obtained by Leppert et al. (1987). The mutant RB1 allele according to this analysis is indicated by the symbol rb, and the wild-type allele is indicated by a plus sign (+).

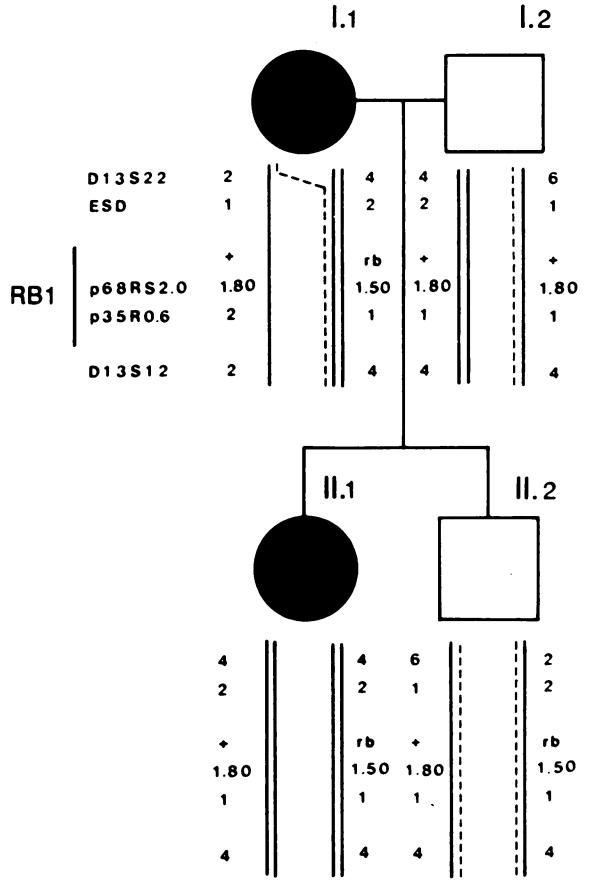

Figure 4 Pedigree of part of family Rb-E, showing the segregation of RFLPs within and flanking the RB1 gene. The alleles of the intragenic marker p35R0.6 and the distal four-allele marker D13S12 are numbered from the larger to the smaller allele. Other symbols are as in fig. 3. For clarity, only part of the whole pedigree is shown. Association of the mutant RB1 allele to the D13S22 haplotype 4, the ESD allele 2, the 1.50-kb p68RS2.0 allele, the p35R0.6 allele 1, and the D13S12 allele 4 was deduced from phenotypes observed in the parents, sisters, and brothers of I.1.

the p35R0.6 allele 1, the D13S22 haplotype 4 (see the legend to fig. 3), the ESD allele 2, and the D13S12 allele 4. The unaffected son II.2 has inherited the 1.50-kb p68RS2.0 allele associated with the mutation predisposing to retinoblastoma. Again, proximal as well as distal markers (see fig. 4) have been used to rule out the possibility of an intragenic crossing-over in the meiosis leading to II.2. The closest informative distal marker in this case was D13S12 at approximately 14 cM (authors' unpublished results). No crossovers were detected between RB1 and the flanking markers. Between the proximal markers D13S22 and ESD, however, a maternal crossover was detected. To infer on the basis of com-

bined haplotypes, II.2 in family Rb-E must have inherited from his mother the part of the chromosome 13 homologue carrying the mutation. The crossover thus appears not to be intragenic, which implies that II.2 has inherited the mutation predisposing to retinoblastoma. On ophthalmological inspection no signs of retinoblastoma development were detected in this boy by the age of 3 years. Therefore, this analysis reveals another example of nonpenetrance of a mutation predisposing to retinoblastoma.

Discussion

Development of the eye tumor retinoblastoma requires the functional loss of both alleles of the retinoblastoma gene at the RB1 locus in band q14 of chromosome 13 (Cavenee et al. 1983). In the hereditary form of retinoblastoma a mutation having functionally eliminated one allele is present in the germ line. The nature of this mutation may vary, from a gross deletion to a point mutation. The same applies to the mutation of the second allele occurring in a retinoblast. By high-resolution banding techniques, microscopically detectable rearrangements of 13q14 can be observed in lymphocytes and fibroblasts from about 10% of patients (Turleau et al. 1985).

Small deletions escaping microscopic detection may be revealed in some cases by DNA analysis with genomic RB1 probes or with RB1 cDNA probes. In 19 pedigrees analyzed, we have been able to detect one such deletion. Wiggs et al. (1988) have detected inherited deletions involving one of the RB1 alleles in three of 19 pedigrees. Horsthemke et al. (1987), using the exon-containing probe H3-8, detected an intragenic deletion in three of 11 unrelated individuals with hereditary retinoblastoma. Thus, in a total of seven of 49 pedigrees a direct detection of deletions involving the RB1 gene was shown by the use of intragenic probes.

An alternative approach to detect directly mutational deletions or rearrangements within the RB1 gene is the hybridization of cDNA to long DNA fragments generated by restriction enzymes recognizing rare cleavage sites and separated by PFGE. An advantage of this method is that any deletion or rearrangement that generates qualitative changes in the restriction pattern can be detected. This kind of analysis was applied to family Rb-W (fig. 2*b*). The RB1 cDNA clone pG3.8M detected no aberrant *Sac*II fragment in DNA from the affected family members I.2, II.1, and II.2. Under the conditions used, aberrant restriction fragments that differ from the normal fragment by 10% or more in length can be discerned. This implies that if in family Rb-W a deletion would also be present at the second allele, it must be smaller than approximately 75 kb. For the direct detection of small mutations (point mutations and minute deletions) RNase protection studies can be used as an approach (Dunn et al. 1988).

The isolation of the highly informative intragenic VNTR marker p68RS2.0 and of other intragenic markers has markedly facilitated the detection of carriers of mutations predisposing to retinoblastoma. Still, 19 of 69 meioses (in four of 19 families) could not be analyzed using the intragenic probes alone. Informative flanking markers are necessary to analyze these meioses. In this study inclusion of our D13S22 markers caused an increase of the informativity, from 15 of 19 families to 18 of 19 families.

A summed lod score for linkage between the retinoblastoma trait and the RB1 markers obtained in this study and by Wiggs et al. (1988) is presented in table 2. From such combined data, one might be able to get an estimate of the maximum possible frequency of intragenic crossing-overs within the retinoblastoma gene. No case of intragenic crossing-over has been observed, either in 48 meioses informative for markers within the RB1 gene in this study or in 71 informative meioses in families with an established diagnosis of retinoblastoma who were studied by Wiggs et al. (1988). On the basis of these intragenic markers, a conservative estimate of the error rate in diagnosis of a predisposition to retinoblastoma is less than 4% (95% confidence interval). For unaffected individuals, such as III.3 in family Rb-K or II.2 in family Rb-E, who have inherited a haplotype (including alleles from intragenic markers) associated with a predisposing mutation, exclusion of an intragenic crossing-over was possible by the use of flanking markers that identified these individuals as carriers of nonpenetrant mutations. Of course, this is very important for the assessment of the risk of retinoblastoma for the next generation, but it may also be important in reference to a possibly increased risk for carriers themselves to develop second primary tumors.

It has been estimated that about 10% of the mutations predisposing to retinoblastoma are nonpenetrant (Vogel 1979). From the two-hit model (Knudson 1971) it can be predicted that statistically a fraction of carriers of a mutation predisposing to retinoblastoma will never become affected. In carriers all retinal cells have a mutated RB1 allele. In asymptomatic carriers the second somatic mutation, affecting the remaining normal RB1 allele, has not occurred in any of these retinal cells. In a small number (fewer than 2%) of mutation car-

riers, clinically benign retinal lesions or "retinomas" occur (Connolly et al. 1983). Most likely, retinoma arises when the second mutation occurs in an almost terminally differentiated retinal cell (Gallie et al. 1982). Individuals with retinoma can only be detected on ophthalmologic inspection. They may therefore usually pass for normal individuals but are able to transmit to their offfspring the mutation predisposing to retinoblastoma. Thus, they resemble true asymptomatic carriers of a nonpenetrant mutation. Both asymptomatic carriers identified in the present study did not show any signs of retinoma.

Matsunaga (1976) proposed a "host resistance" model to account for the variability of penetrance in familial cases of retinoblastoma. In this theoretical model unaffected carriers are resistant to tumor formation, whereas bilaterally affected carriers are susceptible and unilaterally affected carriers have intermediate susceptibility. Another mechanism explaining a decreased penetrance has been proposed by Knudson (1983). Deletion of an extensive chromosome 13 region containing the RB1 locus is a common mechanism in tumor development, as evidenced by loss of heterozygosity (Cavenee et al. 1983). If, however, an allele lethal at the cellular level would be present at some other locus on the chromosome 13 homologue containing the mutation predisposing to retinoblastoma, occurrence of a deletion including this locus at the other chromosome 13 copy would lead to cell death. In that case no tumor would result. In families in which such a lethal allele is associated with an RB1 mutation, the number of affected individuals would therefore be smaller than expected. Dryja et al. (1984) suggested a similar explanation for the observation that the incidence of bilateral retinoblastoma in patients with a constitutional 13q14 deletion was lower than that in nondeletion patients. A retinal cell that becomes homozygous for a substantial deletion may be nonviable because it lacks essential genes.

DNA analysis offers the possibility of discriminating, in the unaffected offspring from obligate carriers, noncarriers from asymptomatic carriers of the mutation predisposing to retinoblastoma. We have identified two carriers of a nonpenetrant mutation in 48 meioses informative for one or more intragenic RB1 markers, of which 27 have led to an affected individual. Thus, in our material 7% (two of 29) of transmitted mutations are not expressed (95% confidence interval 1%–22%).

As demonstrated by the recent results of both Dryja's group and our own, DNA analysis offers a promising approach to the presymptomatic diagnosis of *hereditary* retinoblastoma. The variety of possible mutations makes the use of DNA markers the proper method by which to follow the inheritance of the predisposition to retinoblastoma through further generations. Risk predictions should, however, still take into account the occurrence of nonpenetrance.

Acknowledgments

We are grateful to Drs. H. Monasch, and A. Petrakova for their help in collecting blood samples and to the families for their cooperation. We are indebted to Dr. T. P. Dryja for making available his genomic retinoblastoma probes before publication. We acknowledge the gift of the ESD probe from Dr. J. Squire, the retinoblastoma cDNA probes from Dr. Y.-K.T. Fung, and probes WC25 and WC83 from Dr. D. James at the Ludwig Institute for Cancer Research (Montreal branch). We thank Dr. W. K. Cavenee for his hospitality and Dr. M. F. Hansen for expert advice on the PFGE technique during a stay by H.S. in Montreal. This work was financially supported by the Netherlands Cancer Foundation grant GUKC 83-13, by the International Union Against Cancer (Yamagiwa-Yoshida Memorial International Cancer Study Grant), and by the Foundation of Paediatric Oncology, Groningen.

References

Bowcock AM, Farrer LA, Herbert JM, Agger M, Sternlieb I, Scheinberg IH, Buys CHCM, et al (1988) Eight closely linked loci place the Wilson disease locus within 13q14-q21. Am J Hum Genet 43:664–674

Carritt B, Welch HM, Perry-Jones NJ (1986) Sequences homologous to the human *D1S1* locus present on human chromosome 3. Am J Hum Genet 38:428–436

Cavenee WK, Dryja TP, Phillips RA, Benedict WF, Godbout R, Gallie BL, Murphree AL, et al (1983) Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature 305:779–784

Cavenee W, Leach R, Mohandas T, Pearson P, White R (1984) Isolation and regional localization of DNA segments revealing polymorphic loci from human chromosome 13. Am J Hum Genet 36:10–24

Chaum E, Ellsworth RM, Abramson DH, Haik BG, Kitchin FD, Chaganti RSK (1984) Cytogenetic analysis of retinoblastoma: evidence for multifocal origin and in vivo amplification. Cytogenet Cell Genet 38:82–91

Chu G, Vollrath D, Davis RW (1986) Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science 234:1582–1585

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Connolly MJ, Payne RH, Johnson G, Gallie BL, Allderdice PW, Marshall WH, Lawton RD (1983) Familial, EsD-linked, retinoblastoma with reduced penetrance and variable expressivity. Hum Genet 65:122–124

Dryja TP, Cavenee W, White R, Rapaport JM, Petersen R, Albert DM, Bruns GAP (1984) Homozygosity of chromosome 13 in retinoblastoma. N Engl J Med 310:550–553

Dunn JM, Phillips RA, Becker AJ, Gallie BL (1988) Identification of germline and somatic mutations affecting the retinoblastoma gene. Science 241:1797–1800

Ellsworth RA (1969) The practical management of retinoblastoma. Trans Am Ophthalmol Soc 67:462–534

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert D, Dryja TP (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323:643–646

Fung Y-K T, Murphree AL, T'Ang A, Qian J, Hinrichs SH, Benedict WF (1987) Structural evidence for the authenticity of the human retinoblastoma gene. Science 236:1657–1661

Gallie BL, Ellsworth RM, Abramson DH, Phillips RA (1982) Retinoma: spontaneous regression of retinoblastoma or benign manifestation of the mutation? Br J Cancer 45:513–515

Horsthemke B, Barnert HJ, Greger V, Passarge E, Hopping W (1987) Early diagnosis in hereditary retinoblastoma by detection of molecular deletions at gene locus. Lancet 1:511–512

Knudson AG (1971) Mutation and cancer: statistical study of cancer. Proc Natl Acad Sci USA 68:820–823

——— (1983) Model hereditary cancers of man. Nucleic Acids Res 29:17–25

Lalande M, Dryja TP, Schreck RR, Shipley J, Flint A, Latt SA (1984) Isolation of human chromosome 13–specific DNA sequences cloned from flow sorted chromosomes and potentially linked to the retinoblastoma locus. Cancer Genet Cytogenet 13:283–295

Lathrop GM, Lalouel JM (1984) Easy calculations of lod scores and genetic risks on small computers. Am J Hum Genet 36:460–465

Lee W-H, Bookstein R, Hong F, Young L-H, Shew J-Y, Lee EY-HP (1987) Human retinoblastoma susceptibility gene: cloning, identification and sequence. Science 235:1394–1399

Leppert M, Lathrop GM, O'Connell P, Nakamura Y, Cavenee WK, Cartwright P, Lalouel JM, et al (1987) Extension of a primary linkage map of chromosome 13. Cytogenet Cell Genet 46:648

Matsunaga E (1976) Hereditary retinoblastoma: penetrance, expressivity and age of onset. Hum Genet 33:1–15

Mitchell CD, Cowell JK (1988) Molecular evidence that the esterase-D gene lies proximal to the retinoblastoma susceptibility locus in chromosome region 13q14. Hum Genet 81:57–60

Scheffer H, Penninga D, Goor N, Pearson PL, Buys CHCM (1986*a*) A four-allele RFLP identified by an anonymous single copy clone at 13q21-13qter (HGM8 assignment no. D13S12). Nucleic Acids Res 14:3148

Scheffer H, van den Berg JHM, Penninga D, Tan KEWP, Der Kinderen DJ, Monasch H, Houwen RJ, et al (1987) Genetic linkage analysis of hereditary retinoblastoma and Wilson disease with chromosome 13 markers. Cytogenet Cell Gent 46:688

Scheffer H, Van der Lelie D, Aanstoot GH, Goor N, Nienhaus AJ, Van der Hout AH, Pearson PL, et al (1986*b*) A straightforward approach to isolate DNA sequences with potential linkage to the retinoblastoma locus. Hum Genet 74:249–255

Sparkes RS, Sparkes MC, Wilson MG, Towner JW, Benedict WF, Murphree AL, Yunis JJ (1980) Regional assignment of genes for human esterase D and retinoblastoma to chromosome band 13q14. Science 208:1042–1044

Squire J, Dryja TP, Dunn J, Goddard A, Hofmann T, Musarella M, Willard HF, et al (1986) Cloning of the esterase D gene: a polymorphic gene probe closely linked to the retinoblastoma locus on chromosome 13. Proc Natl Acad Sci USA 83:6573–6577

Turleau C, De Grouchy J, Chavin-Colin F, Seger J, Schlienger P, Leblanc A, Hage C (1985) Cytogenetic forms of retinoblastoma: their incidence in a survey of 66 patients. Cancer Genet Cytogenet 16:321–334

Van Ommen GJB, Verkerk JMH (1986) Restriction analysis of chromosomal DNA in a size range up to two million base pairs by pulsed field gradient electrophoresis. In Davies KE (ed) Human genetic diseases: a practical approach. IRL, Oxford, pp 113–133

Wiggs J, Nordenskjold M, Yandell D, Rapaport J, Grondin V, Janson M, Werelius B, et al (1988) Prediction of the risk of hereditary retinoblastoma, using DNA polymorphisms within the retinoblastoma gene. N Engl J Med 318:151–157

Vogel F (1979) Genetics of retinoblastoma. Hum Genet 52:1–54