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Original Paper

Constitutional Nonsense Germline Mutations in the *RB1* Gene Detected in Patients with Early Onset Unilateral Retinoblastoma

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The 'two-hit' hypothesis for the development of the childhood eye cancer, retinoblastoma (Rb), predicts that bilaterally affected individuals will carry germline mutations. The second suggestion is that patients with early presentation of unilateral tumours also carry predisposing mutations. We have used SSCP analysis to study the 27 individual exons of the *RB1* gene in constitutional DNA from 3 patients whose tumours were treated under the age of 12 months. Bandshifts on SSCP gels were detected in 2 of these patients which, on sequencing, were shown to be a C → T transition converting a CGA_{arg} to a TGA_{stop} codon in exon 17 and an 8 bp deletion in exon 20 resulting in a downstream stop codon. The mutations seen in these patients are reminiscent of those seen in patients with hereditary Rb and confirms that at least some early onset unilateral cases carry constitutional mutations, which has important implications for genetic screening and counselling of these individuals. Copyright © 1996 Elsevier Science Ltd

Key words: retinoblastoma, *RB1*, gene mutations, unilateral Rb, early onset, genetic counselling

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INTRODUCTION

INDIVIDUALS PREDISPOSED to the development of the children's eye cancer, retinoblastoma (Rb), carry heterozygous germline mutations in the *RB1* gene [1–4]. Approximately 10–15% of patients already have a family history of tumours where the malignant phenotype segregates as an autosomal dominant trait [5, 6]. In familial cases, it is possible to use polymorphic DNA markers [1, 7] to follow the inheritance pattern to determine who is carrying the predisposing mutation [7, 8], and to offer prenatal screening to 'at risk' individuals [9, 10]. The remaining 85–90% of patients, however, present as apparently sporadic cases and, in the absence of a family history, it is not possible, using linkage studies, to determine who is carrying germline mutations and who is not. In a mathematical treatise of Rb, Knudson [11] formulated a two-hit hypothesis which, with modification in subsequent years [12, 13], predicted that carriers of germline mutations would develop multiple, bilateral tumours at an earlier age than would sporadic cases, the majority of whom would present with a single tumour focus in one eye only. The reasoning behind this hypothesis is that, by inheriting a mutation in the *RB1* gene, only a single additional mutation in the normal, homologous gene must occur in an immature retinal cell for tumour initiation. Since the chances of this second random mutation occurring is high, then more than one cell in the developing retina will be affected and tumorigenesis begins immediately, accounting for the early age of onset. However, we know empirically that, occasionally, in Rb families, predisposed individuals only develop a single tumour, although usually at an earlier age [5, 6]. This is explained by the fact that, because of its random nature, the second hit follows a Poisson distribution allowing for single events and, in rare cases, for no second mutation at all which is described as "incomplete penetrance". On the basis of this hypothesis, all bilaterally affected individuals should carry constitutional heterozygous predisposing mutations in the *RB1* gene, and population screening programmes have given preliminary in-

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dications that this is true [3, 4, 14]. The second prediction of the two-hit hypothesis is that patients with an early age of onset of tumours, or more accurately an early age of detection, are also likely to be carriers of germline *RB1* mutations. The mean age of diagnosis for bilateral/hereditary tumours is 5–7 months compared with 24–30 months for sporadic cases [6]. This suggestion has important implications for the genetic screening and counselling of *RB1* patients since, if those with early diagnosis of unilateral disease account for the majority of the hereditary unilateral cases, then screening efforts can be concentrated on them. In this report, we demonstrate the presence of constitutional mutations in the *RB1* gene in a proportion of patients diagnosed early with unilateral Rb.

MATERIALS AND METHODS

DNA was prepared from isolated peripheral blood lymphocytes of 3 patients whose tumours were treated under the age of 12 months, using standard phenol chloroform extraction procedures [15]. The individual 27 exons and the promotor region were amplified using the primers described by Hogg and colleagues [16] and subjected to SSCP as described previously [16]. To reduce their size, PCR products were digested with selected restriction enzymes which produced DNA fragments of 250 bp or less [16]. The restriction enzymes were added directly into the PCR reaction and left at 37°C (65°C for Taq 1) overnight. The initial PCR reaction and the digested DNA were analysed on 2% agarose gels and SSCP gels were only run if the initial PCR product was free of non-specific bands and complete digestion could be demonstrated. DNA fragments showing altered mobility when compared with adjacent samples were sequenced by separating single stranded DNA using streptavidin coated magnetic beads and Sequenase as described by Hogg and colleagues [16].

RESULTS

Of the three cases examined, two showed abnormal bandshifts on SSCP gels. On sequencing, these were demonstrated to be a C → T transition in exon 17 converting an arginine codon to a stop codon (Figure 1) and an 8 bp deletion in exon 20 (Figure 2). The arg → stop mutation does not affect a restriction enzyme site and so is only detectable using SSCP, and is one of the most common mutations found in patients with germline mutations [14]. Included in the 8 bp deletion is an insertion of a T nucleotide, and so the deletion involves only 7 bp of the normal sequence (Figure 2) and results in the generation of a downstream stop codon, which is again one of the class of mutations frequently seen in patients with a hereditary predisposition to Rb. Thus, we have confirmed the prediction that these patients carry those types of mutation which are part of the spectrum seen in patients predisposed to Rb and which will normally lead to a more severe form of the disease in subsequent generations.

DISCUSSION

During the past ten years, we have offered a genetic screening service for Rb patients in the U.K. Until the cloning of the *RB1* gene, this effort concentrated on the quantitative analysis of the levels of the esterase D enzyme which was designed to detect constitutional 13q14 deletions [17, 18] in both affected individuals and their families. During this programme, samples from over 500 patients were recruited to the study, which was later extended to linkage analysis, where there was a family history of tumours [19] and, more recently, mutation analysis in patients with bilateral or hereditary disease [2, 14, 16, 20]. Over half the patients recruited to the original studies were only unilaterally affected and showed no evidence of a family history. Since the mutations which give rise to Rb occur as random somatic events (a single event in hereditary cases and both events in sporadic cases), it would be expected that there will be patients with late presentation of single unilateral tumours who carry germline mutations as well as sporadic cases which have early presentation of tumour. Draper and colleagues [6] presented an extensive survey where the age of detection of tumours in unilaterally affected individuals

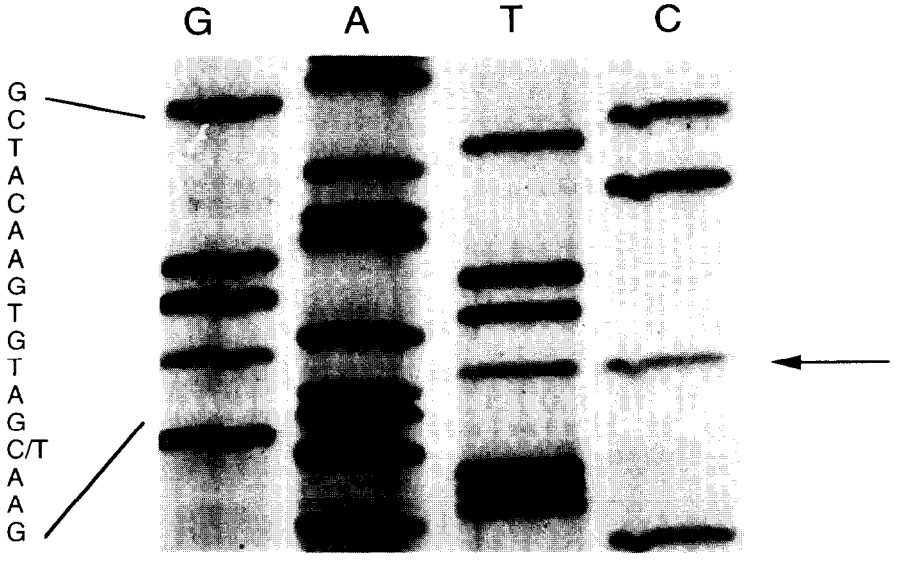

Figure 1. DNA sequencing ladder from exon 17 showing the heterozygous C → T change resulting in an arg → stop codon change.

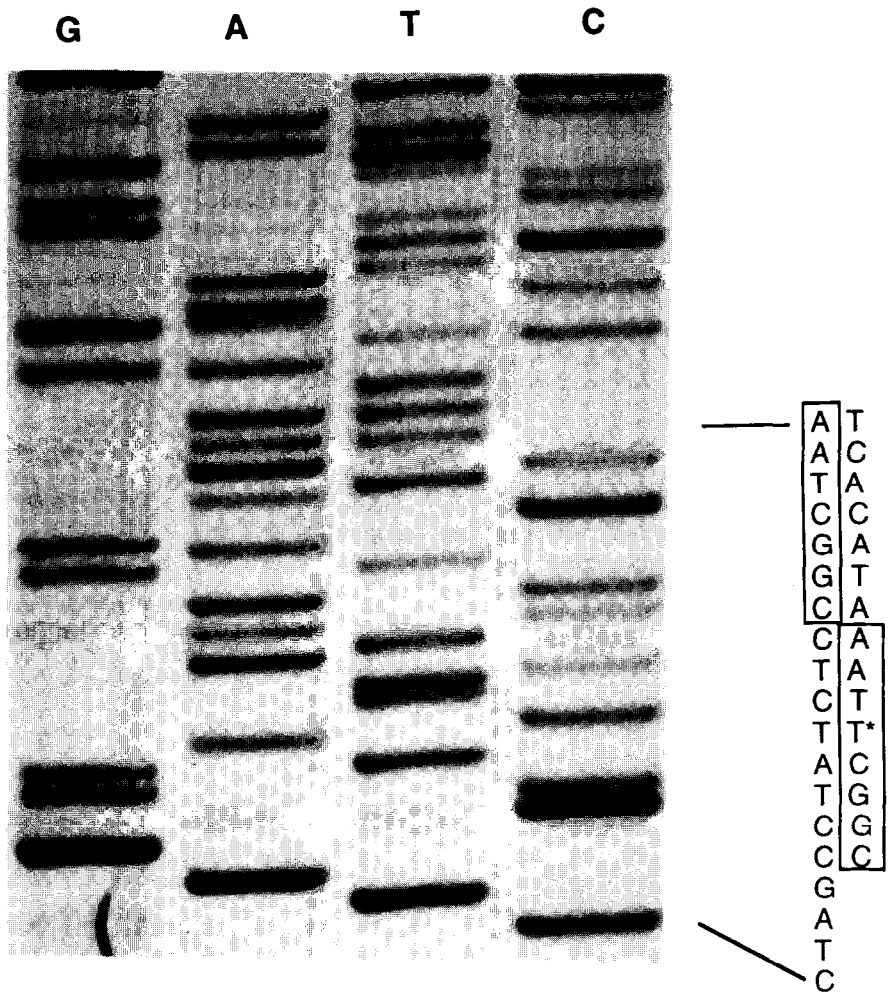

Figure 2. DNA sequencing ladder from exon 20 showing an 8 base pair deletion. Because this mutation is heterozygous, both sequencing ladders are superimposed on each other, one lagging by 8 bp. In the generation of the deletion, an insertion of a 'T' nucleotide has also occurred (shown by * on the right).

with a family history of Rb was compared with that of sporadic cases with unilateral Rb. Of the familial cases, 30% were detected before 6 months with 61% presenting before 18 months. By contrast, of the sporadic cases, only 5% presented before 6 months and 28% before 18 months. Since we wanted to test the hypothesis that it was the early presentation unilateral cases which might preferentially carry germline mutations, we selected those individuals who presented before 12 months to screen for *RB1* mutations. When the age of diagnosis was analysed in our series, we could only identify 3 patients who presented with a tumour under the age of 12 months, with the vast majority showing late onset (>24 months) of tumour development. This observation is in keeping with the estimate that only 2% of unilaterally affected patients will have affected children and hence must carry germline mutations [6]. To determine whether any of the 3 patients in our series with early diagnosed tumours carried a germline mutation, we used the single strand conformational polymorphism (SSCP) technique [21] to search for mutations in each of the 27 exons of the *RB1* gene which we have already been used successfully in the analysis of the *RB1* gene in tumours [20] and hereditary cases [2, 14, 16]. The 3 patients in our series whose tumours were identified early have been followed for more than 10 years and none of them subsequently developed a tumour in the contralateral eye.

We confirmed the prediction that these patients carry the types of mutation which are part of the spectrum seen in patients predisposed to Rb and which will normally lead to a more severe form of the disease in subsequent generations. For these families, this is an important distinction to make since there are other rare families where the majority of affected individuals may only have a single tumour or only demonstrate other "mild" forms of the disease, such as regressed tumours or incomplete penetrance [19]. In these families, those individuals predicted by linkage analysis to be predisposed to tumorigenesis can be shown to carry the predisposing mutation which is typically either a missense mutation [22], an in-frame deletion [23] or one affecting the promotor region of the *RB1* gene [24, 25]. We demonstrated in one such case that the missense mutation only partially inactives the *RB1* gene [26] which presumably allows most of the retinal precursor cells to escape malignant transformation. Thus, when offering genetic counselling to these families, the relatively low penetrance of this type of mutation must be taken into account. In contrast, the mutations found in early onset, unilaterally affected individuals, because they generate stop codons, would be pre-

dicted to carry a risk of the development of a more severe form of the disease in subsequent generations. This observation confirms the suggestion [11] that unilaterally affected patients with early presentation of tumour are more likely to be carrying germline mutations than individuals who present later. From our series of 250 patients with unilateral tumours, 5 (2%) would be predicted to carry germline mutations. Unfortunately, because of the referral bias in our series [27], many unilaterally affected patients from other regions in the U.K. have not been included in the study and so, before any statistical correlation can be established, it will be necessary to carry out more extensive surveys on unilaterally affected individuals. Our findings do, however, have important implications for routine mutation screening and genetic counselling programmes since, given limited resources, it may not be possible to analyse all cases of Rb in an exon-by-exon search for mutations. By demonstrating that it is the patients with early presentation of unilateral disease who are more likely to carry mutations, and if this is generally true, this will ultimately streamline genetic screening. If only 1/25 unilaterally affected individuals carry predisposing mutations then the remaining 24/25 unilaterally affected individuals can be given a low risk for tumorigenesis and a low priority for mutation screening because of their extremely low risk of being mutant gene carriers. One potential drawback with analysing the *RB1* gene sequence in lymphocytes from sporadic cases, is the possibility that the patients are mosaic for the mutation and so they may not carry the defect in the germline. Mosaicism has been reported following cytogenetic analysis of lymphocytes from patients with 13q deletions [28, 29], but systematic searches have not yet been performed in patients where constitutional mutations have been detected.

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