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Original Paper

Age at Diagnosis to Discriminate those Patients for whom Constitutional DNA Sequencing is Appropriate in Sporadic Unilateral Retinoblastoma

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***RB1* gene constitutional mutations were studied using 'exon-by-exon' sequencing in a series of 17 patients with sporadic unilateral retinoblastomas. Constitutional *de novo* germline mutations were detected in 4 patients. The age at diagnosis of retinoblastoma in all these cases was lower (mean 10.8 months; range 5–18) than in cases in which constitutional mutations were not found (mean 31.7 months; range 19–42). These results strongly indicate that age at retinoblastoma diagnosis may be a major factor for discriminating patients for whom a search for *RB1* gene constitutional mutations could be justifiable in sporadic unilateral retinoblastomas.** © **1998 Published by Elsevier Science Ltd. All rights reserved.**

Key words: *RB1* gene, mutation detection, sporadic retinoblastoma, early onset
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INTRODUCTION

RETINOBLASTOMA IS an ocular childhood tumour which is familial in approximately 10% of cases and sporadic in approximately 90%. Approximately 40% of sporadic cases are caused by *de novo* germline mutations. The majority of hereditary cases can be identified by the occurrence of bilateral or multifocal tumours. However, up to 15% of children with unilateral sporadic tumours also have a constitutional *RB1* gene mutation that can be inherited by their children and can increase the risk of a second cancer in the patient [1, 2]. Therefore, the identification of *de novo* germline mutation carriers is crucial. One way to detect them among patients with sporadic unilateral retinoblastomas is by direct identification of constitutional mutations.

The most accurate methods for detecting *RB1* mutations are molecular analyses at the DNA level, particularly using 'exon-by-exon' sequencing. These techniques are complex, time consuming and expensive. Thus, given limited resources in routine mutation screening, it may not be possible to examine all patients with sporadic retinoblastoma.

It is well recognised that hereditary retinoblastomas occur at an early age. It has been suggested by Cowell and Cragg that age at tumour diagnosis may be an important factor to discriminate sporadic cases who merit searches for constitutional *RB1* gene mutations [3]. Two constitutional *RB1* gene mutations were detected when 3 patients with sporadic retinoblastomas diagnosed under the age of 12 months were studied [3]. However, Lohmann and colleagues, looking at age at enucleation and the presence of mutation in peripheral blood lymphocytes in a larger series of unilateral sporadic patients, did not find a significant difference between the age at operation of the 'mutation present' and 'mutations absent' groups [4]. There are no other reports correlating age at first tumour diagnosis and occurrence of mutations detected at the DNA level in sporadic unilateral retinoblastoma patients.

Herein, we describe the results of studies on the occurrence of constitutional mutations in the *RB1* gene, detected using 'exon-by-exon' sequencing, in relation to age at tumour diagnosis in a series of 17 unselected sporadic retinoblastoma cases diagnosed in our centre.

PATIENTS AND METHODS

Patients

A series of 17 unselected families with one child affected by unilateral sporadic retinoblastoma were referred by the Ophthalmology Department, Medical University, Szczecin, Poland. All cases were unilateral at the time of diagnosis and

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Table 1. *Age at tumour diagnosis and occurrence of mutations in patients with unilateral sporadic retinoblastomas*

Patient no.	Sex	Age at tumour diagnosis (months)	Length of follow-up (months)	Mutations
1	F	5	96	45867 G→C
2	F	5	29	65386 C→T
3	M	15	96	1862 G→A
4	F	18	55	76889→77027 del
5	F	19	36	–
6	F	19	60	–
7	M	24	144	–
8	F	24	36	–
9	M	30	108	–
10	M	32	72	–
11	F	34	120	–
12	F	36	72	–
13	M	36	48	–
14	M	36	34	–
15	M	38	156	–
16	M	42	54	–
17	M	42	36	–

–, not detected.

sporadic: that is no other cases of retinoblastoma and other typically associated tumours were recognised in the family. Patients with tumour of the second eye diagnosed within 12 months of follow-up were excluded. Diagnosis was established by current ophthalmological and histopathological criteria. The age at tumour diagnosis was variable (mean 26.8 months, range 5–42).

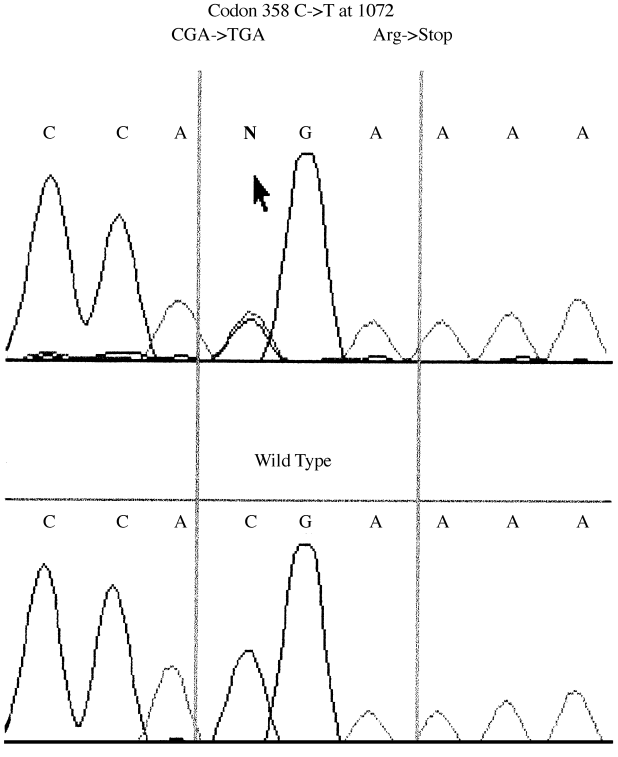

Figure 1. Constitutional germline *de novo* mutation in exon 11 found in patient no. 2; sequence chromatograms showing the identified mutation compared with wild-type sequences.

DNA isolation and sequence analysis

High molecular weight DNA was isolated from whole blood lymphocytes according to standard phenol–chloroform extraction. The individual 27 exons, promotor and acceptor regions were amplified using the primers described by Lohmann and colleagues [5], except the primers for promotor (5′-CCTGGAAGGCGCCTGGACCC-3′, 3′-TCCCCCG-CCGGCAACTGAG-5′). Polymerase 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 terminator protocol (PE Applied Biosystems, Warrington, U.K.). Sequencing reactions were analysed on an Applied Biosystem 373A sequencer.

RESULTS

RB1 gene mutations were detected in 4 of 17 cases studied (Table 1, Figure 1). All diagnosed alterations were *de novo* germline mutations, because they were not found in parents of affected children. In none of the above patients were mutations in the *RB1* gene detected in siblings. The age at diagnosis of retinoblastomas due to *de novo* germline mutations was 5 (case 1), 5 (case 2), 15 (case 3) and 18 (case 4) months. The mean age at diagnosis in this group was 10.8 months. In patients in whom *RB1* gene constitutional mutations were not detected tumours were diagnosed later, with a mean age at diagnosis of 31.7 months (range 19–42).

DISCUSSION

Early diagnosis of retinoblastoma makes low morbidity treatment possible based on focal laser and cryotherapy, whilst delayed diagnosis necessitates enucleation followed by chemotherapy and radiation [1]. Since not only familial, but also sporadic bilateral or unilateral retinoblastomas may be heritable [1, 2, 6], it is recommended that infant relatives of all affected patients are screened. Screening consists of a large number of frequently performed retinal examinations, including five to six examinations under anaesthetic during the first year of life [1]. Many family members can be spared invasive, stressful and costly ophthalmological examinations provided DNA analyses of the *RB1* gene are applied.

Exclusion of carrier status can be achieved by haplotype analyses or direct detection of the mutation [5, 7]. The most sensitive technique for direct detection of *RB1* gene mutations is ‘exon-by-exon’ sequencing. Molecular techniques in medicine are relatively expensive. However, it has been calculated that DNA tests can be economically justified in families with sporadic retinoblastomas by lowering the number of screening examinations. Noorani and associates calculated the cost of family screening in familial and sporadic bilateral retinoblastomas and found a 4-fold decrease in cost if DNA techniques were applied [7].

The probability of finding germline *RB1* gene mutations in familiar and sporadic bilateral cases is up to 90% if all available molecular techniques are applied [7]. This value is much lower in sporadic unilateral cases because only 10–15% of such patients can transmit disease to their children [2, 8, 9]. Thus, it is reasonable to expect that the application of molecular methods in this group of patients will actually increase the total cost of health care. In order for molecular tests to be economically justifiable, it seems crucial to search for pedigree and clinical features identifying subgroups of patients

with unilateral sporadic retinoblastoma likely to have the *RB1* gene mutation.

It is reasonable to hypothesise that to discriminate patients for screening for germline *RB1* gene mutations, age at diagnosis of unilateral sporadic retinoblastomas might be a powerful factor [3]. According to Knudson's two-hit hypothesis, tumours in patients with constitutional mutations occur at an early age [6]. This was borne out in studies in retinoblastoma patients—the mean age at diagnosis for bilaterally hereditary tumours is 5–7 months compared with 24–30 months for sporadic cases [8–10]; 50% of bilateral tumours are diagnosed by the age of 1 year, whilst only up to 15% of unilateral cases are diagnosed at this age [2].

The correlation between age at retinoblastoma diagnosis and the frequency of constitutional mutations in sporadic unilateral cases has not been established [3, 4, 11–13]. Our series of 17 cases is the second largest series of sporadic unilateral retinoblastoma cases in which a correlation between age of tumour diagnosis and occurrence of *RB1* constitutional gene mutations has been evaluated.

The results of our analyses strongly suggest that age at diagnosis may be a major factor in identifying a subgroup of patients affected by unilateral sporadic retinoblastoma with a particularly high frequency of constitutional mutations. In the 4 cases with *RB1* germline mutations, age at diagnosis ranged from 5 to 18 months (mean 10.8) whereas in the 13 cases without detectable DNA abnormalities, retinoblastoma was diagnosed later, between 19 and 42 months (mean 31.7). Our studies are based only on sequencing of peripheral blood lymphocytes. Certainly, there would be greater accuracy if numerous additional analyses, such as karyotyping, Southern blotting, search for mosaicism and tumour tissue studies, were performed. However, because the large majority of mutations is detectable if 'exon-by-exon' sequencing is applied, it is reasonable to accept that the correlation reported in our series of cases is correct. Our results are in agreement with Cowell and Cragg, who detected two germline mutations (one deletion and one substitution) in 2 of 3 patients with retinoblastomas diagnosed under the age of 12 months [3]. Our suggestion is not supported by the results of Lohmann and colleagues who, in 39 cases studied, found *RB1* germline abnormalities in 3 cases operated upon at an early age (2, 11 and 18 months) and in 3 cases in which enucleation was performed later (36, 50 and 60 months) [4]. Yandell and associates did not detect constitutional mutations in 3 patients with sporadic unilateral tumours of which two occurred at the ages of 12 and 13 months [11]. However, if all 13 reported *RB1* germline mutations in sporadic unilateral cases are summarised, 10 (77%) were found in patients with tumours diagnosed at an early age (2–18 months).

Thus, on the basis of existing data from small series and a knowledge of hereditary cancer pathogenesis, it seems justified to develop larger studies on the correlation between the occurrence of *RB1* gene constitutional mutations and the age of tumour onset in sporadic retinoblastomas. It is possible that these investigations will indicate the limit of the age of tumour onset below which molecular analyses are effective and, therefore, economically justified in finding constitutional *RB1* gene mutations in patients with sporadic unilateral retinoblastomas.

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