

Molecular Studies of Loss of Heterozygosity in Retinoblastoma

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Abstract

Retinoblastoma (Rb) is a paediatric intraocular tumour in which predisposition can be inherited. Cases of Rb tumours can be divided into three types: familial cases, sporadic bilateral cases and sporadic unilateral cases. Familial and sporadic bilateral cases are usually categorised as hereditary while sporadic unilateral cases as non-hereditary. In both familial and non-familial forms of Rb, loss of heterozygosity of the Rb locus has been reported although its frequency in tumours has not so far been accurately determined. The cloning of the gene responsible for retinoblastoma (Rb1) has facilitated DNA studies and genetic counselling of patients. We have examined forty-five cases of retinoblastoma at five intragenic sites of the Rb1 locus, namely intron 1/BamHI, intron 17/XbaI, intron 24/TthIII, intron 25/DraI and Rb1.20 VNTR. Thirty-six out of the forty-five cases (80%) were informative for these markers. Comparison of results between genomic DNA from peripheral blood and from tumours revealed that loss of heterozygosity of alleles could be detected in 50% of cases studied in which tumour samples were available. Investigation of parental origin of retained alleles showed that in all these cases, the paternal alleles were preferentially retained. The analysis of the genetic origin of mutations predisposing to retinoblastoma can facilitate new approaches for identifying recessive mutant genes that lead to cancer as well as to provide a conceptual basis for accurate prenatal predictions of cancer predisposition.

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Introduction

Retinoblastoma (Rb) is a malignant tumour which arises in the retinal cells. It is the most common intraocular childhood cancer with an onset usually before four years of age. It affects 1 in 20 000 newborns. Most cases of retinoblastoma are sporadic and occur due to non-hereditary somatic mutations. However, 40% are hereditary and show autosomal dominant inheritance. Hereditary cases of retinoblastoma are due to germ-line mutations, with 10% being inherited and 30% being newly transmitted.

The retinoblastoma gene (Rb1) is a recessive gene which is located at chromosome 13 band q14. It was first

cloned in 1986¹ and is more than 200 kb in size with an mRNA transcript of 4.7 kb. Mutations in the Rb1 gene results in non-functional gene product which causes retinoblastoma. It has been reported that mutations in the Rb1 gene also play an important role in development of other cancers e.g. small cell lung cancer,^{2,3} bladder cancer,^{4,5} and breast cancer.^{6,7}

Retinoblastoma arises as a consequence of the inactivation of both alleles in Rb1 locus as proposed by Knudson⁸ and Comings.⁹ Thus complete loss or inactivation of the Rb1 gene is associated with tumorigenesis. In all bilateral cases, the first mutation is present in the germ-line whereas in unilateral cases, it occurs in a

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somatic retinal cell. The second mutation is always a somatic event. The phenomenon of loss of heterozygosity (LOH) as a somatic event in mammalian cells was first reported in Rb tumours.^{10,11} Both hereditary and non-hereditary cases undergo LOH, resulting in the retention of mutant allele and loss of normal allele.¹² LOH may be the process that actually initiates cancer.¹³

Thus, by comparing the constitutional cell (genomic) and tumour genotypes with the use of restriction fragment length polymorphisms DNA markers in the Rb1 gene, it is possible to determine whether LOH involved the whole chromosome, or all or only part of the alleles. Total LOH is said to occur when the whole chromosome or all of the Rb1 alleles are absent, while partial LOH is due to the absence of only part of the alleles. Homozygosity or heterozygosity within the Rb1 locus can thus be determined.^{10,14,15}

The objective of this study was to use markers in the Rb1 gene to determine LOH by comparing their presence in both genomic and tumour DNA samples through linkage analysis using five markers, namely intron 1/*Bam*HI, intron 17/*Xba*I, intron 24/*Tth*III1, intron 25/*Dra*I and Rb1.20 VNTR. These markers were chosen because they were known to be highly polymorphic^{16,17} and could be analysed easily by the polymerase chain reaction (PCR) technique.

Materials and Methods

Patients

A total of forty-five retinoblastoma patients were studied. Of these, fourteen cases were provided with tumour samples. These included three bilateral cases and eleven unilateral cases. All of these patients did not have other affected siblings or a family history of retinoblastoma.

Genomic DNA Preparations

Ten millilitres of peripheral blood were collected under aseptic techniques into ethylenediaminetetra-acetic acid (EDTA) anticoagulant tubes. The genomic DNA was extracted using standard phenol-chloroform method. The DNA was dissolved in Tris/EDTA buffer and stored at 4°C for further analysis. For polymerase chain reaction work, DNA was diluted to 100ng/μl.

Linkage Analysis

Amplification by polymerase chain reaction (PCR) was carried out for markers intron 1/*Bam*HI,¹⁸ intron 17/*Xba*I,¹⁹ intron 24/*Tth*III1,¹⁹ and intron 25/*Dra*I²⁰ in 50 μl reaction volume consisting 200 ng DNA, 50 pmol of each of the forward and reverse primer, 10X Taq-polymerase buffer (500 mM KCL, 100 mM Tris-HCL, 1% Triton X-100), 0.4 mM of each of the dNTPs and 1 unit of Taq-polymerase. The reaction mix was overlaid with 1 to 2 drops of mineral oil. Amplification was

performed using the following conditions: denaturation at 94°C for 1 minute, annealing at 52°C to 63°C for 2 minutes and extension at 72°C for 2 minutes. After 35 PCR cycles, the products were digested by appropriate endonucleases using conditions specified by manufacturer. The digested DNA fragments were resolved by electrophoresis on 3% NuSieve gels. The Rb1.20 VNTR²⁰ consists of variable number of {CTTT(T)}_n (n = 14-26) repeats and occurs 54 bp from the 3' end of the exon 20. The 5' primer was first end-labelled using radioisotope γATP³³ and T4 polynucleotide kinase. PCR was then carried out using similar conditions above. The PCR products were then resolved on 6% denaturing polyacrylamide gels containing 6M urea. The gels were then fixed and exposed to autoradiography.

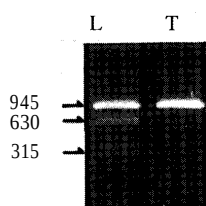
Results

In thirty-six out of the forty-five patients, it was found that the maternal and paternal origin of the alleles in each genotype could be distinguished using the five markers tested. Eighty per cent of these patients were thus informative for these markers. Eight out of the fourteen tumours available from the retinoblastoma patients showed a LOH at different loci when the constitutional and tumour genotypes were compared. The PCR-amplified region in intron 17 contains a restriction fragment-linked polymorphism exhibiting an *Xba*I site in one allele. In the heterozygous condition, PCR-amplification of this region followed by digestion with *Xba*I resulted in one large DNA fragment of 945 bp, as well as two other fragments of molecular weights 630 bp and 315 bp. This region was informative in two of eight cases (Fig. 1). The PCR-amplified region in intron 1 contained a *Bam*HI site. In the heterozygous condition, PCR-amplification of this region followed by digestion with *Bam*HI resulted in three DNA fragments of 180 bp, 130 bp and 50 bp. This region was informative in one of eight cases (Fig. 1). PCR-amplification of intron 25 region followed by digestion with *Dra*I resulted in fragments of 167 bp, 129 bp and 38 bp in heterozygous condition. This restriction site was informative in five of eight cases studied (Fig. 1). Figure 1 also shows the analysis in the Rb1.20 VNTR region in which several alleles representing several polymorphic repeats can occur. Resolution of these PCR products on 6% polyacrylamide gels revealed two alleles in the heterozygous condition. Loss of heterozygosity was observed in all cases. Among these three cases, only one was a bilateral case.

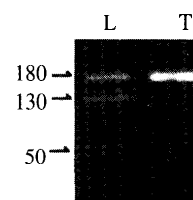
Figure 2 shows the pedigree analysis of the cases studied. In all these cases, there was a preferential retention of the paternal alleles.

Discussion

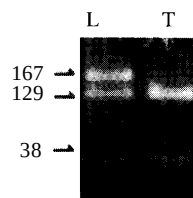
The mechanisms for LOH in Rb1 include mitotic non-



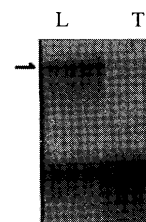
(a) Digestion of the 945 bp polymerase chain reaction (PCR) products in the heterozygous condition by *Xba*I results in three bands of 945 bp, 630 bp and 315 bp.



(b) Digestion of the 180 bp PCR products in the heterozygous condition by *Bam*HI results in three bands of 180 bp, 130 bp and 50 bp.



(c) Digestion of the 167 bp PCR products in the heterozygous condition by *Dra*I results in three bands of 167 bp, 129 bp and 38 bp.



(d) PCR amplification of a region containing a variable number of bp repeats in intron 20 of the *Rb* locus. The position of the missing allele is indicated by an arrow.

Fig. 1. Heterozygosity at the retinoblastoma (*Rb*) locus at polymorphic regions of intron 17(a), intron I(b), intron 25(c) and intron 20(d). Loss of one allele is seen in tumour DNA (T) as compared to lymphocyte DNA (L).

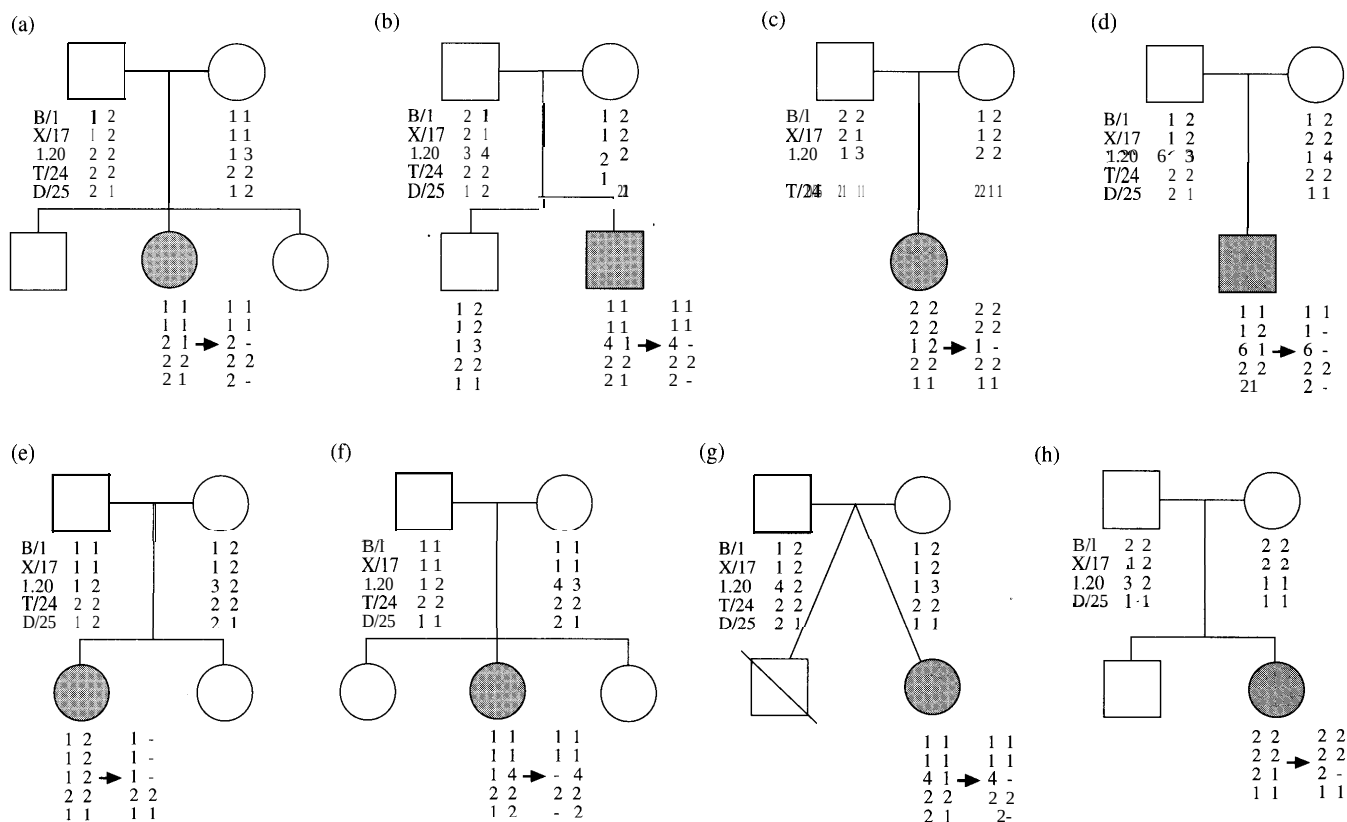


Fig. 2. Pedigree analysis of families in which loss of heterozygosity was found in tumour DNA as compared to the lymphocyte DNA. Loss of maternal allele was only found in family (f), the remaining had lost the paternal alleles.

disjunction with loss of the wild type chromosome resulting in hemizyosity at all loci on chromosome 13; non-disjunction and reduplication of the mutant chromosome resulting in homozygosity at all loci on the chromosome; mitotic recombination between the Rbl locus and the centromere, resulting in heterozygosity at loci in the proximal region of the chromosome and homozygosity throughout the rest including the Rbl locus; and also some other more regionalised event like gene inactivation, deletions and point mutations.^{10,21}

Forty-five cases were examined at five intragenic sites of Rbl locus and thirty-six were found to be informative. Eight out of the fourteen tumours showed partial LOH of alleles as there was absence of some of the alleles in the tumour DNA. The remaining cases which did not show LOH could be due to the non-informativeness of the five intragenic markers used in this study. Of the eight Rb cases that were informative in LOH, only one involved bilateral tumours and in this case the paternal allele was retained. Of the remaining seven cases that were all unilateral, it is interesting to note that six cases preferentially retained the paternal allele. The significance of this has been postulated to be linked to the tumour suppressor function of the Rbl wild type gene. Loss of wild type maternal allele would activate the mutation on the paternal allele. A higher mutation rate is thought to occur in the paternal alleles due to the larger number of cell divisions involved in spermatogenesis than in oogenesis,²² and also weaker paternal age effect.^{23,24} There have been previous reports that there was no evidence that the paternal Rbl allele is preferentially retained in non-hereditary retinoblastoma although for hereditary cases, there is a preferential retention of paternal allele.^{25,26} A study of LOH on chromosome 13 in relation to the age at operation for tumour removal of Rb patients with non-hereditary Rb tumours was also reported by Kato and coworkers.²⁷ They found that the age of patients at the time of operation in cases of tumours with loss of paternal allele was significantly lower than the age of patients at operation for removal of tumours with loss of maternal allele. Such study may help in the understanding of the delayed development of retinoblastoma associated with loss of maternal allele in chromosome 13. It has also been previously reported that the frequency of LOH on chromosome 13 in non-hereditary Rb tumours is significantly higher than that in hereditary Rb tumours, suggesting that LOH could be the initial somatic event in the tumorigenesis of non-hereditary tumours.²⁸ Preferential retention of paternal allele has also been reported in other cases such as osteosarcoma.^{29,30} Although the number of informative tumour cases in this study is small, the detection of the phenomenon of LOH in these cases confirms the concept of loss or inactivation of both wild type alleles for tumorigenesis to occur in retinoblastoma. It should prove

useful to study the mechanisms of LOH in all the above cases to determine whether partial or whole chromosome loss had occurred by using markers flanking the RB1 locus and other extragenic polymorphic sites on chromosome 13.

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