

ELECTRONIC LETTER

A T to C mutation in the polypyrimidine tract of the exon 9 splicing site of the *RB1* gene responsible for low penetrance hereditary retinoblastoma

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Inactivation of both alleles of the retinoblastoma susceptibility gene (*RB1*), localised on chromosome band 13q14, is the crucial event for the development of retinoblastoma, a malignant tumour that originates from embryonal retinal cells.^{1,2} Germinal mutation of one allele leads to a predisposition to retinoblastoma. Tumour development is initiated by inactivation of the second allele. In most families, the tumour predisposition segregates as an autosomal dominant trait with high (90%) penetrance³ and affected subjects usually develop bilateral multifocal tumours. Rare instances of familial retinoblastoma with low penetrance and variable expressivity have been described. In such families, patients develop retinoblastoma later than classical familial cases, there is a high rate of unilateral cases, and phenotypically normal obligate carriers are observed.⁴

METHODS AND RESULTS

A family with several cases of retinoblastoma has been identified (fig 1). Four members of the family were affected; three had unilateral retinoblastoma (III.5, IV.12, and IV.13), diagnosed at 12, 12, and 24 months, respectively, and one had bilateral retinoblastoma (IV.6), which developed at 11 months. Thus, with the exception of patient IV.13, the age of onset was not delayed compared to an average of less than 12 months in the majority of familial retinoblastoma. There were no cases of spontaneous osteosarcoma in this family, a secondary tumour recurrently found in patients with predisposition to retinoblastoma.⁵ However, the retinoblastoma of

patient IV.6 was treated by radiotherapy (45 Gy) and 10 years later an osteosarcoma occurred in the irradiated field. It is well established that a germline mutation in the *RB1* gene dramatically increases the probability of developing a radiation induced osteosarcoma.⁶ After ophthalmic examination of unaffected family members, no case of retinoma/retinocytoma, which may represent benign proliferation or tumour regression, was found. Altogether, these observations suggest the presence of a germline *RB1* mutation linked to incomplete penetrance of the tumour predisposition. To aid genetic counselling, the characterisation of this mutation was performed.

The germline mutation of patient IV.6 was investigated by denaturing gradient gel electrophoresis (DGGE) using RNA from peripheral blood (experimental conditions are available on request). For 13 of the 14 segments covering the entire coding sequence, no heteroduplex band was observed, indicating the absence of mutation (not shown). For the region covering exon 9, two RT-PCR products were observed, the wild type (320 bp) and a mutant (242 bp) (fig 2A). The sequencing of this mutant product showed the deletion of exon 9. The removal of this 78 bp exon creates an in frame mRNA lacking codons 287 to 313. To determine whether the RNA deletion was the result of a genomic deletion or a splicing mutation, the genomic DNA spanning exon 9 was sequenced. A heterozygous T→C transition at position -10 in intron 8 was observed (fig 2B). The mutation is located in the polypyrimidine tract of the 5' acceptor splicing site of exon 9.

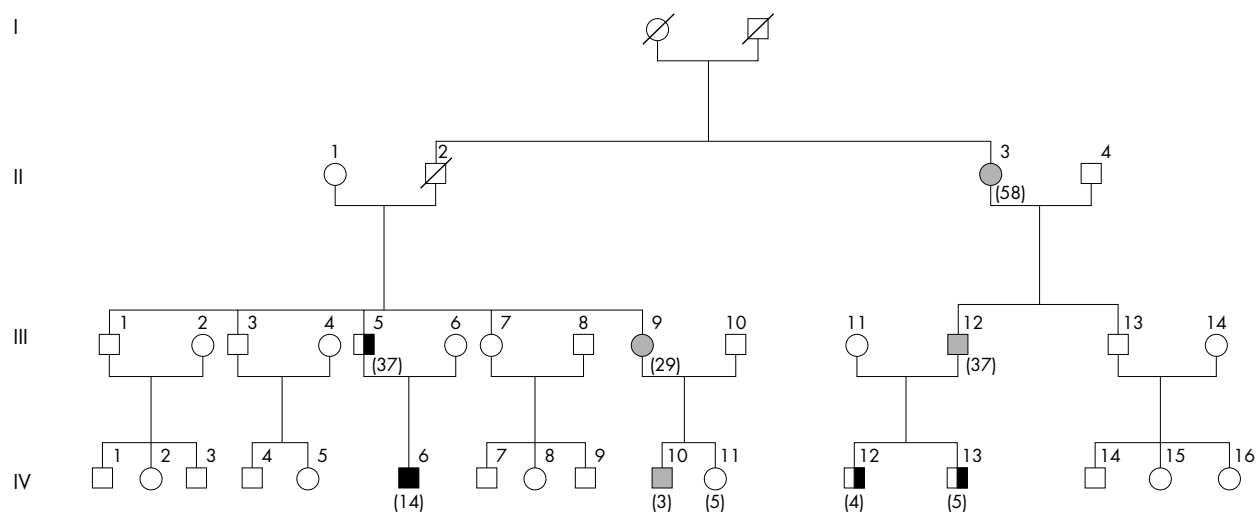


Figure 1 Pedigree of the family. Half blackened symbols denote unilateral retinoblastoma and completely blackened symbol denotes bilateral retinoblastoma. Shaded symbols denote unaffected carriers. Values in brackets indicate the present age of the subject (in years). Samples were obtained after informed consent from the patients or parents.

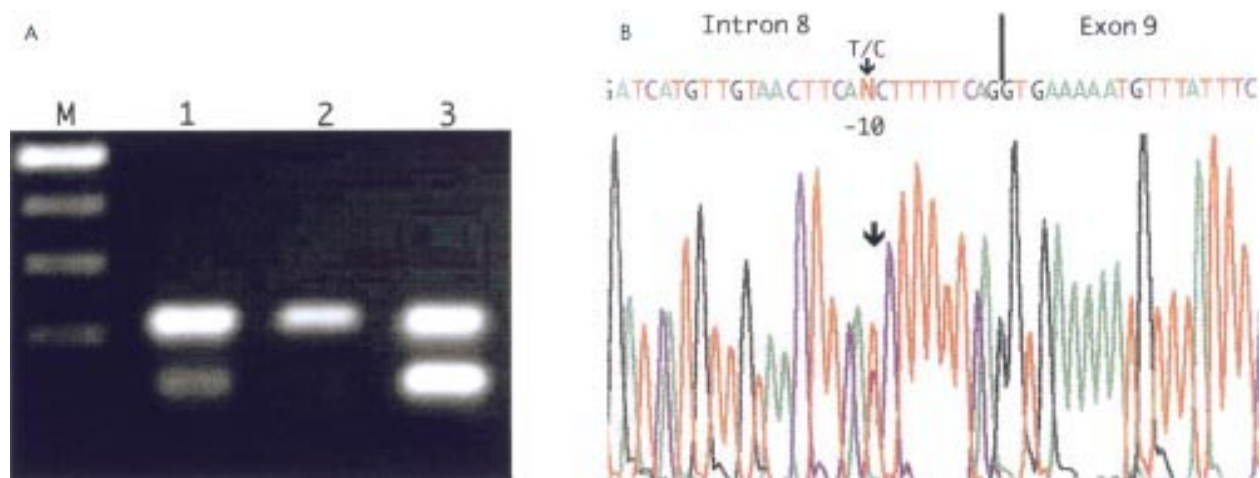


Figure 2 (A) RT-PCR analysis of mRNA. (1) Patient IV.6 peripheral blood. (2) Healthy control donor peripheral blood. (3) Patient IV.12 retinoblastoma. cDNA was prepared by reverse transcription using random oligonucleotide priming and the Superscript II System (Invitrogen) according to the manufacturer's protocol. RB1 cDNA was amplified with primers (5'-TTCATCAAGGTTACTTTTCGTTGTT CT-3' and 5'-AGAAATTATTGAAGTT CTCTG-3'). PCR conditions were 40 cycles at 94°C (30 seconds), 55°C (30 seconds), and 72°C (50 seconds). PCR products were analysed on a 1% agarose gel in the presence of ethidium bromide. M: molecular weight marker. The additional band observed in 1 and 3 corresponds to the deletion of exon 9. (B) Partial sequence of the genomic DNA from patient IV.6 in the intron 8-exon 9 region. DNA from peripheral blood was amplified with primers (5'-TGCATTGTCAAGAGTCAAG-3' and 5'-TTCACCACAATTCTACTTGG3'). The heterozygous mutation was observed at position -10 in intron 8. An identical result was obtained for patient IV.12 (not shown).

This T→C transition was previously found in the retinoblastoma DNA of patient IV.6 and interpreted as a polymorphism.⁷ The mutation was also found in the radiation induced osteosarcoma of patient IV.6 and in the retinoblastoma of patient IV.12. The exon 9 deleted mRNA was present in the retinoblastoma (fig 2A), but could not be studied in the osteosarcoma for which no mRNA was available. No other mutation was found, either by screening the gene using denaturing high performance liquid chromatography (DHPLC), multiplex PCR, or sequencing the whole cDNA and the promoter region of the gene (not shown). In addition, this mutation was not found by DHPLC in 200 DNA samples from unrelated healthy donors (not shown).

The mutation was searched for in the peripheral blood of all the living members of the family (fig 1). All affected members (III.5, IV.6, IV.12, and IV.13) and two living unaffected obligate carriers (II.3 and III.12) had the mutation and expressed the exon 9 deleted mRNA. In addition, an unaffected member (III.9) and her 3 year old unaffected son (IV.10) were also carriers and expressed the mutated mRNA. No relevant differences were observed in the level of expression of the two alleles between affected patients and healthy carriers. The other members of the family displayed only the wild type allele. Taking into account the obligate carrier II.2, who had died, and one obligate carrier in the first generation, the disease-eye ratio (DER) score, defined as the ratio of the number of diseased eyes and the number of subjects at risk, was 0.5. This value is among the lowest reported for low penetrance familial retinoblastoma.⁸ Thus, the mutation in intron 8, which segregates with the affected members of the family via unaffected obligate carriers, displays the characteristics of a predisposing mutation.

DISCUSSION

In families with low penetrance retinoblastoma, a high rate of in frame deletions, missense mutations, and mutations in the promoter is observed, whereas in the majority of the families with germline mutations, nonsense and frameshift mutations are mainly found.⁹ It has been suggested that the mutated genes encode an altered RB1 protein that leads to reduced or deregulated levels of wild type protein or to a defective protein, which retains partial wild type activity.^{8 10-12} In our case, a similar situation seems to exist. When translated, the

mRNA creates an in frame protein (Δ 9pRB1) lacking 26 amino acids from the N-terminal region. Published data suggest that the amino terminus of the RB1 protein (pRB1) plays an important role as a site for interactions with targets or regulators of pRB functions.^{13 14} However, contradictory data exist about the properties of the N-terminally truncated pRB1. In several experimental systems, the truncated proteins have enhanced growth suppressor activity compared to wild type protein.^{15 16} On the other hand, studies using transgenic mice indicate that N-terminally partially deleted RB1 proteins are insufficient for complete tumour suppression.¹⁷ Furthermore, another family with low penetrance retinoblastoma, with a deletion encompassing exon 4, has been described.¹⁸ Thus, N-terminally truncated pRB1, such as Δ 9pRB1, must be unable to provide the tumour suppressor function of the complete protein in some cases. However, the actual effect seems to depend on the biological context and could be related to the availability of possible tissue specific associated factors. In our case, it is not at present possible to establish if the truncated mRNA species has lost the RB1 wild type function or expresses a partially defective RB1 product.

The T→C mutation, localised in the polypyrimidine tract of the 5' acceptor splicing site of exon 9, is expected to be at the origin of the deletion of the exon. This polypyrimidine tract is required for efficient spliceosome assembly and efficient splicing of the pre-mRNA.¹⁹ It has been shown that the deletion of one T, or its substitution by another base in the tract, is sufficient to strongly decrease or abolish the recognition of the splicing site and prevent the splicing of the exon.²⁰⁻²² In our case, the presence of the full length and truncated mRNA species in both normal tissues and in the retinoblastoma does not allow us to establish whether the mutated allele is partially expressed with correct splicing. The biological relevance of such a mutation has been shown in only a few cases of genetic disease.^{23 24} Three mutations, in addition to the present one, have been described in a polypyrimidine tract in families with low penetrance retinoblastoma.⁹ In two of these cases, no other mutation was identified in addition to the intronic mutation. These three mutations were assumed to be non-pathological variants, since it was not possible to establish their actual role in retinoblastoma predisposition owing to the lack of available RNA.²⁵ It is noticeable that, as for the mutation in intron 8 described in this paper, mutations in the

polypyrimidine tract of intron 14 (two cases) and intron 21 (one case) could be at the origin of deletion of exons 15 and 22, respectively.²⁵ This may give rise to in frame deletions in the corresponding mRNA and to the formation of truncated inactive proteins. Thus, these three mutations may also be the actual predisposing mutations in these families with low penetrance retinoblastoma. We conclude that intronic mutations in the polypyrimidine tract of *RB1* exons must be looked for and taken into consideration in the screening of families with low penetrance retinoblastoma.

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