Experimental Eye Research 146 (2016) 313–317

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Research article

Genotype-phenotype correlation in the presentation of retinoblastoma among 149 patients

Shahar Frenkel [a, *, 1], Ofira Zloto [a, 1], Michal Sagi [b], Avishag Fraenkel [b], Jacob Pe'er [a]

[a] Department of Ophthalmology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel
[b] Departments of Human Genetics and Metabolic Diseases, Hadassah-Hebrew University Medical Center, Jerusalem, Israel

ARTICLE INFO

Article history:
Received 17 February 2016
Received in revised form
25 March 2016
Accepted in revised form 4 April 2016
Available online 8 April 2016

Keywords:
Retinoblastoma
Genotype-phenotype
Genetic counseling
Germline mutations
RB1

ABSTRACT

In this work, we describe the association between a germline *RB1* mutation and disease presentation characteristics of retinoblastoma. The study evaluates a retrospective cohort of 164 of the 295 patients with retinoblastoma who were treated at a single center between 1988 and 2013 and who were referred for genetic evaluation. Peripheral blood was evaluated for *RB1* mutations via Multiplex Ligation-dependent Probe Amplification (MLPA), sequencing, and detection of recurrent CpG transition mutations. Patients with an *RB1* mutation were compared to patients without a mutation, regarding epidemiological factors and clinical presentation. Genetic analysis was completed for 149 patients. An *RB1* mutation was identified in 76 children (51.0%) including 90.0% of the bilateral patients, and 19.8% of the unilateral unifocal patients (24.7% if we include the unilateral multifocal cases). The most common mutations were a stop codon (38.2%), a splicing error (19.7%) and a large deletion (15.8%). The mutation type correlated only with sex (Likelihood ratio, $p = 0.0240$) and with macular involvement (Likelihood ratio, $p = 0.0591$ and Fisher's exact one tail test $p = 0.0459$ for more macular involvement if there are germline mutations). It did not correlate with laterality, with the reason for referral, or with diagnosis age. However, identification of a mutation was more common in babies diagnosed under one year of age (Likelihood ratio, $p < 0.0001$). In conclusion, we were surprised that our genetic tests have also found mutations in 24.7% of patients with unilateral retinoblastoma in addition to most of the bilateral children. These unilateral patients with a germline mutation have an increased risk for other cancers throughout their lives, and their first-degree relatives have an increased risk for retinoblastoma. Therefore, genetic testing for RB1 mutation should be offered to all patients, including the unilateral cases.

1. Introduction

Retinoblastoma is the most common intraocular malignancy in children. According to the Surveillance, Epidemiology and End Results (SEER) Program database, the tumor occurs in 11.8 per million children in ages 0–4 years in the United States (Broaddus et al., 2009). There are no sex or race differences in incidence (Broaddus et al., 2009). Most of the cases were diagnosed before the patients were 2 years old, and 95% of the cases were reported before the patients were 5 years old (Broaddus et al., 2009).

RB1 is a tumor suppressor gene located on chromosome 13q14 (Cowell, 1989). Retinoblastoma results when both *RB1* alleles are mutated and become inactive, and it occurs in two genetic forms: hereditary or sporadic. 40% of the cases are hereditary, autosomal dominant with high penetrance (Shields et al., 1991). In the hereditary form, the first mutation occurs early in development and affects all germline cells. However, this mutation alone doesn't cause retinoblastoma. The tumorigenesis starts when a second "hit", which occurs later on, affects the undamaged allele, and results in retinoblastoma when the second hit occurs in the developing retina cells (Knudson et al., 1975). The sporadic form arises from two spontaneous mutations affecting both alleles in the same somatic cells of the retina (Dyer and Bremner, 2005; Knudson et al., 1975).

Due to the widespread distribution of mutation-containing cells patients with bilateral or unilateral multifocal disease may pass the

* Corresponding author. Specialized Ocular Oncology Service, Department of Ophthalmology, Hadassah-Hebrew University Medical Center, POBox 12000, Jerusalem, 91120, Israel.
E-mail address: shahar.frenkel@gmail.com (S. Frenkel).
[1] Both authors contributed equally as first authors.

http://dx.doi.org/10.1016/j.exer.2016.04.002
0014-4835/

disease on to their children. However, a unilateral disease can be either sporadic (with a somatic mutation) or hereditary (with a germline mutation). In most of the cases, patients with a bilateral disease are diagnosed earlier than patients with a unilateral disease, in any stage of the disease (Rubenfeld et al., 1986).

Patients with the heritable disease are at an increased risk compared with the general population to develop other tumors through their life (Kleinerman et al., 2007; Wong et al., 1997). The most common second tumors are osteogenic sarcoma and soft tissue sarcomas. These patients are also at increased risk to develop malignant melanoma and benign and malignant neoplasm of brain and meninges (Eng et al., 1993). The patients with the sporadic form do not have this increased risk (Kleinerman et al., 2007; Wong et al., 1997). Therefore, patients with the hereditary mutation are monitored for second tumors through their life.

Our center is a national ocular oncology referral center in Israel. In recent years, we refer all patients with bilateral or unilateral-multifocal retinoblastoma for genetic counseling with molecular genetic testing for germline mutations in the *RB1* gene. Patients with unilateral retinoblastoma have also been sent to genetic counseling, but less adamantly than the bilateral patients. We also recommend parents and siblings of patients with a germline mutation in *RB1* gene to undergo these tests.

The purpose of our study was to describe the association between the detection of an *RB1* mutation and disease characteristics in the presentations of patients with retinoblastoma. The outcome of this study can clarify who should be recommended to undergo genetic counseling.

2. Methods

2.1. Patients and blood samples

This retrospective study included a cohort of 295 patients who have been diagnosed with retinoblastoma and treated at the Israeli national referral center (Hadassah-Hebrew University Medical Center, Jerusalem, Israel) between 1988 and 2013. All of them were referred to genetic counseling and *RB1* mutation analysis. However, only 164 patients attended genetic counseling and underwent testing for *RB1* mutations. In the 11 families in which two relatives were found to have the same mutation only one of them was entered into the statistical analysis to avoid bias in the evaluation of the diagnosis age and the presenting symptoms. The Hadassah-Hebrew University Medical Center Institutional Review Board approved the use of patients' data.

2.2. Molecular analysis

Genomic DNA was extracted from peripheral blood using standard techniques. When required and available, genomic DNA was also extracted from paraffin embedded tumor tissue using standard techniques. Detection of large deletions/duplications was performed with Multiplex Ligation-dependent Probe Amplification kit (MLPA RB1 P047, MRC- Holland). Sequencing of exons 1–26 (including promoter sequence to −290) was performed with specific primers located in the introns (primer sequences available upon request) and the Big Dye dideoxy terminator kit using the ABI 3130 sequencer and software (Applied Biosystems). Detection of recurrent CpG transition mutations performed by Allele Specific-PCR designed for each mutation. PCR products were run on 3% Nusieve Agarose gel visualized with UV light. In one case, samples were sent for analysis in Prof. Brenda Gallie's laboratory (The Hospital for Sick Children, Toronto, Canada) where it was diagnosed as a case of *MycN* amplification.

2.3. Statistical analysis

The group with mutations was compared to the group without mutations in the following parameters: bilateral or unilateral disease, multifocal disease, sex, the age of diagnosis, macular involvement (a tumor arising within the macular area), and the reason for referral. Distribution analysis of mutations' type was performed.

Statistical analysis including distribution analysis, correlations (Pearson) and associations (likelihood ratio), and a stepwise regression model to evaluate what most affects the diagnosis age was performed with JMP Statistical Discovery Software 7.0 (SAS Institute, Cary, NC, USA).

3. Results

Out of the 295 retinoblastoma patients in our database, 164 followed the recommendation to receive genetic counseling and analyze the blood for *RB1* mutations. Results of the genetic evaluation were completed for 149 (90.8%) of them (the study group).

There were similar numbers of girls (75, 50.3%) and boys (74, 49.7%). Sixty of the children (40.3%) had bilateral disease, 89 (59.7%) had unilateral disease of them 8 (5.4%) had unilateral multifocal (UniM) disease. Among the unilateral cases, there was a similar distribution of the laterality of the involved eye (RE 46.9%, LE 53.1%). In 11 children, there was a family history of retinoblastoma (only a single representative of each family was entered into this study group).

The mean (±SD) age at diagnosis was 18.5 ± 19.8 months (range 1–132), median diagnosis age 13 months. Children presenting with a bilateral disease were diagnosed earlier (8.7 ± 2.3 vs. 24.6 ± 2.0 months (mean ± SD), $p < 0.0001$) (Fig. 1). Children were referred for an abnormal pupillary reflex (mostly leukocoria) in 43.0% of cases, for strabismus in 28.5%, for a combination of leukocoria and strabismus in 9.8%, for complaints of visual disturbance in 4.1%, and for other reasons in 13.8% (e.g. an abnormal finding in a routine examination, or when evaluating repeated events of a red eye).

The distribution of the severity grouping of the eyes according to the International Classification of Retinoblastoma (ICRB) (Linn Murphree, 2005) was similar between eyes (Fig. 2) with a quarter of the eyes grouped as B or E, 10% as group A, and 15% as either C or D. Interestingly, when comparing the groups of the bilateral cases between eyes, the right eye tended to have a higher, worse group ("mean" C vs. B, paired Wilcoxon $p = 0.1328$).

Documentation of whether the macula was involved or not was available for 117 patients and was positive in 40 (34.2%) cases. In this subset of patients it was present in 25.0% of the unilateral cases, in 38.8% of at least one eye of the bilateral cases, and in 6/8 (75.0%) of the unilateral multifocal cases (43.9% of the bilateral and UniM combined). This distribution indicates a significant association between having a germline mutation and macular involvement (Chi Square likelihood ratio $p = 0.0591$, and Fisher's exact one tail test $p = 0.0459$ for more macular involvement if there are germline mutations).

Genetic analysis of peripheral blood identified an *RB1* mutation in 76 children (51.0%). Eight children (five unilateral and three bilateral cases) had mosaicism of 10–30%. In one boy who was diagnosed with an eye filled with tumor at the age of 2 months, and in whom genetic analysis of peripheral blood did not identify an *RB1* mutation, we also analyzed tissue from the enucleated eye. The eye was enucleated after two courses of chemotherapy, and analysis indicated that the tumor had *MycN* amplification rather than an *RB1* mutation.

The most common mutations were a stop codon (38.2%), splicing errors (19.7%) and large deletions (15.8%) (Table 1). The