Application of PCR amplification of DNA from paraffin embedded tissue sections to linkage analysis in familial retinoblastoma

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Abstract

A family segregating for the retinoblastoma predisposition gene has been analysed using the polymerase chain reaction to exclude their son as being an affected gene carrier. The unusual feature of this family is that the affected child, who would ordinarily have been used to establish phase in a linkage study, died as a result of developing a second tumour some years ago. The only tissue available from this child was a paraffin embedded, formalin fixed histopathological specimen from the second tumour. It was possible to isolate DNA from this tissue and amplify the DNA flanking two polymorphic restriction enzyme sites to establish alleles which cosegregated with tumour predisposition. Archival material can now be used to offer families such as this prenatal screening to provide informed genetic counselling.

Retinoblastoma (Rb) is the most common intraocular tumour of children. In its familial form the tumour phenotype segregates as an autosomal dominant trait.¹ Rb arises as a result of mutations in both copies of the Rb gene.² Most cases are apparently sporadic, owing to random key mutations in both genes; in hereditary cases (25 to 40%), however, the first mutation is transmitted through the germline and only the second mutation occurs as a random somatic event. Subjects carrying the Rb predisposition gene are also at a significantly higher risk than the general population for development of a number of other tumours, especially osteosarcoma and soft tissue sarcomas.^{3,4}

Through the analysis of rare constitutional chromosomal deletions^{5,6} and classical linkage studies using

the adjacent esterase D gene,⁷⁻⁹ the Rb gene was mapped to chromosome region 13q14 and these observations eventually led to its isolation.¹⁰⁻¹² The gene, called RB1, is 4.7 kb long and spans 200 kb of genomic DNA.¹²⁻¹⁴ The 4.7 kb mRNA comprises 27 exons and structural rearrangements have been observed within the genomic sequence and mRNA of approximately 30% of tumours. The cDNA cannot be used directly in linkage studies, because it does not identify polymorphic sites for restriction enzymes. Therefore a series of unique sequence, intragenic DNA probes were isolated¹⁵ allowing gene tracking in families and the detection of apparently unaffected Rb gene carriers.¹⁶ The sequence for the entire Rb cDNA is now available^{13,17} and the flanking intron sequences around two of the polymorphic sites have also been determined.¹⁸⁻²⁰ This means that linkage analysis in Rb families can be performed using the polymerase chain reaction (PCR).

The intragenic DNA probes are used in our laboratory for pre- and postnatal screening for Rb. However, between 10% and 15% are either uninformative for these probes or DNA samples have not been available from key family members. Here we present one such family, whose first child would normally have been used to establish linkage phase but who died before DNA analysis was available.

Materials and methods

DNA PREPARATION

DNA from whole blood samples was prepared using standard phenol/chloroform extraction followed by ethanol precipitation.²¹ DNA from 10 µm thick paraffin embedded tumour sections was prepared by a method modified from Shibata *et al.*²² Firstly, individual paraffin sections were dissolved in 500 µl of xylene. The tissue was recovered by centrifugation and then washed twice in ethanol and desiccated for 0.5 to 2 hours. The DNA extraction solution consisted of 100 mmol/l Tris-Cl, 4 mmol/l EDTA pH 8.0, and 0.5 mg/ml proteinase K. After 12 to 18 hours at 37°C, this solution was boiled for seven minutes and 1 to 10 µl used for PCR.

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PCR BASED DETECTION OF POLYMORPHIC SITES

*Bam*HI site

Two primers (table), one from exon 1 and the other from intron 1 of the human Rb gene, were used to amplify a genomic DNA fragment containing the polymorphic *Bam*HI site. In conventional Southern blotting experiments this polymorphism is identified using the M1.8 DNA probe. PCR was carried out in a total volume of 50 µl containing approximately 1 µg DNA and Promega buffer consisting of 50 pmol of each primer, 0.2 mmol/l each dNTP (dATP, dTTP, dCTP, dGTP), 50 mmol/l KCl, 10 mmol/l Tris HCl (pH 9.0 at 25°C), 1.5 mmol/l MgCl₂, 0.01% gelatin (w/v), 0.1% Triton X-100, 10% dimethyl sulphoxide (DMSO), and 2 to 3 units *Taq* DNA polymerase (Promega). The reaction mix was overlaid with 50 µl of mineral oil to prevent evaporation. Amplification was performed using a programmable thermal cycler (Techne PHC-1). Amplification conditions consisted of three steps (after an initial 15 minute denaturation step at 96°C): denaturation at 96°C for 20 seconds, annealing at 60°C for 20 seconds, followed by an extension step at 72°C for 60 seconds. On completion of 30 cycles the mineral oil was removed by chloroform extraction. The amplified product was then digested overnight with *Bam*HI. DNA fragments were resolved by electrophoresis through 2% agarose gels.

*Xba*I site

A genomic DNA fragment containing the polymorphic *Xba*I site (21.8 kb downstream of exon 17) was amplified using primers (table) from intron 17 of the human Rb gene. Using conventional Southern blotting procedures this polymorphism is identified by the PRO.6 DNA probe. The PCR conditions were the same as those used for the *Bam*HI site except that the PCR mix did not contain DMSO and the annealing temperature was 50°C. The amplification product was digested overnight with *Xba*I. DNA fragments were resolved by electrophoresis through 1.4% agarose gels.

Results and discussion

Prenatal screening for carriers of the RB predisposition gene is now relatively routine using a panel of unique DNA sequences derived from within the genomic

sequence of RB1.^{15 23 24} The polymorphic variants identified using these probes are sufficiently common in the population to offer a prediction of carrier status in 85 to 90% of families²⁴ (unpublished observations). To date, there have been no reported recombinations between the intragenic probes and the Rb phenotype, allowing predictions to be made with 95% confidence. There are, however, still families for whom this service is not available. Approximately 8 to 10% of families will be uninformative using the available probes because family members transmitting the predisposition to tumour development are homozygous at all of these loci. The application of new technology looking at short variable number tandem repeats (VNTRs) around exon 20 of the Rb gene^{18 25} or at single base pair polymorphic sites by direct sequencing²⁶ offers the possibility of prenatal screening in these cases. There are some families, however, where prenatal screening is not an option because, for a variety of reasons, tissue samples from key family members are not available. One such family is the subject of this report.

The pedigree of family RB-29 is shown in fig 1. At the age of 18 months the mother developed a tumour in the right eye which was enucleated. There was no previous history of Rb in this family. There was no history of Rb on the paternal side of the family either. At the age of 2 years the first born child, a girl, was treated for bilateral Rb with radiation. The tumour regressed but six months later a rhabdomyosarcoma arose in the right cheek and eventually caused the patient's death.²⁷ Although a rare subtype of this tumour, this second malignancy was part of the group frequently seen in Rb gene carriers.^{3 4} The second child, a boy, was born in 1987 and the family was referred to us for genetic screening. The mother was shown to be heterozygous using the M1.8 and PRO.6 DNA probes described by Wiggs *et al.*¹⁵ The boy had

Figure 1 Pedigree of family RB-29.

Sequence of PCR primers used to amplify polymorphic restriction enzyme sites within the RB1 gene.

Polymorphic site	Primers	
	Sense	Antisense
<i>Bam</i> HI	5'-CAGGACAGCGGCCGGAG-3'	5'-CTGCAGACGCTCCGCCGT-3'
<i>Xba</i> I	5'-TTCCAATGAAGAACAATGG-3'	5'-GCAATTGCACAATCCAAGTT-3'

Figure 2 PCR amplification of the polymorphic *Bam*HI site from DNA samples from the members of family RB-29 (above). In all cases a dominant band of the expected size, ± 200 bp long, is seen as well as fainter, non-specific bands in some cases. The amplified DNA from each patient was digested with *Bam*HI (below). The marker lane (M) in each case contains the Gibco BRL 1 kb ladder. The father (lane 1) is homozygous for the lower allele but the mother (lane 2) is heterozygous. Although a weak residual upper band is seen in this example, in DNA extracted from the tissue section representing the affected dead child (lane 3), the dominant bands are those for the lower allele indicating that this patient is homozygous for the allele. The unaffected child (lane 4) is heterozygous indicating that the *Rb* gene is segregating with the lower allele from the mother.

no evidence of *Rb* at the time of referral but, although the peak age for hereditary tumours is 10 to 14 months, he is still at risk for developing a tumour. In any case he could still be an apparently unaffected gene carrier which is known to occur in approximately 10% of cases.¹ To establish phase unequivocally it was essential, therefore, to analyse DNA from tissue from the dead child. No blood samples had been saved and no necropsy was performed²⁷ but during the treatment of the rhabdomyosarcoma a tooth was removed. Attached to its base was a small piece of tumour which had been fixed in formalin and embedded in wax. We were able to obtain tissue sections from this tumour material for analysis.

The DNA sequences flanking the polymorphic restriction enzyme sites in *RB1* identified by probes M1.8²⁰ and PRO.6¹⁹ have been established. Using these sequences, oligonucleotide primers have been designed (see Materials and methods) to analyse the polymorphic sites using PCR. Using DNA from the tissue section from the dead child's second tumour, PCR products around both polymorphic sites were generated. For the *Bam*HI polymorphism an approximately 200 bp genomic DNA fragment

containing the intron 1 splice donor site was amplified (fig 2, top). The polymorphic *Bam*HI site is located within the amplified fragment 50 bp from the 3' end. Thus, fragments of 140 bp and 60 bp are generated after *Bam*HI digestion. Using this polymorphism the results were unequivocal; the mother is heterozygous and the affected daughter homozygous for the lower allele (fig 2, bottom). Since the as yet unaffected son is also heterozygous we would predict that the mutant gene, therefore, is segregating with the lower (140/60 bp) allele. Using the other set of primers (table) an approximately 945 bp genomic DNA fragment containing the polymorphic *Xba*I site was amplified. *Xba*I digestion of this fragment identifies two alleles; an upper allele 945 bp long and a lower allele consisting of two fragments 630 bp + 315 bp long. At the *Xba*I locus (fig 3) the mother, the father, and the surviving child are all heterozygous. The dead child was homozygous for the lower allele indicating that the *Rb* mutation is segregating with this allele.

The DNA extracted from the tissue sections was degraded, in the size range of 50 to 1500 bp (fig 4). Owing to the cross linking of the DNA with formalin during the fixation process, smaller sizes are over-represented although this did not present a problem in analysing the 180 bp sequence flanking the *Bam*HI polymorphism, yields of PCR product being relatively good. For the larger 945 bp fragment, however, PCR product yields were somewhat smaller. We interpret this to mean that, because of the fragmented nature of the DNA removed from the formalin fixed tissue, there are fewer intact molecules of the appropriate size in the DNA used as template in the PCR reaction. The DNA thus generated was used directly for restriction enzyme digestion where, although left for six to 24 hours, digestion was sometimes incomplete,

Figure 3 Segregation of the alleles for the polymorphic *Xba*I site in family RB-29. The father (lane 1) and mother (lane 2) are both heterozygous as is the unaffected child (lane 3). The dead affected child (lane 4) is homozygous for the lower allele. The *Rb* gene is therefore segregating with the lower allele from the mother. M=marker lane containing 1 kb DNA ladder (Gibco-BRL).

Figure 4 Size range of DNA extracted from the formalin fixed, paraffin embedded tissue. One tenth of the extracted DNA (lane 1) was run on a 0.6% agarose gel and stained with ethidium bromide. A smear is seen between 200 bp and 3 kb. The marker lane (M) contains DNA from a 1 kb ladder (Gibco-BRL).

as shown in fig 2. We have noted this same finding in several other cases where subjects are known to be homozygous for the lower allele from conventional Southern blotting/radiolabelled probing. The relative intensity of the band caused by DNA molecules resistant to digestion, however, was significantly weaker than in 'true' heterozygotes (fig 2). Repeated amplification and digestion of DNA isolated from different tissue sections showed that, in some cases, this upper band was absent. It is possible, therefore that this finding varies in relation to the purity of the extracted DNA.

It is possible that the tumour tissue used in this analysis is not truly representative of normal tissue from the patient. Loss of heterozygosity is frequently reported in retinoblastoma tissue, osteosarcomas, and soft tissue sarcomas.^{17 28-30} However, it is consistent with the theory of 'exposure' of recessive alleles in these childhood tumours that the mutant allele is

retained.^{26 31-33} This must also be expected to be the case in the rhabdomyosarcoma used in this study if loss of heterozygosity had occurred. Using the PRO.6 polymorphisms, however, since both parents are heterozygous their daughter could have been constitutionally heterozygous, inheriting the upper allele from the father. Whether their daughter is homozygous for the lower allele or it has been reduced to homozygosity in this case, in the tumour the result is still the same; the tumour predisposition gene cosegregates with the lower allele. From our linkage analysis using the *Bam*HI polymorphism our prediction is that it is the lower allele which cosegregates with the mutant allele. Here loss of heterozygosity is not relevant to the argument since the father is homozygous and must contribute the lower allele to his children. Since the mother also contributes the lower allele, associated with the mutant gene, her daughter could not have been constitutionally heterozygous. Since both parents are heterozygous for the PRO.6 polymorphism, carrier status can only be confirmed or otherwise in 50% of future offspring but results will be unequivocal for the *Bam*HI polymorphism. Taken together these tests will allow counselling of this family in the future.

The use of DNA from formalin fixed tissue in PCR has improved our capability to analyse mutations in Rb families. As long as DNA is available from key dead family members linkage phase can be established. The identification of the specific mutations in apparently sporadic cases will soon be possible via direct sequencing of the amplified DNA. Tumour tissue from many patients now approaching child bearing age exists as fixed material in pathology archives. Using this material and the methods described here, accurate genetic counselling is now available for many of these families.

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