Application of linkage analysis to genetic counselling in families with hereditary retinoblastoma

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SUMMARY Six families with retinoblastoma in more than one member were investigated with DNA markers linked to the retinoblastoma locus because direct analysis had not disclosed the gene defect. In all of the families we could identify the affected chromosome and predict the genetic risk with a high level of confidence (90 to 99%). In one patient the test helped to detect tumour development earlier than usually possible. Several subjects were found not to carry a mutation, thus obviating frequent ophthalmological examinations under anaesthesia as would be necessary otherwise. These results show that linkage analysis can be successfully applied to genetic counselling in families with hereditary retinoblastoma.

Retinoblastoma (Rb) belongs to a group of childhood tumours predisposition to which can be inherited as an autosomal dominant trait.¹ The gene locus has been assigned to the long arm of human chromosome 13, region 1, band 4.² Two genetic events are assumed to be required to initiate tumour formation.³

In hereditary retinoblastoma (40% of cases), the patients carry a germinal mutation at one of the two homologous Rb loci in all their cells. Retinoblastoma develops from a retinoblast that has lost the wild type allele on the homologous chromosome, either by mitotic non-disjunction, mitotic crossing over, gene conversion, deletion, or point mutation during the development of the retina.⁴ Mitotic non-disjunction and crossing over occur in approximately 50% of tumours and lead to hemi- or homozygosity for all or part of chromosome 13.⁵

Patients with hereditary retinoblastoma usually have bilateral or multifocal unilateral retinoblastoma. However, a small number of these patients develop only one tumour or do not have tumour development at all.

In about one-third of patients with hereditary retinoblastoma, the first mutation has been transmitted from an affected parent. In this situation, sibs and offspring have a genetic recurrence risk of 50%. In the other patients with hereditary retinoblastoma, a new mutation has occurred spontaneously in a

parental germ cell. In these families offspring, but not sibs, of the patient are at increased risk.

In about 60% of all retinoblastoma patients tumour formation results from two independent somatic mutations in the same retinoblast. This is non-hereditary retinoblastoma, which is always unifocal and unilateral, and neither sibs nor offspring are at an increased risk.

Unfortunately these different genetic forms of retinoblastoma and the different genetic risks they confer cannot be readily distinguished in clinical practice. This leads to uncertainties about the genetic risk, which may be over- or underestimated.

A predictive test that would distinguish persons destined to develop retinoblastoma from those who will not would obviously be of great help for genetic counselling and early ophthalmological diagnosis. Advances in molecular genetics during the last few years have led to the development of molecular probes for the Rb gene⁶⁻⁸ and other loci on chromosome 13.⁹⁻¹² Some of these probes can detect directly a certain proportion of deletions at the Rb locus,^{6-8 13 14} but most mutations escape direct analysis at present.

In families not amenable to direct analysis of the gene defect, an indirect diagnosis can be achieved if the affected chromosome can be identified through genetic linkage analysis. Only one attempt using this approach to predict the development of retinoblastoma has been reported previously.¹⁵ In the meantime, much closer markers have become

available. We have studied nine families with hereditary retinoblastoma and in three a micro-deletion was directly detectable.¹⁶ Here we describe indirect diagnosis based on linkage analysis in the six families not amenable to direct gene analysis.

Methods

PATIENTS

Families with hereditary retinoblastoma were ascertained either through the Retinoblastoma Clinic of the Department of Ophthalmology or the Genetics Clinic of the Department of Human Genetics, University of Essen, between March 1986 and May 1987. The diagnosis of retinoblastoma had been established by current ophthalmological and histological criteria.

DNA ANALYSIS

Total genomic DNA from whole blood and from retinoblastomas obtained after enucleation was digested with appropriate restriction enzymes. The DNA fragments were separated by gel electrophoresis, transferred to nylon membranes, and hybridised with the appropriate probes as described previously.¹⁴ The strategy for the use of the DNA probes (table) involved two stages. In the first stage, only 13q14 probes were used. Families found not to be informative for any of these markers were then investigated at more distant loci.

RISK CALCULATIONS

Risk estimates were calculated by hand and checked using the LINKAGE programme on a Compaq 286 personal computer.¹⁷ For computer analysis we assumed autosomal dominant inheritance, a pene-

trance of 0.9, a gene frequency of 0.00005, and a sex difference in recombination of 3.8.¹⁸

Results

Six families with retinoblastoma in more than one member were studied by genetic linkage analysis. As shown in the figure, all six families were informative for at least one 13q14 marker or two more distant but flanking markers.

FAMILY 1 (MI-0192)

Subject II.2 is a 25 year old male without any signs of retinoblastoma. However, owing to reduced penetrance he had to be assumed to have a 5% risk of carrying a mutation. DNA analysis revealed that he has inherited from his affected mother the chromosome 13 not carrying the mutation, unless two crossovers have occurred between the two marker loci 7D2 and 9D11. Since the chance of a double crossover is $0.05 \times 0.4 = 0.02$, and only 10% of carriers do not develop the disease, he has a risk of $0.02 \times 0.1 = 0.002$. Thus, his actual risk of being a carrier and transmitting the mutation is much lower (approximately 0.2%) than the original estimate based on formal genetics. This information is important for his family planning.

FAMILIES 2,3, AND 4 (FE-0006, MO-0155, AND OL-0162)

These three families, who have small children with retinoblastoma, have asked about the risk for future children and the availability of a predictive genetic test. Family FE-0006 is informative for the intragenic H3-8 *Hind*III restriction fragment length polymorphism.¹⁴ The mutation segregates with

TABLE Details of the probes used.

Probe	Chromosomal localisation	Recombination fraction used for counselling			Enzyme	PIC	Ref No
		Males	Females	Combined			
Hu10	13q12-14	0.1	0.3		<i>Xmn</i> I	0.29	18
					<i>Eco</i> RI	0.25	18
7F12	13q12-14	0.07	0.21		<i>Taq</i> I	0.36	18
					<i>Msp</i> I	0.36	18
G14E1.9	13q14			0.1	<i>Dra</i> I	0.34	11
7D2	13q14			0.05	<i>Taq</i> I	0.14	18
					<i>Dra</i> I	0.35	19
ESD	13q14			0.01	<i>Ap</i> aI	0.27	12
					<i>Ban</i> II	0.35	*
				0.01	<i>Hind</i> III	0.05	14
H3-8	13q14				<i>Bgl</i> II	0.18	18
Hu26	13q22	0.18	0.4		<i>Taq</i> I	0.24	18
9D11	13q22	0.18	0.4		<i>Msp</i> I	0.37	18
1E8	13q31-34	0.2	0.47		<i>Msp</i> I	0.37	18
HuB8	13q31-34	0.2	0.47		<i>Eco</i> RI	0.24	18
					<i>Hind</i> III	0.28	18

*J Squire, 1987, personal communication.

PIC=polyorphism information content.

FIGURE DNA analysis in six families with hereditary retinoblastoma. The results for the closest informative loci only are given. In subject II.4, family WE-0078, the association of ESD allele 1 with Rb+ was inferred from other markers which are not shown in the figure. Solid symbols indicate affected subjects. T1, T2, tumours; Rb+, wild type allele; Rb-, mutant allele. The arrows indicate the subjects in whom DNA diagnosis was performed.

allele 2, and the absence or presence of this allele in a subsequent offspring would predict the absence or presence of the mutation. Since recombination between the mutation and the polymorphic site in the Rb gene is very unlikely, the confidence interval of a diagnosis would be about 99%.

In families MO-0155 and OL-0162, tumour material was also available for DNA analysis. Although knowledge of the tumour genotype was not essential for the diagnosis in these two cases, the loss of constitutional heterozygosity in two tumours allowed the linkage phase to be established easily. Taking the chance of a double crossover between probes 7F12 and HuB8 into account, the confidence interval of a DNA diagnosis in family MO-0155 would be 98%. Since family OL-0162 is informative for the closely linked markers ESD and H3-8, here

the confidence interval of a DNA test would be 99%.

Interestingly, one of the two tumours present in the left eye of subject II.1, family MO-0155, had maintained constitutional heterozygosity (T1), whereas heterozygosity had been lost in the other tumour (T2). This proves that different somatic mutations have led to the formation of these two tumours.

FAMILIES 5 AND 6 (WE-0078 AND ES-0083)
These two families had newborn babies and requested a predictive DNA test on umbilical cord blood. Family WE-0078 was informative for the closely linked marker ESD. Child III.1 was found to have inherited the affected chromosome at a confidence level of 99%. He was first examined four

weeks after birth and found to have two small tumours in his left eye. The presence of another tumour in his right eye was suspected at that time. Three weeks later a small tumour had developed there. The tumours were successfully treated by light and cryocoagulation, but the patient developed several new tumours, and external beam radiation was necessary to stop tumour growth.

Family ES-0083 was informative for the marker G14E1.9 and the mutation was unlikely to be present in the subject II.3 at a confidence level of about 90%. Up to the age of one year the child has not developed any signs of a retinoblastoma.

Discussion

Using cloned DNA probes linked to the retinoblastoma gene locus on chromosome 13, we have been able to offer predictive genetic tests to six families with hereditary predisposition to this disease. In most cases the confidence level was close to that of direct gene diagnosis, which we have recently performed in three other families.¹⁶ Although probes and techniques are now being developed for detecting point mutations within a gene,^{20,21} most mutations at the Rb locus cannot at present be detected directly. Furthermore, owing to a high mutation rate¹ and negative selection, the mutations are expected to be heterogeneous. In contrast to many haemoglobin mutants, heterogeneity will make it difficult to apply the same approach in many families. Thus, linkage analysis will remain useful in many families despite its drawbacks, such as the need for a full family analysis and the risk of recombination between the marker and the retinoblastoma locus. The latter problem, however, will be overcome as more intragenic RFLPs are being identified.

In the families studied here, a minimum of two subjects were affected by the disease. Indirect DNA diagnosis can also be performed in families with only one affected member provided the patient has bilateral or multifocal unilateral retinoblastoma (that is, carries a germinal mutation) and has at least one child that has passed the age of onset (usually two to three years of age). However, owing to reduced penetrance of mutations at the Rb locus, there is a 10% risk that such a child does carry the mutation, thus rendering indirect DNA diagnosis less accurate in these cases. If tumour DNA can be obtained (from fresh material, cultured cells, or histological specimens), and it is found to be hemi- or homozygous for part or all of chromosome 13, the affected chromosome can be identified directly. This would improve the confidence in diagnosis in these cases and make it possible to perform the diagnosis

in the first child also. Therefore, every effort should be made to save tumour material.

DNA diagnosis in retinoblastoma, either by direct or by indirect analysis of the gene defect, can be helpful for retinoblastoma families in several ways. Some families with several severely affected patients may wish to consider prenatal diagnosis. Newborn infants and young children carrying a predisposing germinal mutation could be examined every two to four weeks immediately after birth. Children found not to be at risk could be spared unnecessary ophthalmological examinations, which in very young children require general anaesthesia. Finally, clinically unaffected sibs and offspring planning to start a family of their own may want to know that they really do not carry the mutation at the Rb locus. Our results show that these questions can now be answered in many families with a relatively high level of confidence.

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