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Risk Assessment of Recurrence in Sporadic Retinoblastoma Using a Molecular-based Algorithm

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RESUME

Le rétinoblastome (Rb) est une tumeur provenant des cellules rétiniennes progénitrices des photorécepteurs. C'est la tumeur pédiatrique maligne la plus fréquente avec une incidence par naissance évaluée entre 1/15'000 et 1/20'000. Les enfants atteints de Rb sont diagnostiqué dans leur grande majorité avant l'âge de 4 ans, soit le temps nécessaire à la différentiation et à la maturation des photorécepteurs et donc à la disparition de la cellule d'origine du Rb. La survie du patient, la sauvegarde oculaire et le pronostic visuel restent excellents pour autant que le traitement ne soit pas différé. Dans sa variante non héréditaire (60%) le Rb est toujours unilatéral et sporadique. Le Rb héréditaire de transmission dominante autosomique (40%), se décline sous toutes les formes, familiale (10%) ou sporadique (30%), que l'atteinte soit unilatérale ou bilatérale.

La majorité des mutations causales sont uniques et distribuées de façon aléatoire sur la totalité du gène RB1 sans région prédisposante. La détection de ces mutations est couteuse et chronophage, tout en présentant un taux de détection relativement bas; surtout dans les cas de Rb sporadiques unilatéraux. Dans le but d'identifier les patients présentant un risque réel de développer un Rb, et de réduire le nombre d'examens sous narcose requis pour le dépistage de la maladie chez les sujets à risque, nous avons développé une stratégie sensible, rapide, efficace et peu couteuse basée sur une analyse de l'haplotype intragénique. Cet algorithme prend en compte a) la perte d'hétérozygotie intratumorale du gène RB1, b) l'origine paternelle préférentielle des nouvelles mutations germinales et c) un risque *a priori* dérivé des données empiriques de Vogel. Pendant la période allant de janvier 1994 à décembre 2006, nous avons comparé l'apparition de nouveau Rb parmi la fratrie et la descendance de patient atteints au nombre de nouveaux

cas attendus calculé par notre algorithme. 134 familles ont été étudiées. L'analyse moléculaire a été effectuée chez 570 personnes dont 99 patients âgés de moins de 4 ans et donc à risque de développer un Rb. Parmi cette cohorte, nous avons observé l'apparition d'un cas de Rb, alors que les risques cumulés *a posteriori* calculé par notre algorithme prédisait l'apparition de 1.77 nouveau cas. Dans cette étude, nous avons pu valider notre algorithme prédisant la récurrence de Rb chez les parents de 1er degré de patients atteints. Cet outil devrait grandement faciliter le conseil génétique ainsi que le suivi des patients à risque de développer un Rb, surtout dans les cas ou le séquençage direct du gène RB1 n'est pas disponible ou est resté non informatif.

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RESEARCH REPORT

Risk assessment of recurrence in sporadic retinoblastoma using a molecular-based algorithm

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ABSTRACT

Purpose: Most RB1 mutations are unique and distributed throughout the RB1 gene. Their detection can be time-consuming and the yield especially low in cases of conservatively-treated sporadic unilateral retinoblastoma (Rb) patients. In order to identify patients with true risk of developing Rb, and to reduce the number of unnecessary examinations under anesthesia in all other cases, we developed a universal sensitive, efficient and cost-effective strategy based on intragenic haplotype analysis.

Methods: This algorithm allows the calculation of the a posteriori risk of developing Rb and takes into account (a) RB1 loss of heterozygosity in tumors, (b) preferential paternal origin of new germline mutations, (c) a priori risk derived from empirical data by Vogel, and (d) disease penetrance of 90% in most cases. We report the occurrence of Rb in first degree relatives of patients with sporadic Rb who visited the Jules Gonin Eye Hospital, Lausanne, Switzerland, from January 1994 to December 2006 compared to expected new cases of Rb using our algorithm.

Results: A total of 134 families with sporadic Rb were enrolled; testing was performed in 570 individuals and 99 patients younger than 4 years old were identified. We observed one new case of Rb. Using our algorithm, the cumulated total a posteriori risk of recurrence was 1.77.

Conclusions: This is the first time that linkage analysis has been validated to monitor the risk of recurrence in sporadic Rb. This should be a useful tool in genetic counseling, especially when direct RB1 screening for mutations leaves a negative result or is unavailable.

Keywords: Sporadic, retinoblastoma, recurrence, linkage analysis, genetic counseling

INTRODUCTION

Retinoblastoma (Rb) is the most common pediatric ocular malignancy and the most prevalent eye cancer around the world.[1] Rb originates from progenitors of retinal sensory cells, most probably along a cone lineage,[2,3] with an estimated incidence between 1/18,000 and 1/20,000 live births.[4,5] Affected children will develop Rb very early in life, during the period of cellular differentiation until the age of 4 years. Rb is still a mutilating, blinding and sometimes lethal disorder. There are two forms of Rb; familial and sporadic, the latter being the most widespread and accounting for more than 85% of the cases.

Rb was a model for Knudson's two hit hypothesis.[6] In his initial hypothesis, he stated that two mutational events were required for the initiation of Rb. Later, it was shown that these two mutational events inactivate both alleles of a single gene. Current knowledge indicates that mutations affecting both alleles of the Rb susceptibility gene (RB1) are a prerequisite for the development of this tumor.[6–8] In most patients with sporadic unilateral Rb, the two RB1 gene mutations that initiate tumor development are somatic events, and none are present in DNA from constitutional cells.[9–11] In fact 10–15% of them carry either homogeneous or mosaic mutations (see the description of the algorithm below). Patients with sporadic bilateral or familial retinoblastoma are heterozygous for an RB1 gene mutation that was either inherited from an affected parent or that occurred de novo in parental germline cells.

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Management of affected individuals also takes into account genetic counseling to relatives at risk. Genetic counseling of sporadic Rb can either be based on empirical risk calculations[12] or on the segregation of RB1 mutant alleles. In the former case, identification of disease recurrence relies on systematic examination under anesthesia (EUA) of all first degree relatives irrespective of their genetic predisposition from birth until 4 years of age; while in the latter, only the RB1 mutant carriers are investigated. Unfortunately, the underlying technology that leads to the oncogenic mutation is only available to limited number of patients around the world and is still expensive and time-consuming. Additionally, because the inactivating mutations are highly heterogeneous and distributed along the entire coding sequence of the gene, some 90% of sporadic unilateral Rb and 12–25% of sporadic bilateral Rb have undetected mutation despite all mutational screening efforts,[13–16] leaving more than 50% of families without molecular diagnosis and accurate genetic counseling of the disease. This prompted us to develop and validate an algorithm based on linkage analysis in a large population at risk to improve genetic counseling and disease follow-up in comparison to classical systematic EAU in patients at risk with sporadic Rb. Thus, the primary goal of molecular testing is to identify individuals with significant risk while avoiding repeated examinations in those with minimal risk.

MATERIALS AND METHODS

Patients

All consecutive patients with uni- or bilateral sporadic Rb including their first degree relatives seen between January 1994 and December 2006 at the Jules Gonin Eye Hospital were enrolled. Patients were referred from several countries, mostly Switzerland, Italy, Portugal and Greece. Written informed consent was obtained from all patients and members of the family using a consent form approved by the Swiss Federal Office of Public Health (035.0003-48) for Clinical Research and following the tenets of the Declaration of Helsinki.

All patients at risk were defined as siblings or children of affected Rb patients younger than 4 years of age. They were followed until the age of 48 months and automatically assigned a follow-up under narcosis at 1, 3, 6, 10, 15, 21, 28, 36 and 42 months as customary. Occurrence of new patients with Rb among this population was noted and compared to the predicted number of expected cases using our algorithm.

From January 1994 to July 2003, molecular characterization of RB1 alleles was performed using intra/extragenic sequence polymorphisms such as restriction fragment length polymorphisms (RFLP), variable number of tandem repeats (VNTR) sequences, and microsatellites, as listed in Table 1. From August 2003 to December 2006, polymorphic short tandems (STR) were used as recommended by the Best Practice Guidelines of the European Molecular Genetics Quality Network (EMQN) for linkage analysis of RB1 (Table 2).[17] The calculated risks obtained with our algorithm were then cumulated to allow comparison with the observed occurrence of retinoblastoma in this cohort.

Molecular Testing

Hybridization to cDNA Probe

Genomic DNA from peripheral blood leukocytes and tumoral tissue (from fresh tumors) was digested with 80U of HindIII. The resulting fragments were separated onto 0.8% agarose-gel, and blotted onto nylon membrane.[18] The filters were hybridized overnight at 42°C with the p4.95BT probe labeled with digoxigenin. After removal of the unbound probe, the bands were detected using a chemiluminescent substrate for alkaline phosphatase.[19]

Analysis with Polymorphic Markers

The polymorphic markers used in this study are shown in Tables 1 and 2. Three of the RFLPs were studied by PCR-amplification of the fragments, and the digestion of the product was electrophoresed in polyacrylamide or agarose gel and detected by silver-staining with slight modifications, or ethidium bromide. The VNTR marker is detected by Southern blotting.

The analysis of microsatellite markers is carried out by PCR amplification and electrophoresis in an automated sequencing machine.[20]

TABLE 1 Location of the different polymorphic markers.

Polymorphic marker	Probes	Location
Apa I	ESD 14.I.I	ESD
BamHI	123M I.8	Intron 1
Kpnl	95HS 0.5	Intron 4
Hind III	H3-8	Exon 4
Xba I	88R 2.5	Intron 17
Xba I (I-20 microsatellites)	88R 2.5	Intron 20
RsaI (VNTR)	68RS 2.0	Intron 17
Tth III I (AspI)	35R0.6	Intron 24

TABLE 2 Polymorphic short tandems (STRs) within and linked to the retinoblastoma (RB1) gene.

Marker name	Location	Accession
D13S161	Centromeric to RB1 (1.1Mb)	Z16802
D13S287	Centromeric to RB1 (1.0Mb)	Z24331
D13S164	Centromeric to RB1 (0.05Mb)	Z16858
D13S153	RB1: intron 2	Z16494
Rb1.20	RB1: intron 20	
D13S1307	Telomeric to RB1 (0.25Mb)	Z51671
D13S165	Telomeric to RB1 (0.9Mb)	Z16900
D13S273	Telomeric to RB1 (1.25Mb)	Z23383

Description of the Algorithm

We designed an algorithm based on haplotype characterization of both RB1 alleles applicable to all families. This algorithm combines segregation analysis using RB1 polymorphic markers[21] and is based on: (a) a priori risk derived from empirical data by Vogel,[22] (b) preferential paternal origin of new germline mutations,[23,24] (c) analysis of loss of heterozygosity (LOH) in the tumor,[25] (d) and disease's penetrance of 90% in most cases,[15] although it can vary significantly according to the mutation's type.[14]

In bilateral sporadic cases of Rb, risks have been empirically calculated by Vogel as follows: the a priori recurrence risk is 6% for siblings, whereas the a priori transmission risk is 50% for the offspring.[22] If LOH is present in the tumor, the haplotype linked to the predisposing germline mutation can be identified. This in turn allows us to accurately predict the risk (Fig. 1A):

- $2 \times 6\% = 12\%$ risk if the retained haplotype is passed to the sibling, versus 1/20,000 (exclusion) if not.
- $2 \times 50\% = 100\%$ risk if the retained haplotype is transmitted to offspring, versus 1/20,000 (exclusion) if not.

If LOH is not detected or if no tumoral material is available for analysis, the risk will be modified according to inheritance of grandparental alleles (Fig. 1B):

- $2 \times 6\% = 12\%$ risk for the sibling if the same parental alleles are transmitted; $2 \times 6 \times 90\% = 10.8\%$ if the paternal allele is transmitted (preferential paternal origin of new germline mutations in 90% of cases;[23] $2 \times 6 \times 10\% = 1.2\%$ if the maternal allele is transmitted and 1/20,000 if both alleles are distinct.

In unilateral sporadic Rb, the a priori recurrence risk is 1% for siblings, whereas the a priori transmission risk is 6% for the offspring. If LOH is present in the tumor, the haplotype linked to the predisposing germline mutation can be identified. This in turn allows the accurate prediction of the risk (Fig. 1C):

- $2 \times 1\% = 2\%$ risk if the retained haplotype is passed to the sibling, versus 1/20,000 (exclusion) if not.
- $2 \times 6\% = 12\%$ risk if the retained haplotype is transmitted to offspring, versus 1/20,000 (exclusion) if not.

If LOH is not detected or if no tumoral material is available for analysis, the risk will be modified according to inheritance of grandparental alleles (Fig. 1D). In unilateral Rb, the probability of a de-novo event occurring preferentially in the paternal germline is lower than the 90% reported in bilateral cases. An estimate of this probability can be derived from the study of Schüler.[24] This study found that 54% of constitutional

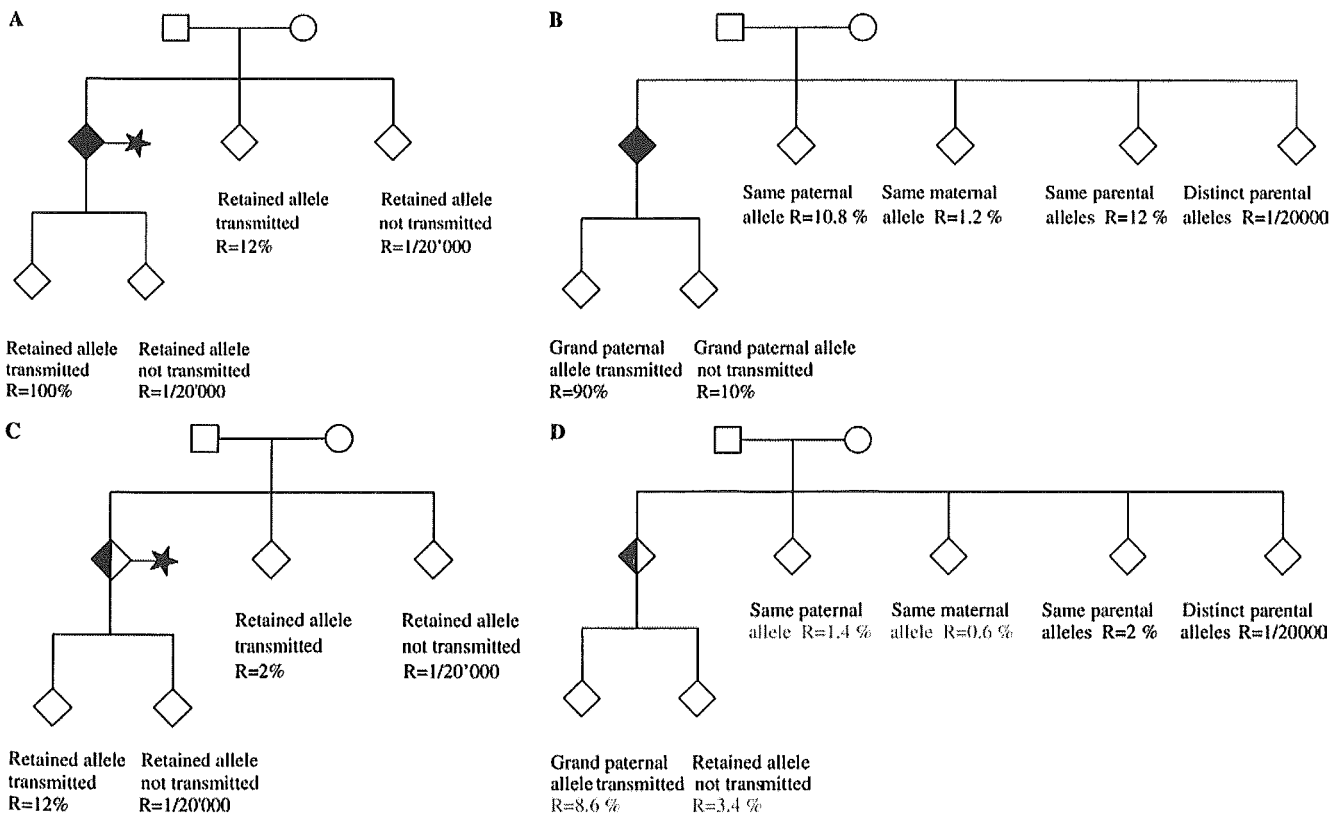

FIGURE 1 (A) Presence of loss of heterozygosity (LOH). Modification of risk in offspring (a priori risk=50%) and in siblings (a priori risk=6%) of bilaterally affected proband. (B) Absence of LOH. Modification of risk in offspring (a priori risk=50%) and in siblings (a priori risk=6%) of bilaterally affected proband. (C) Presence of LOH. Modification of risk in offspring (a priori risk=6%) and in siblings (a priori risk=1%) of unilaterally affected proband. (D) Absence of LOH. Modification of risk in offspring (a priori risk=6%) and in siblings (a priori risk=1%) of unilaterally affected proband.

RB1 mutations in sporadic unilateral Rb were de novo, 29% mosaics in nature, and 17% transmitted from an unaffected parent (incomplete penetrance, two from the father and two from the mother). Therefore, the preferential paternal origin in cases of sporadic unilaterally affected patients can be estimated at $90\% \times 54 + 50\% \times 46 = 72\%$ [90% (preferential paternal origin) of 54% (de novo mutants) + half (absence of preferential paternal origin) of 46% (mosaic and transmitted mutants)]:

- $2 \times 1\% = 2\%$ risk if the same parental alleles are transmitted to the sibling, $2 \times 1 \times 72\% = 1.4\%$ if the paternal allele is transmitted, $2 \times 1 \times 28\% = 0.6\%$ if the maternal allele is transmitted and 1/20,000 if both alleles are distinct.
- $2 \times 6 \times 72\% = 8.6\%$ risk in case of inheritance of the grandpaternal haplotype in offspring, versus $2 \times 6 \times 28\% = 3.4\%$ for the grandmaternal.

Finally the new modified calculated risk of Rb's recurrence was adjusted to a penetrance of 90%.[15]

The siblings and offspring of affected patients were then classified into two categories: (a) low-risk (≤1.08 %), and (b) significant-risk (>1.08%).

RESULTS

A total of 304 kindreds with sporadic Rb were enrolled and analyzed. Out of these 304 index patients, 134 had first degree relatives and were included. Seventy-nine (59%) Rb patients were unilateral and 55 (41%) bilateral. Linkage analysis was based on the segregation of the RB1 allele in a total of 570 individuals. There were 168 first degree relatives of the index patient. Eighty-four out of 146 siblings, 15 out of 22 offspring who were younger than 48 months of age at the time of the linkage analysis and older than 48 months of age by December 2006, were enrolled. Mean age was 26.9 ± 17.2 months (range from birth to 47 months of age). Mean follow up time was of 60.8 ± 15.7 months.

Classical Clinical Follow-up

During this time, one new case of Rb was diagnosed from among the sibling's group at 8 months of age; from a significant-risk relative Rb's recurrence of $1.4 \times 90\% = 1.26\%$ upon our algorithm, 6 months after the first examination and linkage analysis. It was a unilateral Rb due to a R255X mutation, stage C (ABC classification) that was successfully cured by conservative therapy consisting of chemo-, thermo-, and cryo-therapy without radiotherapy. The remaining patients were followed until the age of 4 years and did not develop Rb. All the patients ($n = 69$) older than 4 years were also examined clinically and none of them had Rb.

TABLE 3 New cumulated calculated recurrence risk of retinoblastoma.

Number of patients	Low risk	Significant risk	Cumulated calculated risk (%)
Siblings	61	23	116.2
Offspring	8	7	61.3

Expected Occurrence of New Patients with Rb using our Algorithm

Linkage analysis was informative in 129 (96%) families. Molecular analysis of tumor tissue identified 23 LOH out of 38 (61%). Sixty-one of these siblings were classified as low-risk of which 21 were at no risk given the inheritance of an entirely different haplotype than the proband and 23 patients were considered at significant risk.

Eight of 15 offspring were at minimal risk of which one was at no risk. Seven patients were of significant risk. The data are summarized in Table 3.

Applying our algorithm to this cohort of patients, we expected 1.77 new declared cases of Rb (cumulated risk of recurrence: 1.162 for the siblings group and 0.613 for the offspring) during the pre-defined follow-up period. It should be noted that none of the patients with a different haplotype to the index patient developed Rb and that the new Rb patient occurred in the group at significant risk.

DISCUSSION

Retinoblastoma is a malignant tumor that originates from progenitors of retinal sensory cells. Affected children will therefore develop Rb very early in life, during the period of cellular maturation. In contrast, Rb is extremely rare in adults, where it sometimes arises from retinoma, a related benign lesion.

Survival rate and prevention of blindness in Rb patients depend on accurate and early diagnosis. If Rb is newly diagnosed in a family, examination of the retina is mandatory in all relatives at risk to exclude the presence of retinal tumor. Genetic counseling is required to identify relatives with an increased risk. If relatives at risk are still in early childhood, repeated EUAs are mandatory. Therefore, the primary goal of molecular testing is to identify high-risk individuals while avoiding repeated examinations (a total of nine EUAs is necessary to cover the period of potential retinal oncogenesis). Classically, genetic counseling of families with sporadic Rb has been based on a priori risk derived from the compilation of empiric data.[22] The a priori risk of recurrence for siblings of sporadic bilateral Rb patients is 6% and 50% for offspring. In sporadic unilateral Rb, these risks are 1% and 6% respectively. All patients at risk are then placed under a rigorous program of ophthalmoscopic EUA, despite the fact that only a fraction of them has inherited the Rb

predisposition. To overcome the genetic burden in relatives of index Rb patients, it has been proposed more recently to screen directly for the presence of the disease-causing RB1 mutation. Noorani demonstrated that identification of the oncogenic mutation is cost-effective when compared to conventional screening consisting of repeated EUAs.[13] However, their study was based on molecular scanning of the 180-Kb RB1 gene, which is hampered by a low yield of only 40–45% (80–90% in bilateral cases and 10–15% in unilateral cases). In other words, a significant proportion of families are left without molecular diagnosis. This suggests that, nowadays, no single methodology will be fully sensitive, accurate and economic.

In the current study, an algorithm based on linkage analysis identified patients with true risk, except in situations where a sporadic unilateral Rb patient is a low proportion mosaic for a large RB1 gene deletion. In such a case the risk would be overestimated in the sibling, whereas the risk prediction in the offspring would tend towards either overestimation or loss of informativity. This strategy is efficient (informative in 96% of the families) and can be universally applied. More importantly, the expected recurrence risk of Rb in relatives was in accordance with the natural observed recurrence rate in our cohort without false negative cases. Applying our algorithm, we would have been able to avoid unnecessary EUA surveillance in 69 of 99 (69.7%) patients, with a total of 362 EUAs circumvented. Therefore patients at significant risk should be automatically assigned to a follow-up under narcosis until 4 years of age, although, Abramson reported that the majority of the Rb is diagnosed before the age of 28 months.[26] While in minimal-risk patients, this heavy surveillance can be replaced by a regular ophthalmoscopy without anaesthesia.

RFLP and VNTR analysis are less used and our institution has been primarily proceeding with direct mutation screening since 2006. However, when necessary, we are using polymorphic short tandems (STR) as recommended by the Best Practice Guidelines of the European Molecular Genetics Quality Network (EMQN) for indirect testing of RB1.[17] This algorithm represents a good alternative when mutational screening failed to identify the disease-causing mutation. It is also a cost-effective approach in centers with limited financial resources. In addition, linkage analysis has never been validated for the detection of risk recurrence in sporadic cases of Rb and the present algorithm proved to be reliable in a long-term follow up to modify the a priori risk of Rb transmission.

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