Germline mutations in retinoma patients: Relevance to low-penetrance and low-expressivity molecular basis

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Purpose: To study phenotype-genotype correlation in patients who have retinoma, which is a benign tumor resembling the post irradiation regression pattern of retinoblastoma (RB).

Methods: We selected patients who had retinoma and positive family history for RB and patients who had retinoma in one eye and either retinoma or RB in the other eye. The study included 22 patients with available DNA: 18 from 11 families and four sporadic cases. DNA was extracted from peripheral blood leukocytes. The RB1 gene was screened by DHPLC and direct sequencing of the promoter and all the exons.

Results: We identified 17 occurrences of 11 distinct germline mutations in two sporadic and in 15 familial cases (nine families). The 11 identified mutations were located in exons 1, 10, 11, 13, 14, and 19 to 23. Four of the identified mutations were not previously reported, including g.64407delT, g.153236A>T, g.156743delTCTG, and g.162078delA. Eight out the 11 mutations were truncating and three were nontruncating (missense). There was no correlation between the type of mutation and the number of tumor foci per eye (RB or retinomas). Highly heterogeneous intrafamilial expressivity was observed.

Conclusions: To our knowledge, this study is the largest series of mutations of consecutive retinoma patients. The present data suggest that the type of inherited mutations underlying retinoma is undistinguishable from RB related ones, i.e., largely dominated by truncating mutants. This finding is in contrast with the RB1 genotypic spectrum of mutations associated with low-penetrance RB, i.e., nontruncating mutants. The molecular mechanism underlying low-penetrance and attenuated expressivity (retinomas) appeared to be distinct.

Mutations of the RB1 gene can result in either malignant retinoblastoma (RB; OMIM 180200) or benign retinoma [1]. Retinoma mimics the postirradiation regression pattern of RB [1-4]. On histology, necrosis and mitoses are absent in contrast with RB [5]. According to Knudson’s observation, both alleles of the RB1 gene must be inactivated to develop a tumor [6]. Hereditary germline mutations account for 40% of cases and nonhereditary somatic mutations for the remaining 60% [7]. For hereditary cases, mode of inheritance is autosomal dominant; 90% of germline-mutation carriers develop RB or retinoma (high penetrance) with most of them presenting multiple tumors in both eyes (high expressivity). Nevertheless, low penetrance is observed when germline mutation carriers do not develop RB and reduced expressivity when only unilateral RB or retinoma occurs [8,9]. To better describe low-penetrance RB families with regard to both penetrance and expressivity, Lohmann et al. [9] introduced the disease-eye ratio (DER), which is the ratio of the number of eyes containing tumors to the number of mutation carriers in a family. Typically, diseased-eye ratios are less than 1.0 in low-penetrance families and 1.5 or greater in full-penetrance families [9].

In 70% of RB cases loss of the wild-type allele, or loss of heterozygosity happens through mitotic recombination or nondisjunction with or without concomitant duplication of the mutated chromosome [10]. If the predisposing mutation is truncating, one could expect that loss of heterozygosity would lead to complete loss of functional protein and thus to RB with high penetrance and expressivity [8,9,11,12]. In contrast, the molecular basis of low-penetrance RB is dominated by nontruncating mutations [8,9,11,12]. Functionally, the RB1 mutations causing low-penetrance RB were shown either to reduce the level of expression of normal RB1 protein or produce a mutant RB1 protein that is only partially inactivated [8,9]. However, recent insights strongly call into question this assumption with the report of a chain-terminating mutation in RB1 exon 1 in a large low-penetrance family with unilateral RB and retinoma [13].

In this report, we correlate the clinical features of 17 retinoma patients to their underlying RB1 germline mutations. Results are discussed in light of the recent advances regarding the molecular basis of RB oncogenesis.

METHODS

The study was conducted in accordance to the tenets of the Declaration of Helsinki. Out of more than 500 patients with RB and their first-degree relatives treated between 1964 and 2008, we selected patients who had retinoma and a positive
family history of RB or patients who had retinoma in one eye and either RB or retinoma in the other eye. Patients were referred to us either from university eye clinics and private Swiss practitioners or from neighboring European countries. All patients were examined and treated at the Retinoblastoma Clinic of the Jules-Gonin Eye Hospital, Lausanne, Switzerland. All patients and their first-degree relatives underwent full ophthalmic examination and were documented by fundus photographs and ultrasonography. Fluorescein and indocyanine angiography was performed in selected patients. Previously described diagnostic criteria for retinoma and phthisis were used to identify the retinoma subpopulation.

| Region       | Primer | Sequence 5′-3′                  | Amplicon [bp] | Annealing [°C] |
|--------------|--------|--------------------------------|---------------|---------------|
| promoter     | PromF  | CTGGAGCCACGGCAGGTTCT            | 340           | 61            |
|              | PromR  | GTTTTGCGGCGCATGACCTT            | 340           | 56.4          |
| RB exon 1    | 1F     | CCGGTTTTTCTCATAGGGAGTTTG       | 331           | 54.3          |
|              | 1R     | TTGGCCCGCCCTCTACGCAC           | 281           | 52.8          |
| RB exon 3    | 3F     | CAGTTTTAATCATAGTTACCAG         | 281           | 54.3          |
|              | 3R     | AGCATTTCTCTACTATTAC            | 305           | 55            |
| RB exon 5    | 5F     | CATGGAAGAAACTACATGAC           | 194           | 54.3          |
|              | 5R     | CACCAAAAAGATTATCTGGA           | 222           | 54.3          |
| RB exon 7    | 7F     | CCTGCCATTTTCTCTCTAC           | 256           | 55            |
|              | 7R     | ATGTGTTGTGATCCACTAGAC         | 380           | 50.8          |
| RB exon 9    | 9F     | TGCTCTTGCAAGTTACAG            | 222           | 56            |
|              | 9R     | AGTCTGACCAATATTTCCC           | 291           | 56            |
| RB exon 10   | 10F    | TCTTTAATGAAAATCTGTTG          | 245           | 54.2          |
|              | 10R    | GATATCTCTAAAGTGCAAAACTG        | 310           | 52.5          |
| RB exon 11   | 11F    | GAGACAACAGAAGGATTATAC         | 342           | 54.3          |
|              | 11R    | CGTGAACAAATCTGAAAACC          | 212           | 58.9          |
| RB exon 12   | 12F    | GGCAGTGTATTTGAAGATAC          | 273           | 56            |
|              | 12R    | AACACTACGTGTTAGATGG           | 335           | 56.4          |
| RB exon 13   | 13F    | CTTATGTTGATGTGTTG             | 305           | 55            |
|              | 13R    | TATACGAACCTGGGAAAGATG         | 273           | 52.5          |
| RB exon 14   | 14F    | GTGATTTTCTCTAATAAGCAGG        | 310           | 50.8          |
|              | 14R    | TGCTCCAGCCACTCTCAGTCT          | 366           | 55            |
| RB exon 15+16| 15/16F | CATGCTGACAGAAAATAAGGG         | 339           | 56            |
|             | 15/16R | AGCATTCCTCTCCTCTAACC          | 340           | 56.4          |
| RB exon 17   | 17F    | AAAATAATCTCACCCTGCAAGGG       | 281           | 54.3          |
|              | 17R    | TTATGAAAAACACCTTCAAC          | 287           | 54.2          |
| RB exon 18   | 18F    | TGCTACCGGAAAATATGAC          | 313           | 56            |
|              | 18R    | CTTATTTGCTCTGATTG             | 313           | 56            |
| RB exon 19   | 19F    | ATAATCTGTGATCTTTACG          | 273           | 56            |
|              | 19R    | AAAGAAACATGATTGAACCC         | 335           | 56.4          |
| RB exon 20   | 20F    | AAAGAGTGGTGAGAAAGAGG         | 328           | 54            |
|              | 20R    | CAGTTAACAAGTAAAGTTAGGG        | 340           | 56.4          |
| RB exon 21   | 21F    | AAACCTTCTCTTTTTTGGGCG        | 313           | 56            |
|              | 21R    | TACATAAATAGCTGACAGC          | 313           | 56            |
| RB exon 22   | 22F    | TAATATGCTCTTCTACAGTCT         | 222           | 56            |
|              | 22R    | TTAATGTTTTTTTGGGAGCC         | 287           | 54.2          |
| RB exon 23   | 23F    | ATCTAATGTAATGGTGCTCC         | 273           | 52.5          |
|              | 23R    | CTGGGATAAAAAATATCCCC         | 273           | 52.5          |
| RB exon 24   | 24F    | GAATATGTTTTTCTCGTGGTTC       | 297           | 54.2          |
|              | 24R    | GGTGTGTTGAAATCTGCGTTG         | 297           | 54.2          |
| RB exon 25   | 25F    | GGGTGTCAATCTGAGAAAC         | 209           | 52.8          |
|              | 25R    | AGAAATTTGGGTAATAGGCC         | 209           | 52.8          |
| RB exon 26   | 26F    | AGTAAGTCATCGAAAAGACATC       | 237           | 54.2          |
|              | 26R    | AAGCAGAG Acer TCTGCGCA      | 237           | 54.2          |
| RB exon 27   | 27F    | CGCACCAGTTGAGCATGAG         | 237           | 54.2          |
DNA was extracted from blood leukocytes and used for PCR amplification. Genetic analysis was performed at the Institut de Recherche en Ophtalmologie. Previously described denaturing high-performance liquid chromatography (DHPLC) [14] and sequencing were used. Amplification was performed in a thermal cycler (GeneAmp 9700; Applied Biosystems, Foster City, CA), in a total volume of 30 μl. Each primer was fluorescently labeled, and the product was separated on an automated sequencer (ABI XL3100; Applied Biosystems). Sequences were aligned using the Chromasversion 2.23 (Technelysium, Tewantin, Australia). Screening for large deletions was performed by haplotype analysis using RBL1 flanking microsatellites D13S161, S13S164, D13S153, D13S1307, and D13S273. One primer was fluorescently labeled, and the product was separated on an automated sequencer (ABI XL3100; Applied Biosystems).

RESULTS

Out of the 22 selected patients, 18 were familial from 11 families and four were sporadic. Out of the 18 familial cases, we identified mutations in 15 cases, which were from nine families, and we found mutations in two of the four sporadic cases. Thus, we had 11 index cases in total with proven germline mutations. Patient clinical features and mutation descriptions are detailed in Table 2.

DISCUSSION

We studied 22 patients with retinomas and detected RBL1 mutations in 17 (15 familial cases and in 2 sporadic) of them. The 15 familial cases belonged to nine families that were not low-penetrance ones (DER 1.1 to 2). In accordance with the results of Sanchez-Sanchez et al. [13], the observation of retinoma with predisposing nonsense germline mutation does not support the hypothesis that truncating mutations do not cause retinoma. Indeed, truncating mutations were found in 70% of retinoma patients in this study. We tried to determine if a certain type of mutation could cause higher expressivity leading to multiple tumor foci in each eye, but there was no correlation between the type of mutation and the number of tumor foci per eye (RB or retinoma). In our cohort most mutations were either nonsense mutations, duplication or deletions. Although we did not analyze the consequences of the observed mutations at the RNA level, we can reasonably assume that they represent truncating mutations. The case of the two remaining mutations, R46K and E737D, is more complicated. They could represent true missense mutations or could affect splicing of the nearby intron. Their pathogenicity is not questionable, as they have been reported to cause bilateral RB [15]. We have previously described the L42RfsX25 mutation located in exon 1 [11]. Alternative translation might be the mechanism by which different levels of expressivity are present within this family (F1, Figure 1).

In contrast with the family studied by Sanchez-Sanchez [13], all nine families in our series had DER above 1.0, suggesting absence of low-penetrance. That the mean number of retinoma foci per eye was 1.88±1.5. Four of the identified mutations were not previously reported (Table 2).
Table 2. Clinical Features and Mutations Description of 17 Affected Patients with Hereditary Retinoblastoma and/or Retinoma Harboring RB1 Mutations.

| Patient number | Family number | Age at diagnosis | Disease-eye ratio | Right eye foci | Left eye foci | Mutation location | DNA Alteration | Protein Alteration |
|----------------|---------------|------------------|-------------------|----------------|---------------|-------------------|----------------|-------------------|
| 1              | F1            | 43 y             | 1.3               | -              | 1 re          | 1                 | g.2179_2183dupGGACC | L42RfsX25        |
| 2              | F2            | 2 y              | 1.1               | 2 re           | Rb            | 1                 | g.2196G>A         | R46K             |
| 3              | F2            | 4 y 2 m          | 1.1               | -              | 1 re          | 1                 | g.2196G>A         | R46K             |
| 4              | F2            | 62 y             | 1.1               | -              | 1 re          | 1                 | g.2196G>A         | R46K             |
| 5              | F3            | 33 y             | 1.7               | 4 re           | 2 re          | 10                | g.64407delT*      | 348X             |
| 6              | F4            | 5 y 8 m          | 1.8               | 3 re           | 1 re          | 11                | g.65386C>T        | R358X            |
| 7              | F4            | 8 m              | 1.8               | 2 re           | Rb            | 11                | g.65386C>T        | R358X            |
| 8              | F4            | 30 y 8 m         | 1.8               | 1 re           | 1 re          | 11                | g.65386C>T        | R358X            |
| 9              | S             | 6 y 10 m         | -                 | 1 re           | 1Rb           | 13                | g.73843C>T        | Q436X            |
| 10             | F5            | 35 y 10 m        | 1.4               | 2 re           | 1 re**        | 14                | g.76403C>T        | R445X            |
| 11             | S             | 39 y             | -                 | 2 re           | 1 re**        | 19                | g.153236A>T*      | K615X            |
| 12             | F6            | 1 year 6 m       | 2                 | Rb             | 1 re          | 20                | g.156743delTCTG*  | 675X             |
| 13             | F7            | 32 y 2 m         | 2                 | 1 re           | 2 re          | 21                | g.160834G>C       | E737D            |
| 14             | F8            | 40 y 2 m         | 1.7               | 1 re           | 1 re          | 22                | g.162078delA*     | A7666X44         |
| 15             | F8            | 36 y 11 m        | 1.7               | 3 re           | 1 re          | 22                | g.162078delA*     | A7666X44         |
| 16             | F9            | 8 y 9 m          | 1.3               | 7 re           | 5 re          | 23                | g.162237C>T       | R787X            |
| 17             | F9            | 1 y 10 m         | 1.3               | Rb             | 1 re          | 23                | g.162237C>T       | R787X            |

All retinoma patients are presented in this table. All familial retinoma patients belong to families which are not low-penetrance ones with DER>1. There is no correlation between the number of tumor foci and the type of mutations. Identified mutations were distributed over several exons. Abbreviations: sporadic (S), familial (F), year (y), month (m), retinoblastoma (Rb), retinoma (rc), deletion (del), frameshift (fs), novel mutation (*), malignant transformation into retinoblastoma (**).
germline mutations in retinoma cases. That we did not detect mutations in two families and two sporadic cases might be due to the technology applied that would not detect copy number changes of exons or most mosaicism. However, the 73% of \(\text{RB1}\) mutations (11 mutations found in 15 participants who had bilateral or familial RB) detected by the technologies applied suggests that the spectrum of mutations associated with retinoma is the same as for RB. Further work is needed in collaboration with other laboratories to use other mutation screening technologies to identify mutations in the remaining cases and to analyze the consequences of the observed mutations at the RNA level. This is beyond the intended scope of the present work. Although no statistical analysis was possible in this study, in accordance with other published series [4,16], we propose that retinoma should not be considered a form of attenuated expressivity [17-22], but rather the result of variable expressivity occurring in a penetrance independent manner.

Gallie et al. [23] have hypothesized that the stage of cell maturation at which the second \(\text{RB1}\) mutation occurs is determinant for the phenotype expression. We observed one case in this series, Patient 11, who underwent malignant transformation of his retinoma, at an adult age. This observation has been previously reported by others [2,4,23, 24] and may support the stage of cell maturation theory with the understanding that a primitive cell could have remained latent for many years before becoming activated in the eye of an adult [23]. Another mechanism has been proposed to understand attenuated expressivity represented in this study by retinoma. Recent histopathological analysis of eyes enucleated for RB showed retinoma tumors adjacent to both normal retina and RB tumors in up to 15.6% (20/128), suggesting clonal progression from a normal cell to a benign one and finally to a malignant cell [16]. Other researchers have [16] reported molecular evidence of this clonal progression, showing that retinomas and RBs were homozygous null for \(\text{RB1}\), could share the same mutation of whatever class, including stop codon, and that retinomas expressed senescence proteins maintaining them in an arrested state. Retinomas displayed genomic changes such as gain of oncogenes and genomic instability to a lesser degree than RB, which is thought to develop by escaping the senescence state of retinomas [16]. This hypothesis of increasing genomic instability for the development of RB is in accordance with the results we present. Unfortunately, we were not able to study the type of the second-hit mutation, which might be determinant, too, in cell destiny.

Conclusions: RB development understanding remains a challenging but mandatory task due to the life-threatening complications it can induce. Its causative gene, \(\text{RB1}\), has opened the way to the two-hit theory [6], the limitations of which have been highlighted by the study of retinoma, a benign tumor. For a long time, retinoma has been considered to be part of the low-expressivity as well as low-penetrance
presentation and thought to be caused by less severe inherited mutations. We have shown in this series that even severe inherited mutations segregating in families with bilateral RB patients can also cause retinoma. Recently, it has been shown that RB emerges from retinoma after accumulation of genomic changes, whereas retinoma develops after homogeneous loss of RB1 [16]. Thus, what we used to consider as low or attenuated expressivity should be revisited as a step in the cell pathway to malignant tumor development. Genetic counseling, treatment, and follow-up recommendations may highly be influenced by such advances in RB and retinoma development understanding.

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