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Genetic testing in Tunisian families with heritable retinoblastoma using a low cost approach permits accurate risk prediction in relatives and reveals incomplete penetrance in adults

Hajer Ayari Jeridi [a,*], Hédi Bouguila [b], Birgit Ansperger-Rescher [c], Olfa Baroudi [a], Imen Mdimegh [a], Ines Omran [a], Khaoula Charradi [a], Hssan Bouzayene [a], Amel Benammar-Elgaaïed [a], Dietmar R. Lohmann [c]

[a] Laboratoire de Génétique Immunologie et Pathologies Humaines, Faculté des Sciences de Tunis, Université de Tunis EL MANAR, Campus universitaire, Tunis 2092, Tunisia
[b] Institut Hédi Rais d'Ophtalmologie de Tunis, Tunisia
[c] Eye Cancer Research Group, Institut für Humangenetik, Universitätsklinikum Essen, Germany

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ABSTRACT

Heritable retinoblastoma is caused by oncogenic mutations in the *RB1* tumor suppressor gene. Identification of these mutations in patients is important for genetic counseling and clinical management of relatives at risk. In order to lower analytical efforts, we designed a stepwise mutation detection strategy that was adapted to the spectrum of oncogenic *RB1* gene mutations. We applied this strategy on 20 unrelated patients with familial and/or *de novo* bilateral retinoblastoma from Tunisia. In 19 (95%) patients, we detected oncogenic mutations including base substitutions, small length mutations, and large deletions. Further analyses on the origin of the mutations showed mutational mosaicism in one unilaterally affected father of a bilateral proband and incomplete penetrance in two mothers. In a large family with several retinoblastoma patients, the mutation identified in the index patient was also detected in several non-penetrant relatives. RNA analyses showed that this mutation results in an in-frame loss of exon 9. In summary, our strategy can serve as a model for *RB1* mutation identification with high analytical sensitivity. Our results point out that genetic testing is needed to reveal or exclude incomplete penetrance specifically in parents of patients with sporadic disease.

1. Introduction

Retinoblastoma (MIM 180200) is a childhood ocular cancer that originates from the developing retina. Two mutational events that functionally incapacitate both alleles of the *RB1* tumor suppressor gene are critical for the development of most retinoblastomas (Knudson, 1971; Lee et al., 1987; Thériault et al., 2014). Clinically, retinoblastoma may affect one or both eye (unilateral and bilateral retinoblastoma, respectively). Some patients have close relatives who have also developed retinoblastoma (familial retinoblastoma) but most patients have not (sporadic retinoblastoma). The most important factor that determines the kind of clinical presentation is the timing of the first mutational event (Carlson and Desnick, 1979; Lohmann et al., 1997). In familial retinoblastoma the first mutation has occurred in some ancestor and is transmitted via the germline. Family members that are heterozygous for the mutant allele have a risk to develop retinoblastomas. Each tumor focus is initiated by an independent somatic inactivation of the second *RB1*. Because of this two step mechanism, occurrence of this tumor in families shows an autosomal dominant mode of inheritance (heritable retinoblastoma). Individuals heterozygous for a mutation that occurred *de novo* in the germline of one of the parents also have heritable retinoblastoma although they present as sporadic cases. Individuals with heritable retinoblastoma have a greater than usual lifetime risk for development of primary neoplasms other than retinoblastoma (Eng et al., 1993; Marees et al., 2008). Patients in whom the first step of mutational inactivation of the *RB1* gene has occurred in a retinal progenitor cell have non-heritable retinoblastoma because the mutant allele is not present in their germline cells. In some patients, the first mutational event has occurred *de-novo* as post

* Corresponding author. Tel.: +216 23820172; fax: +216 71807432.
E-mail addresses: hager.ayajeri@yahoo.fr, hajer_a21@yahoo.fr (H. Ayari Jeridi).

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zygotic event during the embryonal development (Carlson and Desnick, 1979; Lohmann et al., 1997; Sippel et al., 1998). In these patients, the predisposing mutation is present in only some constitutional cells (mutational mosaicism). If cells that belong to this mutant sector are present among progenitors of retinal sensory cells, tumor foci may develop in consequence of mutational inactivation of the normal allele. Moreover, if cells carrying the predisposing mutation are present in the germline of the patient then the mutant allele may be transmitted to offspring.

In addition to the timing, the location and nature of the predisposing mutation in *RB1* gene significantly influence clinical manifestation. Some oncogenic mutant alleles are consistently associated with bilateral retinoblastoma (Lohmann et al., 1996), while others usually lead to a milder phenotypic expression (unilateral retinoblastoma or no tumor at all). With few exceptions, *RB1* gene alterations resulting in premature chain termination, such as nonsense and frameshift mutations, are associated with bilateral retinoblastoma and complete penetrance (Lohmann et al., 1996; Richter et al., 2003). It is plausible to assume that the observed homogeneity of phenotypic expression is a consequence of complete loss of functional retinoblastoma protein due to nonsense mediated RNA decay (NMD) of the mutant transcript. Splice site mutations at highly conserved positions that result in out-of-frame changes in the mutant mRNA usually result in bilateral retinoblastoma. Other splice mutations may cause milder phenotypic expression (Zhang et al., 2008). Other types of *RB1* gene mutations that are associated with fewer tumor foci or incomplete penetrance are whole-gene deletions, promoter mutations and some missense mutations (Cowell et al., 1996; Lohmann et al., 1994; Onadim et al., 1992; Sakai et al., 1991). Most mutant alleles associated with incomplete penetrance result in diminished expression of a normal protein or in expression of a structurally altered protein that has lost some functions of normal pRb (Otterson et al., 1997).

Identification of the oncogenic *RB1* gene mutation in every patient is indispensable for early detection of asymptomatic relatives who are at risk to develop retinoblastoma (Dunn et al., 1989; Yandell et al., 1989) as well as to provide the necessary knowledge to understand the molecular mechanisms underlying variable phenotypic expression (Houdayer et al., 2004; Lohmann, 1999; Richter et al., 2003). As genetic testing improves the clinical management of retinoblastoma it has become an essential part of contemporary care for patients with retinoblastoma and their families.

Considering the extensive mutational heterogeneity mutation identification is no trivial task. Schedules that define the order of standard analyses can help to lower the analytical efforts and the cost for detection of an oncogenic mutation in an average patient.

In the present study we used such a schedule. We only used standard assays such as parallel sequencing of PCR products and MLPA. From an analysis of the spectrum of oncogenic mutations we derived the order of individual analyses. We have applied this strategy to identify the causative *RB1* germ-line mutations. The results permit to delineate genotype–phenotype correlations in 20 unrelated patients of Tunisian origin and their families.

2. Patients and methods

2.1. Patients

We searched for predisposing *RB1* gene mutations in 20 unrelated Tunisian patients with sporadic bilateral (15 patients) or familial retinoblastoma (5 patients). One case (RB29) of the five patients with familial retinoblastoma was diagnosed with sporadic bilateral retinoblastoma at the beginning of our study but was reclassified as a familial case because retinoblastoma was diagnosed in a sibling later. All patients were recruited, examined and treated at the Institute of Ophthalmology Hedi Raies of Tunis, Tunisia. Diagnosis of retinoblastoma was established by standard ophthalmologic and histological criteria. The initial treatment for all these cases was chemotherapy followed by enucleation. Clinical data for the patients are summarized in Appendix S1, Table 1 – Supporting information. Informed consent for genetic analyses was obtained for each case in accordance with the Declaration of Helsinki. Our study was approved by the ethics committee of Pasteur Institute of Tunis.

2.2. DNA isolation

Samples of whole blood from probands and relatives were collected in standard EDTA blood collection tubes. Fresh tumor tissue was obtained after dissecting enucleated eyes and stored at −80 °C until processing. DNA was extracted from tumor samples and leukocytes using the phenol-chloroform method (Sambrook et al., 1989). DNA concentration was evaluated by spectrophotometry at 260 and 280 nm.

2.3. Sequence analysis

Mutation identification was performed on leukocyte DNA. Further details are in Appendix S1, Supporting information.

2.4. Multiplex Ligation-dependent probe Amplification (MLPA)

The SALSA MLPA probemix P047-B1 *RB1* (MRC Holland, www.mrc-holland.com) was used to detect single- to multi-exon deletions or insertions at the *RB1* locus. The procedure was performed according to the manufacturer's instructions. Further details are in Appendix S1, Supporting information.

2.5. Microsatellite analysis

The short-tandem-repeat (STR) loci STR-890, RBi2, and RB1.20 located upstream, in intron 2, and in intron 20 of the *RB1* gene, respectively, were amplified by multiplex PCR with fluorescently labeled primers. PCR products were analyzed on an ABI PRISM 3130 XL Sequencer. Data analysis was performed with the GeneMarker® software version 1.9 (SoftGenetics, State College, PA, USA).

2.6. Analysis of mRNA

RNA from blood was analyzed to identify the consequences of a non-canonical splice site mutation in the *RB1* gene. PAXgene Blood RNA Tubes (Qiagen, Hilden, Germany) were used to collect 2.5 mL of fresh peripheral blood from the proband RB3, from twelve of her relatives, and from normal control individuals. Total cellular RNA was extracted using the Blood RNA Kit following the manufacturer's protocol (Qiagen, Hilden, Germany). RT-PCR analysis and sequencing was performed as described in Zhang et al. (2008).

2.7. In-silico analyses

Mutations involving splice sites were evaluated by the Analyzer splice tool (http://ibis.tau.ac.il/ssat/SpliceSiteFrame.htm). The maximum entropy model (Yeo and Burge, 2004) was used to compare scores of normal and new AG-splice acceptor sites (MIT MaxEnt Build Server; genes.mit.edu/burgelab/software.html). The diseased-eye ratio (DER) was determined to parameterize the phenotypic expression observed as described in Lohmann et al. (1994).