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Original Paper

Frequency and Nature of Germline *Rb-1* Gene Mutations in a Series of Patients with Sporadic Unilateral Retinoblastoma

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Constitutional *Rb-1* gene mutations were studied in a series of 17 families with isolated unilateral retinoblastoma patients. Peripheral blood lymphocytes were analysed by karyotyping, Southern blot hybridisation, and 'exon by exon' sequencing. Mutations were detected in 4 (24%) of the investigated probands. All mutations were identified by sequencing. No alteration was detected by Southern blotting or karyotyping. In one of our cases with a R358 stop codon mutation, retinoblastoma was unilateral at the time of diagnosis, but a tumour of the second eye was diagnosed after 35 months of follow-up. After exclusion of this case, the frequency of constitutional mutations in our series was 19% (3 of 16 cases). Alterations in our cases without involvement of the second eye included G→A substitution in the promoter region 198 bp upstream of the initiating methionine codon; G→C transversion in the splice donor site at position +1 leading to exon 6 skipping and a 137 bp in-frame deletion, starting 3 bp from the 5′ end of exon 15 to 27 bp from the 3′ end of exon 16. All alterations were germline *de novo* abnormalities.

Key words: retinoblastoma, sporadic, unilateral, constitutional mutations
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INTRODUCTION

RETINOBLASTOMA IS a malignant intra-ocular tumour of childhood occurring in both familial and sporadic forms [1]. All patients with familial or sporadic bilateral retinoblastoma are regarded as carriers of an *Rb-1* gene constitutional mutation. Cases of unilateral sporadic tumours represent a mixture of retinoblastomas caused by either somatic or constitutional mutations [1, 2].

On the basis of surveys of the occurrence of retinoblastoma in offspring, it has been estimated that the proportion of hereditary cases among patients with isolated unilateral tumours is 10–12% according to the most widely cited figures reported by Vogel [1] or only 2% according to Draper and colleagues [3]. These figures are quite different. Mutation frequency assessment is also possible by analysis at the DNA level of *Rb-1* gene constitutional abnormalities in a series of sporadic unilateral cases.

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Retinoblastomas occurring on the basis of constitutional mutations are bilateral in the majority of such cases. However, in some familial retinoblastomas the proportion of unilateral tumours is clearly higher. This suggests that some particular inherited genomic alterations may predispose to unilateral instead of bilateral tumours. The significance of the nature of *Rb-1* gene abnormalities for tumour laterality is not yet known.

To date, literature data on constitutional mutations within the *Rb-1* gene in sporadic unilateral retinoblastomas are very limited. Only 12 patients with such tumours resulting from constitutional mutations have been reported in the literature by Blanquet and associates, 4 cases [4]; Lohmann and colleagues, 6 cases [2] and Cowell and Cragg, 2 cases [5].

The mutation frequency in a series of patients affected by sporadic unilateral tumours has been reported only by three groups: Shimizu and colleagues [6], Blanquet and associates [4] and Lohmann and colleagues [2], who found genome abnormalities respectively in 0% (0/12), 7% (4/56) and 15.4% (6/39) of cases. Such huge discrepancies between these values can result from variable factors including sensi-

tivity of applied techniques and population differences. In order to establish actual mutation frequency, investigations of an additional series of cases are needed.

In some patients with tumours initially unilateral, the second eye may be involved during follow-up. Thus, without long-term clinical observation, a distinction between unilateral and bilateral retinoblastomas cannot be made with appropriate accuracy. Among the aforementioned reports, detailed follow-up data are presented only in 6 cases with mutations reported by Lohmann and colleagues [2] and 2 cases with mutations described by Cowell and Cragg [5].

Herein, we describe the results of our studies in a series of 17 unrelated patients with sporadic retinoblastomas which were unilateral for at least the 24 months of follow-up. These cases were initially examined at the DNA level by complete 'exon by exon' sequencing to verify the hypothesis that age at diagnosis can discriminate patients for whom research for constitutional mutations is appropriate [7]. After extension of our analysis by karyotyping, Southern blotting and RNA studies, we present in this paper more precise data on the nature and frequency of *Rb-1* gene constitutional mutations in sporadic unilateral retinoblastomas.

PATIENTS AND METHODS

Patients

Peripheral blood was obtained from all 17 unselected families with one child affected by unilateral retinoblastoma, referred between 1992 and 1996 by the Ophthalmology Department, Medical University, Szczecin, Poland. All cases were sporadic—that is no other cases of retinoblastoma nor other typically associated tumours were recognised in the family history. The mean age at diagnosis was 26.8 (range: 5–42) months. Patients with tumour of the second eye diagnosed within 24 months of follow-up were excluded, assuming that the probability of second eye involvement, that is late onset bilaterality, in our group was below 10% [8, 9]. The mean length of follow-up was 81.6 months (range: 37–164 months). Diagnosis was established by current ophthalmological and histopathological criteria. In all patients, enucleation had to be performed because the tumours involved more than half of the retina.

DNA isolation and sequence analysis

DNA was isolated from blood samples anticoagulated with EDTA using phenol–chloroform extraction as previously described [7]. The individual 27 exons, promoter and acceptor regions were amplified using the primers described by Lohmann and colleagues [10], except the primers for the promoter (5′-CCTGGAAGGCGCCTGGACCC-3′; 3′-TCCCCCGCCGGCAACTGAG-5′).

Polymerases chain reaction (PCR) products were purified from unincorporated primers and dNTPs by ultrafiltration using a Centricon 30 filtration unit (Amicon Inc., Beverly, Massachusetts, U.S.A.). Both DNA strands were sequenced by applying the Taq cycle sequencing dye termination protocol (PE Applied Biosystems, Warrington, U.K.). Sequencing reactions were analysed on an Applied Biosystem 373A Sequencer.

Southern blot analysis

RNA isolated from peripheral blood lymphocytes of healthy donors was converted into cDNA by reverse transcription using M-MLV Reverse Transcriptase (Promega Corp., Madison, Wisconsin, U.S.A.), and cDNA covering exons 2–27 of the *Rb-1* gene was amplified. The PCR fragment was digested with *Eco*RI enzyme into two fragments (0.8 and 2.9 kb) which were used as probes after purification by electrophoresis on low-melting-point agarose (Nusieve, FMC, Rockland, Maine, U.S.A.) and labelling by dUTP-DIG using Boehringer-Mannheim kit (Mannheim, Germany).

Genomic DNA (15 μg) was digested overnight at 37°C with *Eco*RI, *Eco*RV, *Hind*III and *Sac*I enzymes, electrophoresed on a 0.7% agarose gel (SeaKem, FMC), alkaline denatured and blotted overnight on to Hybond-N membrane (Amersham, Life Science Ltd, Little Chalfont, Buckinghamshire, U.K.). After capillary transfer, DNA was immobilised on a membrane by ultraviolet (UV) cross-linking, hybridised with labelled probes and the blot was developed using Boehringer Mannheim Kit.

Reverse transcription (RT) PCR

To examine the consequences of a potential splice-site mutation identified in one case, patient RNA was isolated from 72-h lymphocyte culture using the method of Chomczynski and Sacchi [11]. cDNA was created using dTA 17 oligonucleotide priming and the M-MLV Reverse Transcriptase Kit (GIBCO, Life Technologies Inc., Rockville, Maryland, U.S.A.) with RNAsin®Ribonuclease Inhibitor (Promega) according to the manufacturer's protocol. PCR amplification was performed using primers: 5′-AGCTACAGAAAAACATACGAAATC-3′; 5′-GTGCAGTTGTTAAAATAGGAAATC-3′. The PCR product of 869 bp was excised from agarose gel, eluted through a 0.47 μm filter unit (Millipore CDRP, Bedford, Massachusetts, U.S.A.) and used as template for cycle sequencing reactions.

Karyotyping

Karyotype analysis was performed on 72-h lymphocyte cultures. Prometaphases were obtained by methotrexate (MTX)–thymidine synchronisation followed by GTG banding. In each case 30 metaphases were analysed and 5 prometaphase karyotyped.

RESULTS

Mutations were detected in 4 of the 17 investigated probands (24%) (Table 1). All mutations were identified by sequencing. Alterations were not detected by Southern blotting and karyotyping.

In case 2, retinoblastoma was unilateral at the time of diagnosis, but a tumour of the second eye has been diagnosed after 35 months of follow-up. After exclusion of this case, the frequency of constitutional mutations in our series was 19% (3 of 16 cases).

Table 1. *Molecular characteristics of identified mutations*

Case	Mutation	Position	Consequence
1	G→C	Exon 6 IVS	Exon 6 skipped
2	G→C	1072 Codon 358 CGA(R)→TGA(X)	Arg→Stop
3	G→A	–/98 from AUC	
4	Deletion 137 bp	3 bp from 3′ end of exon 15 to 27 bp from 3′ end of exon 16	In-frame deletion with creation of new codon

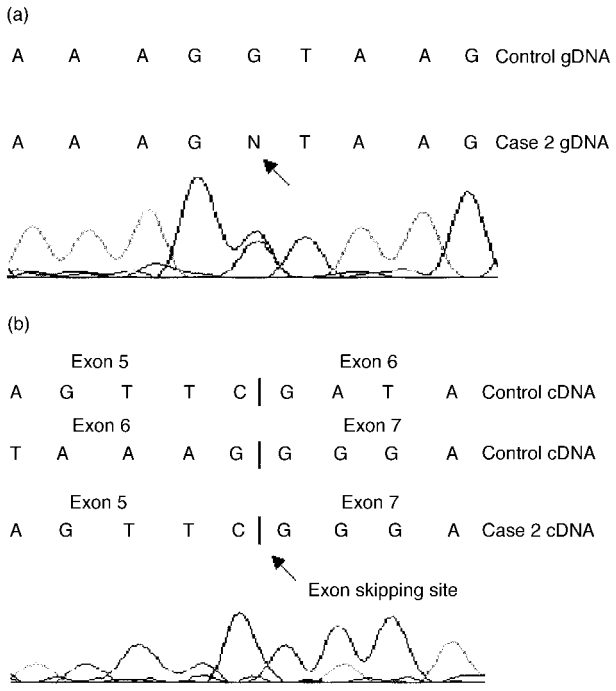

Figure 1. Sequence chromatograms of case 1 showing germline *de novo* mutation—splice donor site transversion G→C (a) leading to exon 6 skipping at the RNA level (b).

Case 3 revealed a sequence variation due to a G→A substitution (g-198 G→A base pairs upstream of the initiating methionine codon). This alteration was not found in any member of the family.

In case 2, sequencing of PCR products for exon 6 indicated a splice donor site transversion G→C (Exon6 IVS + 1G→C) (Figure 1). RT-PCR analysis of RNA from peripheral blood lymphocytes revealed that exon 6 was skipped in the mutant transcript which generated a frameshift mutation with a stop codon TGA created from nucleotides c630-632 of exon 7. Only wild-type sequences were detected in PCR products of exon 6 in all first-degree relatives of this patient. In case 2, sequencing of exon 11 PCR products showed heterozygosity because of a transition c1072C→T. The mutation converts codon 358 from an arginine (CGA) to a stop codon (TGA). Therefore, this mutation results in R357X. The mutation was not identified in any family member.

In case 4, analysis of exon 15–16 PCR products showed a faster migrating band in addition to the normal band, indicating a constitutional heterozygous deletion. Sequencing showed a deletion of 137 bp, starting 3 bp from the 5′ end of exon 15 to the 27 bases from the 3′ end of exon 16. This alteration does not result in the generation of a new stop codon, but creates a new codon composed of the first two bases of exon 15 and one base of exon 16 and causes deletion of 19 amino acids from exon 15 and 16. The newly generated triplet GAT shows altered translation to amino acid as compared with the intact triplet of exon 15 (GAA→GAT with generation of Glu→Asp). This mutation was not detected in either parents or siblings of the patient.

DISCUSSION

In our studies of a series of 16 families with patients affected by sporadic unilateral retinoblastoma, constitutional *Rb-1* gene abnormalities were identified in 3 (19%) cases. These were an alteration of the promoter region (case 3), mutation of the splice site of exon 6 leading to exon skipping and a stop codon (case 1), and an in-frame deletion of exons 15–16 without generation of a stop codon (case 4). In one additional case (case 2) included initially in our series, tumour of the second eye was recognised after 35 months of follow-up. In this patient, a stop codon within exon 11 was identified.

The frequency of constitutional *Rb-1* gene mutations found in our series is similar to the 17% reported by Lohmann and colleagues [2] and higher than the 7% found by Blanquet and associates [4]. Overall, from the only three reports published on sporadic unilateral retinoblastomas [2, 4, current study] constitutional mutations of the *Rb-1* gene were detected in 14% (15 of 111 patients). In comparison with results from epidemiological studies, this value is slightly higher than the 10% found by Vogel [1] and clearly higher than the 2% reported by Draper and colleagues [3]. That the frequency is higher in DNA studies than in epidemiological studies is not unexpected, because low penetrance cases without occurrence of tumours in offspring may be misinterpreted as non-hereditary if only pedigree analyses are used [10, 12]. In order to obtain accurate values of *Rb-1* gene constitutional mutations, further investigations of a larger series of samples from population-based surveys, in different ethnic groups and using highly standardised techniques of DNA/RNA analyses are needed.

Preliminary studies suggest that the frequency of abnormalities detected justify consideration of molecular tests as a diagnostic tool useful in routine clinical practice. Relatives of patients affected by sporadic unilateral retinoblastoma are also at a significant risk of developing eye tumours. Thus, these persons need surveillance with a large number of clinical examinations under anaesthetic from birth to 3 years [13]. If a constitutional mutation is not detected, it is impossible to determine whether this indicates a non-hereditary case, the somatic character of the tumour, mosaicism [14] or limited sensitivity of the molecular techniques. In such situations, surveillance in relatives must be continued [13, 14]. However, once a constitutional mutation in a child with retinoblastoma is found, it is possible to exclude a mutation carrier status in many children in such a family. This can significantly reduce healthcare expenditure. Noorani and colleagues calculated the costs of family screening in familial and sporadic bilateral retinoblastomas finding a 4-fold decrease in costs if DNA techniques were applied [15]. The cost-effectiveness of DNA tests in families with sporadic unilateral retinoblastomas needs precise assessment.

In order to use molecular tests in an economically justified manner, it may be essential to search for clinical features identifying subgroups of patients carrying mutations with particularly high probability. It has been suggested by Cowell and Cragg that the frequency of constitutional *Rb-1* gene mutations is higher in patients with retinoblastomas diagnosed at an early age [5]. Our results are in agreement with this hypothesis because in our series all four tumours with *Rb-1* gene mutations were diagnosed in patients $\leq$18 months of age. The problem of correlation between age at tumour diagnosis and occurrence of DNA abnormalities has been previously discussed [7].

Rb-1 gene alterations found in our studies were *de novo* changes in all three cases with unilateral sporadic retinoblastomas showing constitutional abnormalities. Among cases with sporadic tumours and constitutional mutations,

two subgroups can be distinguished: one with abnormalities carried by several generations—'low penetrance' cases [10, 12, 16] and a second subgroup with *de novo* mutations [2, 17]. The latter subgroup occurs much more frequently according to epidemiological analyses and our results are in agreement with this observation.

The most common form of hereditary retinoblastoma results in the development of multiple tumours in both eyes. In this group, the majority of mutations are predicted to create premature stop codons as a result of point mutations, deletions and/or insertions [17].

In some patients with constitutional alterations of the *Rb-1* gene, the phenotypic presentation is different and characterised by low penetrance of the mutation with only unilateral or unifocal tumours, regressed tumours or no evidence of malignant disease [2, 10, 12, 17]. It has been suggested that in these cases the mutation only compromises gene function rather than abolishing it [16]. It seems that mutations with low penetrance are mainly promoter alterations and missense mutations or in-frame deletions, which do not lead to the creation of stop codons.

To date, the only cases reported in the literature with *Rb-1* gene sporadic unilateral retinoblastomas showing constitutional alterations and with follow-up longer than 24 months (thus, long enough to exclude bilaterality with reasonably high probability) include only five cases described by Lohmann (1 case in his series had only 10 months follow-up) [2], two cases described by Cowell and Cragg [5] and three cases studied in our series. Among 10 of them, abnormalities leading to stop codons were found in four cases only (exon10 c958 C→T, exon17 c1654 C→T, IVS6 + 1G→C, del[c1973^c1980]c1985insT) so less frequently than in bilateral retinoblastomas. Other mutations include promoter alteration (our case 3), missense mutations (samples G1142 and N2408 in the Lohmann series) or in-frame deletions (samples M2920 and probably M6680 in The Lohmann series and our case 4). It can be expected that splice acceptor changes IVS12-G→A in sample M6680 studied by Lohmann also lead to in-frame deletion because usually abnormalities in such a location result in skipping of exon 13 which does not alter the reading frame. Of the 10 mutations described by Lohmann and colleagues [2] and Cowell and Cragg [5] and in this study, four are new alterations not previously detected in sporadic bilateral nor in familial cases (Lohmanns' cases M2920-c1332 G→A and M 6680-IVS 12-G→A; del (c.1973^ c.1980)c.Ins T in Cowell and Cragg studies and our case three). Two types of mutation—our case 1 and Lohmanns' patient G 1142 with c1982 C→T were previously reported in families with low penetrance [16].

The above results suggest that the occurrence of particular *Rb-1* gene alterations, characteristic for sporadic unilateral retinoblastomas, cannot be excluded. Possibly, some of these alterations might be strongly related to unilaterality, whereas others might predispose both to a uni- and bilateral phenotype and their presentation would depend on modifying genomic alterations or environmental factors.

In summary, studies of the frequency and nature of *Rb-1* gene constitutional abnormalities in sporadic unilateral retinoblastomas seems to be of potential practical value for differentiating between hereditary and sporadic cases; for the prognosis of second eye involvement and the age of tumour onset; for the exclusion of relatives from surveillance programmes; and possibly in solving other problems crucial in the management of retinoblastoma patients and their families.

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