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Age at diagnosis of isolated unilateral retinoblastoma does not distinguish patients with and without a constitutional *RB1* gene mutation but is influenced by a parent-of-origin effect

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Abstract

Patients with hereditary cancer are usually diagnosed earlier than patients with non-hereditary tumours. In children with isolated unilateral retinoblastoma, some of whom have a hereditary predisposition, this rule has been subject to debate. We have analysed the clinical manifestation of disease in 188 children with completely resolved mutational status. In 24 (13%) of these patients, testing of blood DNA showed a constitutional *RB1* mutation. The distribution of age at diagnosis was not different between patients with and without a constitutional mutation. However, patients with loss of the maternally inherited *RB1* allele had an earlier age at diagnosis than patients with loss of the paternally inherited *RB1* allele. Our data show that early age at diagnosis does not identify patients with isolated unilateral retinoblastoma that have a higher risk of being carriers of a *RB1* gene mutation. Our findings suggest that, at least in some patients, age at diagnosis is modified by a parent-of-origin effect.

Keywords: Retinoblastoma; Genes; Sporadic retinoblastoma; Genetic screening; Genetic predisposition

1. Introduction

Knudson's two-step mutation hypothesis [1] has been seminal for the identification of the genetic mechanisms underlying hereditary cancer predisposition syndromes. It was developed on the basis of mathematical analyses of data from patients with retinoblastoma. One of the cornerstones of this hypothesis is that age at diagnosis in patients with non-hereditary retinoblastoma is later compared with patients with hereditary retinoblastoma. This assumption is based on the observation that in children with bilateral retinoblastoma, who, with rare exceptions, have hereditary disease, the diagnosis is usually made earlier compared with children with unilateral retinoblastoma, most of whom have non-hereditary retinoblastoma [2]. In many cancers, it has been observed that the age at diagnosis is earlier in patients with a hereditary susceptibility to disease compared with patients with sporadic disease. Therefore, age at diagnosis is often included in the clinical criteria that help to discriminate patients with a hereditary tumour predisposition. The problem of distinguishing patients with and without a hereditary predisposition also arises in children with isolated unilateral retinoblastoma.

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Approximately 10–15% of these children are carriers of an oncogenic *RB1* gene mutation [3,4]. Referring to Knudson's hypothesis it was proposed that age at diagnosis is a criterion that helps to discriminate those children with isolated unilateral retinoblastoma that are more likely to be carriers of an oncogenic *RB1* gene mutation [5,6]. The results of genetic analyses in 3 [6] and 17 [5] patients with isolated unilateral retinoblastoma seemed to support this association. However, in a larger series, which included 68 patients with isolated unilateral retinoblastoma, the age at diagnosis was not earlier in carriers of an oncogenic *RB1* gene mutation [7]. It is important to find out if children with isolated unilateral retinoblastoma show a distinct clinical manifestation depending on the presence of a constitutional *RB1* gene mutation because this will influence decisions in clinical management and risk figures used in genetic counselling. Furthermore, if the distribution of age at diagnosis in patients with unilateral retinoblastoma is not distinctly different in children with a constitutional *RB1* gene mutation this will influence our understanding of the two-step mutation hypothesis.

The rationale for the present study was to resolve this open question. To this end we obtained precise information on the clinical manifestation and the genetic status of children with isolated unilateral retinoblastoma and included only those patients for whom both data-sets were complete. In order to obtain a complete genetic status, mutational analysis of the *RB1* gene was performed in tumour and in constitutional DNA. We employed a set of methods that covers the whole spectrum of *RB1* gene mutations. Such a laborious approach is indispensable because only if the two oncogenic *RB1* gene mutations that are responsible for tumour development in a patient are known, the carrier status can be unambiguously resolved [3,4,8].

2. Patients and methods

2.1. Patients

Since 1986, the Department of Human Genetics at the University Duisburg-Essen has offered a genetic testing service for patients with retinoblastoma. To date, we have received samples from 1144 index patients including 530 (46%) patients with unilateral retinoblastoma. From 331 children with unilateral retinoblastoma, we obtained fresh frozen tumour samples and peripheral blood and the parents requested a mutational analysis to make a molecular risk prediction.

2.2. Mutational analysis

To unequivocally identify the somatic origin of *RB1* gene mutations in patients with isolated unilateral retinoblastoma, genetic testing must start using tumour DNA. As the spectrum of oncogenic alterations in tumours is broad [9], we use a wide range of methods for the identification of *RB1* gene mutations. Specifically, point mutations are detected by single-strand conformational polymorphism (SSCP) [3], denaturing high performance liquid chromatography (DHPLC), and exon-by-exon sequencing [10]. Gross deletions and insertions are determined by quantitative fluorescent multiplex polymerase chain reaction (PCR) [4] and quantitative Real-time PCR. Methylation-sensitive PCR [11] and Real-time PCR [12] are used to identify epigenetic gene silencing [13]. To find out if a mutation detected in the tumour is of somatic origin, DNA from peripheral blood leucocytes is tested by sequencing, quantitative Real-time PCR, or methylation-sensitive PCR, depending on the type of the mutation. To identify allele loss in tumours, we determine the genotypes at the polymorphic short tandem repeat loci RBi2 and RB1.20, which are located in intron 2 and 20 of the *RB1* gene, respectively [14]. The parental origin of alleles is determined by genotyping RBi2 and RB1.20 in constitutional DNA from parents.

2.3. Data analysis

Detailed information on clinical presentation, treatment and follow-up was obtained. The present study focuses on patients with isolated unilateral retinoblastoma who had been treated by enucleation and who did not develop tumours in the remaining eye. In addition, to qualify for the present study, patients had to present with isolated disease. In a previous report on a small set of patients, we analysed the age at enucleation [7], which, in most patients, was not much different from age at diagnosis. For the present study, we re-examined all clinical data from all of the patients and thus were able to establish the date of clinical diagnosis of retinoblastoma. We used a data warehouse software environment (Cognos Series 7.1, Cognos incorporated) to link all clinical and genetic data and to set the stage for data-mining.

Following the exploration of the relationships between clinical and genetic information, relevant data were retrieved and subjected to statistical analyses using the SAS software (version 8.02; SAS Institute Inc., Cary, NC). Because the age distributions deviated from a normal distribution, medians and quartiles were presented instead of means and standard errors. Age distributions between different groups were compared using the Kolmogorov–Smirnov test. If this test detected a difference, two further tests were performed to identify the different types of variance. The test proposed by Brunner and Munzel [15] was applied to test for a difference in location, i.e., for testing whether the values in one group tend to be higher, or lower, than the values of the other

group. The Fisher–Pitman test was applied after a modified Levene transformation using group medians to test for a difference in variability [16]. No multiplicity adjustment is necessary for these stepwise tests [17]. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Patients with and without complete mutational status and distribution of age at diagnosis

Both fresh-frozen tumour samples for genetic testing and complete data-sets with all of the relevant clinical data were available from 219 patients with isolated unilateral retinoblastoma. In 188 (86%) of these patients, we identified both oncogenic *RB1* gene mutations in the tumour DNA (complete mutational status, Table 1). In 16 (7%) patients, DNA extracted from fresh-frozen tumour samples was used up before all of the methods for mutational analysis could be completed. In the remaining 15 (7%) patients, our methods failed to identify both of the mutations linked with tumour development. We detected a mutation in one *RB1* allele in 12 of these 15 tumours. This indicates that inactivation of *RB1* plays a role in the biology of these tumours. It is possible that a second *RB1* gene mutation is present in these tumours that may have been missed by our mutational analysis methods. We found no significant difference in the clinical manifestation in patients with or without a complete mutational status. Specifically, the distribution of age at diagnosis is similar in both groups of patients (median 686.5 days [352.5; 980.5] and 721 days [324; 970], respectively, $P = 0.9978$).

Table 1
Result of mutational analysis and age at diagnosis

	Number of patients	Median age at diagnosis/ days (quartiles)
Mutational status		
Complete	188 (86%)	686.5 (352.5; 980.5)
Incomplete	31 (14%)	721 (324; 970)
RB1 mutation in blood DNA		
Absent	164 (87%)	686.5 (368; 982.5)
Present	24 (13%)	662.5 (287; 938)
Heterozygous	17 (71%)	632 (330; 979)
Inherited	*4*	
Mosaic	7 (29%)	746 (244; 827)
Loss of heterozygosity		
No LOH	61 (29%)	685 (365; 1059)
Homozygous deletion	6 (3%)	609.5 (298; 1103)
LOH	144 (68%)	707.5 (352.5; 950)
Parental origin of the allele lost in the tumour		
Maternal	61 (55%)	482 (293; 830)
Paternal	50 (45%)	865 (516; 1121)

LOH, loss of heterozygosity.

3.2. Identification of patients with a constitutional RB1 gene mutation

In 24 of 188 (13%) patients with a complete mutational status, one of the two mutations identified in the tumour was also detected in constitutional DNA. In 17 of these 24 (71%) patients, the ratio of signals of the normal and mutant alleles in DNA from blood was balanced suggesting that the mutation is present in the heterozygous state. Analysis of DNA from parents showed that 4 of the 17 (24%) patients had inherited the mutation from an unaffected heterozygous parent (2 paternal and 2 maternal transmissions). In the 7 of 24 (29%) patients with a mutation in constitutional DNA, the signal of the mutant allele was significantly weaker compared with the heterozygous samples. This is interpreted as evidence of mutational mosaicism [3,8].

3.3. Age at diagnosis in patients with and without a detectable constitutional RB1 gene mutation

The distributions of age at diagnosis in the 24 patients with and the remaining 164 patients without an *RB1* gene mutation in constitutional DNA are not significantly different (Fig. 1(a), median 662.5 days [287; 938] and 686.5 days [368; 982.5], respectively, $P = 0.7976$). In addition, the distributions of age at diagnosis in heterozygous patients and in carriers that show mutational mosaicism is similar (median 632 days [330; 979] and 746 days [244; 827], respectively).

3.4. Manifestation of retinoblastoma in patients with and without loss of heterozygosity of markers in the RB1 gene

211 patients were heterozygous at the STR-loci RBi2 or RB1.20 and, therefore, informative for investigation of allele loss in the tumour. In tumours from 61 (29%) patients, both *RB1* alleles were retained (no loss of heterozygosity (LOH)) and in another 6 (3%) tumours, the *RB1* locus was homozygously deleted. In tumours from 144 (68%) patients, constitutional heterozygosity was lost for one or both STR-loci. In 111 of these patients, DNA from parents was available and informative at these loci. The allele lost in the tumour was determined to be of maternal and paternal origin in 61 (55%) and 50 (45%) patients, respectively. The distributions of age at diagnosis in the 61 patients without LOH and the 144 patients with LOH are similar (Fig. 1(b), median 685 days [365; 1059] and 707.5 days [352.5; 950], respectively, $P = 0.8890$). However, age at diagnosis in patients with LOH was distinct depending on the parental origin of the allele that was retained in the tumour (Fig. 1(c) and Fig. 2). In patients that lost the maternal *RB1* allele, the median age at diagnosis was 482 days [293; 830], whereas patients with loss of the paternally inherited allele were diagnosed at a mean

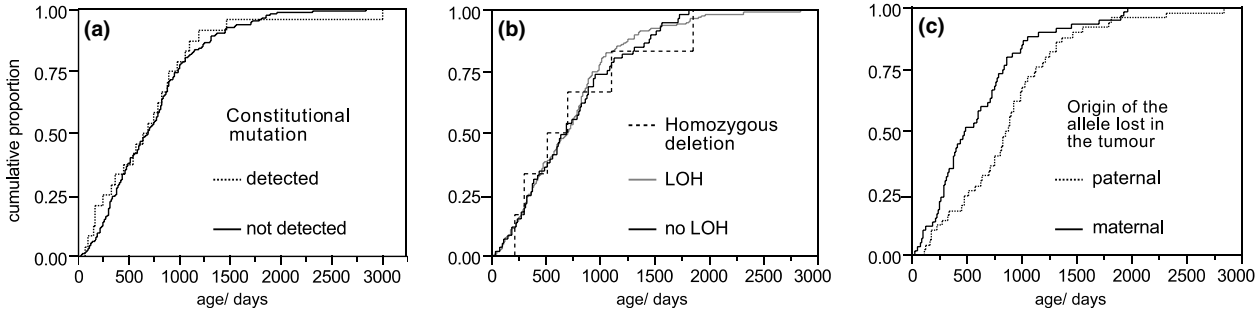

Fig. 1. Comparison of age at diagnosis in children with isolated unilateral retinoblastoma. (a) Distributions of children with or without a *RB1* gene mutation in peripheral blood DNA (Kolmogorov–Smirnov-test $P = 0.7976$), (b) distributions of children with loss of heterozygosity (LOH) and without LOH at the *RB1* locus in the tumour ($P = 0.8890$) or homozygous deletion of the *RB1* gene, (c) distributions of children with LOH in the tumour and loss of the *RB1* allele inherited from the father or mother ($P = 0.0038$).

age of 865 days [516; 1121]. The difference between the two distributions was statistically significant (Kolmogorov–Smirnov test: $P = 0.0038$). This is due to a difference in location (Brunner–Munzel test: $P = 0.0013$) rather than a difference in variability (Fisher–Pitman test after Levene's transformation: $P = 0.5844$).

4. Discussion

We have analysed associations between the clinical manifestation of disease and genetic status in 219 children with isolated unilateral retinoblastoma. All these children presented with sporadic disease and all had been treated by enucleation. In Germany, less than 5% of children with isolated unilateral retinoblastoma are not treated by enucleation. Therefore, the bias introduced by this selection is likely to be small on the population level. The mutational status was fully resolved in 188 patients and in 24 (13%) of them, one of the two *RB1* gene mutations identified in the tumour was also detected in constitutional DNA. This percentage corresponds well with theoretical estimates [18] and with the results of previous molecular analyses [3–5,8].

We did not identify a difference in the clinical manifestation of disease between children with and without a constitutional *RB1* gene mutation. Specifically, the distribution of age at diagnosis was almost identical (Fig. 1(a)). This contrasts with the findings by Zajaczek and collegues [5] who, in a series of 17 children found that all 4 children diagnosed under the age of 18 months were carriers of a constitutional mutation, whereas no mutation was found in any of the 13 children diagnosed at later ages. In our series, constitutional mutations were detected in 10 of 24 (42%) and 69 of 164 (42%) children under and over the age of 18 months, respectively. It is important to note that constitutional mutations may be missed if, as in the analyses by Cowell and Cragg [6] and Zajaczeck and collegues [5] mutational testing is only performed using constitutional DNA. However, our approach does not miss germline mutations in those patients that have a complete mutational status in the tumour. Only 31 (14%) of our patients had an incomplete mutational status. However, it is unlikely that the exclusion of these patients results in a relevant bias because they are only a few and we could show that the distribution of age at diagnosis in these children is not different from that observed for patients with complete mutational status. The fact that both oncogenic mutations were not identified in all tumours examined raises the question as to whether retinoblastoma can develop without biallelic inactivation of the *RB1* gene. However, it must be noted, that in 16 (7%) of these tumours we could not complete testing because the DNA extracted from the tumour sample was exhausted before the full range of analytical methods could be applied. This underscores the need to obtain a sufficient amount of tumour material for genetic testing. In 15 (7%) tumours, we did not find both *RB1* mutations, although tumour DNA was still available. Therefore, we cannot exclude that the development of some retin-

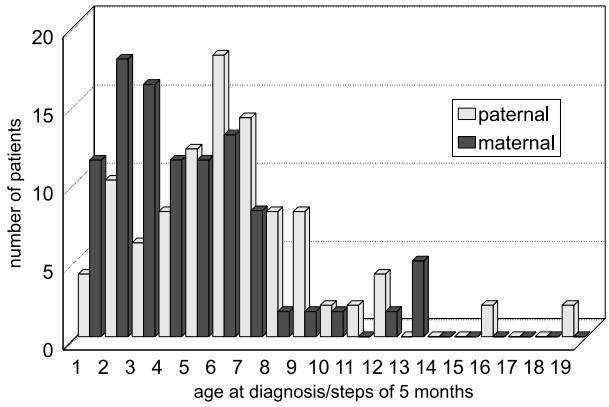

Fig. 2. Histogram of the distribution of age at diagnosis in children with isolated unilateral retinoblastoma with LOH in the tumour and loss of the *RB1* allele inherited from the father or mother. As noted in the legend to Fig. 1(c), the difference is significant ($P = 0.0038$). Each age category on the x-axis has a width of 5 months, i.e., class 1 represents ages < 5 months, 5 months ⩽ age class 2 < 10 months etc.

oblastomas might be triggered by mutations in genes other than *RB1* or by mechanisms other than single gene mutation such as viral infection [19]. As a different aetiology might be accompanied by a distinct biological behaviour, we looked to see if the clinical manifestation of disease was distinct between tumours with and without a complete mutational status, but found no differences. An obvious alternative explanation is that we have missed *RB1* gene mutations in tumours without complete mutational status, despite having employed a broad range of methods. It is becoming increasingly clear that mutations in intron regions can affect normal splicing, even if they are located far away from canonical splice sites. Because of the vast extension of introns in most genes – more that 170 kb in the case of the *RB1* gene – it is often impractical to scan these regions for mutations by sequencing of DNA. To overcome this limitation, screening for abnormally spliced transcripts might be tried and therefore material suited for RNA analysis should be obtained during routine sampling procedures. Of note, special care must be taken because, according to our experience, RNA in retinoblastoma samples is subject to very rapid decay.

In 7 (29%) of the 24 patients with mutant *RB1* alleles present in peripheral blood DNA, the ratio of the signals of mutant and normal alleles indicated that the mutation is present in a mosaic state. It is possible that this figure underestimates the true proportion of mosaic cases because it cannot be excluded that some patients classified as being heterozygous are in fact mosaic. In addition, some patients with no detectable mutation signal in DNA from blood may nonetheless harbour mutant somatic cell lineages. We did not observe a distinct difference in the age at diagnosis between mosaic and heterozygous cases. Of course, the small number of mosaic cases does not allow us to identify minor differences in these distributions.

The number of patients analysed in the present study is almost an order of magnitude higher than that in comparable studies reported by other laboratories. Therefore, we were able to detect more subtle effects in our series. While the age at diagnosis was similar in patients with tumours with and without *RB1* allele loss in the tumour, the age distribution in patients with loss of the paternal allele was distinct from those patients with loss of the maternal allele (Fig. 1(b) and (c)). The comparison of these distributions suggests that this difference might be caused by a subgroup of patients with LOH in the tumour and loss of the maternally inherited *RB1* allele that are diagnosed at young age (Fig. 2). In a few monogenic traits, phenotype expression is dependent on the parental origin of the mutant allele (parent-of-origin effect). Interestingly, a parent-of-origin effect has also been reported in hereditary retinoblastoma [20]. Although this phenomenon was associated only with a specific alteration, a splice mutation in intron 6, the more severe phenotypic expression was also associated with paternally transmitted mutant alleles. The observation in the present series might also be related to the finding of Kato and colleagues [21], who reported a later age at diagnosis in 6 patients with loss of the maternally inherited *RB1* allele compared with 7 patients with paternal allele loss. Notably, the direction of the effect is opposite to that observed by us. Moreover, the effect seems not to be restricted to a subgroup of patients. Nevertheless, the possibility that similar mechanisms are at work must not be dismissed because the patients are derived from different populations – Caucasian in our study and Japanese in the report by Kato and colleagues – and this might have an influence if genetic background plays a role. Clearly, further analyses are needed to support models that explain the biological mechanisms underlying the somatic parent-of-origin effect identified here.

In summary, our data show that the clinical manifestation of disease does not help to select carriers of an oncogenic *RB1* gene mutation among patients with isolated unilateral retinoblastoma. Specifically, it is not justified to restrict genetic testing to children with an early age at diagnosis. In addition, according to the results of our study, it is not correct to modify risk figures in genetic counselling depending on age at diagnosis. Our findings also change the interpretation of the two-step mutation hypothesis [1]. Specifically, our data suggest that tumours in children with unilateral retinoblastoma that carry a constitutional mutation are not initiated earlier than in children without a detectable constitutional mutation. Possibly the timing of the mutational events that inactivate the two *RB1* alleles is less important than assumed in the original formulation of the two-step mutation hypothesis.

Conflict of interest statement

None declared.

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