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Oggetto: Dichiarazione di conformità e richiesta embargo tesi di Dottorato di Ricerca.

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ciclo XXIX
Autore della tesi di Dottorato dal titolo PARENT INHERITANCE OF RB1 HYPOMORPHIC MUTATIONS AND SOMATIC MOSAICISM CAN EXPLAIN LOW PENETRANCE IN RETINOBLASTOMA
Tutore Prof. FRANCESCA ARIANI

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University of Siena

Department of Medical Biotechnologies

Doctorate in Genetics, Oncology and Clinical Medicine

(GenOMeC)

XXIX cycle

Coordinator: *Prof.ssa Alessandra Renieri*

Parent inheritance of *RB1* hypomorphic mutations and somatic mosaicism can explain low penetrance in retinoblastoma

Scientific disciplinary sector: Med/03 – Medical Genetics

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Valentina Imperatore

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1. Introduction

1. Introduction

1.1 Retinoblastoma

Retinoblastoma (RB, OMIM #180200) is the most common intraocular malignancy in children, affecting about 1 in 15,000 to 20,000 live births, which corresponds to about 9000 cases worldwide every year[1, 2, 3]. The disease has no geographic or population hotspots, but it is mostly observed in large populations that have high birth rates, such as Asia and Africa. In these regions, there is the highest mortality (40-70%) of children with RB, compared with 3-5% in Europe, Canada and the USA[4]. Worldwide, RB is responsible for 1% of childhood cancer deaths and 5% of childhood blindness[5]. Retinoblastoma is extremely rare in adults. When the disease occurs in adults, probably it originates from a benign precursor lesion of tumor, called retinoma.

Diagnosis is based on clinical signs and symptoms, which are usually present before the age of five years. Leukocoria (white pupillary reflex) is the first and common sign of RB[6] (Fig 1, left). After the first sign of leukocoria, RB remains intraocular and curable for 3-6 months. The parents can notice this symptom because the pupils of the child's eyes naturally dilate in dim light[4]. If the diagnosis has done too late, RB spreads from the eye and the chances of survival decrease[7]. Other important signs are strabismus (misaligned eyes) (Fig. 1, right), glaucoma, inflammation and poor visual tracking.

Fig. 1: On the left, leukocoria, the first observed symptom in a child with RB. On the right, strabismus, another important sign that can alert parents.

RB is caused by a two-step inactivation of *RB1* alleles. *RB1*, located at 13q14.2[8, 9], is the first characterized tumor suppressor gene. RB occurs in both hereditary and non-hereditaty forms. The most frequent form is the non-hereditary one (60%), with both inactivating events occurring in the retinal cells. In this form, the tumor is confined to a single eye (unilateral) and develops after the first year of life. The hereditary form (40%) is due to a *RB1* germline predisposing mutation and subsequent somatic inactivation of the other allele[10, 11, 12]. In this form, the diagnosis is made earlier and cancers develop as multiple foci (multifocal), usually involving both eyes (bilateral). Hereditary RB is transmitted according to an autosomal dominant pattern with high penetrance (90%). Only 10% of all patients have a positive family history. In these cases, the retina examination in all first-degree relatives of RB patients is very important in order to identify retinomas or retinal scars.

RB patients show an increased risk to develop other tumors (second tumors) outside of the eye such as osteogenic sarcoma, malignant melanoma and soft tissue sarcoma, especially when the children are treated with radiation[13].

Tumor stage, localization/size of the tumor, number of foci, presence of vitreous seeding and the age of the child can influence the treatment of retinoblastoma. On the basis of these factors, therapeutic approaches can include focal cryotherapy, laser surgery, radiotherapy or chemotherapy. Recently, intra-arterial selective infusion of chemotherapy in the ophthalmic artery has been introduced[14]. However, enucleation (removal of the entire eye) is still the standard treatment for advanced intraocular RB, since it is effective in preventing progression to clinical metastatic disease in 95% of cases[15].

1.2 The genetic basis of retinoblastoma

The importance of heredity factors in the etiology of retinoblastoma has been recognized since the 19th century. Initially, all cases were regarded as hereditary. Only later the non-hereditary etiology was recognized for a significant fraction of patients with sporadic RB[16]. In 1971, the study of the genetic basis of RB led *Knudson* to propose the 'two-hit' model of tumour-suppressor gene inactivation[17]. The observation that bilaterally affected individuals were diagnosed at a younger age compared to unilaterally affected individuals without family history, prompted the proposal that the heritable form of the disease results from a germline mutation (the 'first hit', M1) and an acquired somatic mutation (the 'second hit', M2), whereas non-heritable form arises when two somatic mutations are present in the same retinal cell (Fig. 2).

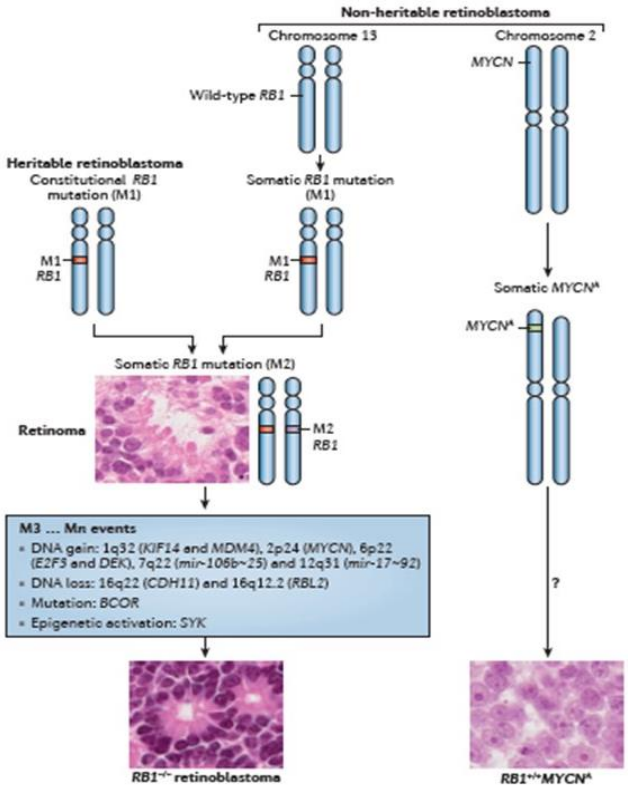

Fig. 2: The figure shows three possible mechanisms of retinoblastoma development. In heritable tumors, the first mutation occurs in germline cells and the second in somatic cells determining retinoma; an additional genetic or epigenetic event leads to tumor development. In the non-heritable form, both mutations occur in somatic cells. In a rare non-hereditable subtype of RB, the first mutation is a somatic amplification of *MYCN* with normal *RB1* (*$RB^{+/+}$MYCN-amplified)*. M1: first mutation; M2: second mutation; *$RB1^{+/+}$*: two normal alleles; *$RB1^{-/-}$*: two mutated alleles. *RBL2*: retinoblastoma like- 2; *CDH11*: cadherin 11; *BCOR*: BCL-6 corepressor; *KIF14*: kinesin famil member 14; *SYK*: spleen tyrosine kinase. *Figure taken from Dimaras H et al., Nature 2015.*

In 1973, *Comings* reinforced this hypothesis by postulating that the two mutations necessary for RB development correspond to the inactivation of two alleles of the same gene[18]. Chromosomal deletions in rare patients with bilateral retinoblastoma, intellectual disability and dysmorphic features suggested that the tumor susceptibility gene was localized at 13q14 locus[8-11, 19]. Comparative analysis of polymorphic markers localized in this candidate region demonstrated the loss of heterozygosity (LOH) in the majority of RB tumours[20]. Soon after, studies focused on this locus led to the identification of *RB1* as the retinoblastoma causative gene[21- 23].

1.3 Additional somatic events involved in retinoblastoma development

The hypothesis of clonal evolution explained the tumor progression. Some of the new cellular populations (generated by genomic instability) showed genetic changes that allowed a selective advantage [24, 25]. Thus, a correlation between specific genetic variations and malignant progression can be establish. In 1999, a new RB development model was proposed by Gallie and collaborators. They asserted that it was necessary another mechanism for exponential RB expansion and so, that only the mutations in both alleles were not sufficient [26]. In RB patients, genomic alterations (such as deletions or duplications) are frequently found in addition to the mutations in both alleles, suggesting that for RB initiation and progression, the extra somatic changes could be critical [27-30].

The identified recurrent genomic variations in retinoblastoma, using CGH array and cytogenetic analysis, are gains on chromosome 6p and 1q and losses of 16q [31, 32, 33, 34]. In 2001, *Herzog* and coworkers investigated chromosomal aberrations on tumors of mutated unilaterally affected patients, demonstrating a significant difference between children with an early or delayed enucleation[35]. In 2003, an association between the loss of 13q and 16 and cancers showing hostile histo-prognostic factors was found by *Lillington*. Conversely cancers with no adverse factors were found associated with -16q. While, -13q and -5q aberrations were associated with metastasis[36].

An accurate analysis of chromosomal abnormalities, and the study of their aftermath on cancer progression, is performed using CGH array (Comparative Genomic Hybridization) with a high resolution[37, 38]. A correlation between DNA copy-number abnormalieties and cancer has been found in several different tumors (such as in prostate, breast and gastric cancer, and in lymphoma)[39- 43]. *Zielinski* and collaborators used array CGH to detect chromosomal imbalances in RB[44]. Four regions have been identified to be frequently alterated limited to overlapping regions on 1q22, 1q32.1q32.2, 2p24.1, and 6p21.33-p21.31. In addition, in a single tumor two high-level amplifications at 1p34.2 and 1p33 were identified. Employing array-CGH technique, our group investigated the role of genomic rearrangements in retinoma-retinoblastoma transition[45]. In one RN, ophthalmoscopically

diagnosed, no rearrangements were detected, while adjacent RB displayed 7 aberrations. In contrast, in the other RN, identified by retrospective histopathological examination, we found 5 rearrangements: 3 in common with adjacent RB and 2 gene-free specific for RN. One rearrangement, dup5p, was tumor-specific and included the S phase kinase-associate protein 2 (*SKP2)* gene, an interesting candidate for malignant transformation since it is involved in senescence regulation.

The array- CGH also allowed to identify both the increased number of several oncogenes such as the mitotic kinesine family member 14 (*KIF14*), the oncogenic microRNA *mir-106b~25* (7q22.1), *mir-17~92* (13q31) and the loss of other tumor suppressor genes like the cadherin 11 (*CDH11*), retinoblastoma – like 2 (*RBL2*)[46]. Mutations in the transcriptional co-repressor BCL 6-co-repressor (*BCOR*) are identified by whole genome sequencing (WGS)[47].

In a number of RB patients, no mutations are detectable suggesting that the mutations can occur during tumorigenesis[48]. In some cases of non-hereditary RB, it is demonstrated an overexpression of oncogenes such as the somatic amplification of the *MYCN* oncogene (~ 2%)[49] (Fig. 2). Several other candidate oncogenes and other tumor suppressor genes have been discovered to be significant in cancerogenesis, such as *MED4*, *MDM4*, *MDM2*, *KIF4*[25].

1.4 pRB structure

The *RB1* gene (GeneBank accession number L11910) includs 27 exons that are spread on 190 kb of genomic sequence on chromosome 13q14[50]. The promoter region presents binding motifs for transcription factors such as Sp1, ATF and E2F but it does not show TATA or CAAT elements. It encodes a 4.7 kb mRNA that is translated into a 928-amino-acid-long protein (pRB) without evidence of alternative splicing[51]. Human and mouse RB proteins show 91% of identity indicating that the *RB1* gene is highly conserved through the species. The encoded RB protein is a nuclear phosphoprotein that, when hypophosphorylated, migrates at 110kD in SDS-PAGE. The protein is constituted by three main areas: the N-terminus, the A/B pocket domains (separated by a spacer region), and the C-terminus (Fig. 3).

The amino-terminal domain (RBN) expands from residue 53 to 355 and is constituted by two additional cyclin folds[52]. This region, interacting with pocket domains[53], plays a role in maintaining the active conformational state of pRB and it is important for the binding of several proteins[54, 55].

Pocket domains (A/B) are conserved through the small family of nuclear proteins that also includes p107 and p130 (encoded by *RBL1* and *RBL2* genes, respectively). These domains can bind to members of the E2F family of transcription factors and other nuclear proteins containing the LxCxEx peptide motif; binding depends on the phosphorylation status at different serine and threonine residues along the protein. The pocket region expands from residue 380 to 787, including the spacer region. The pockets, each resembling a cyclin fold with three additional helices[56], require each other to constitute a functional repressor domain[57]. In fact, they interact with each other by an extensive non-covalent interface such that the pocket folds into a single structural unit (Fig. 3).

The last 150 residues of pRB constitute the carboxy-terminal region (RBC). It is important for growth suppression and contains a nuclear localization signal (NLS) and a cyclin binding motif ([R/K]xL)[58, 59, 60]. The C-terminal region has also an intrinsic non-specific DNA-binding activity and it binds to the nuclear oncogenic proteins c-Abl tyrosine kinase and MDM2[61].

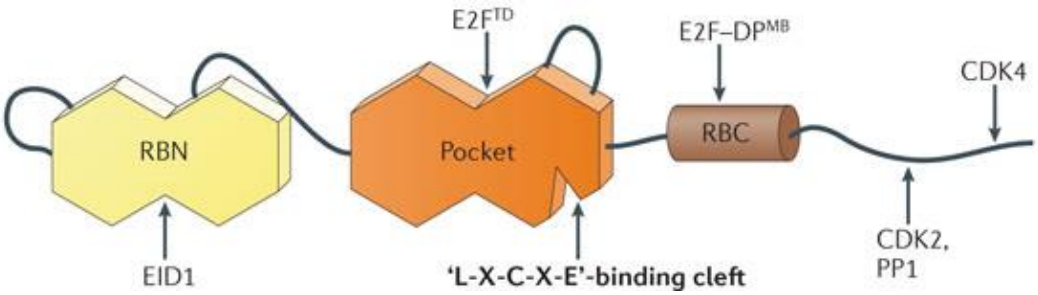

Fig. 3: Schematic representation of pRB. The structure of domains are showed and are indicated the binding sites for interaction with several proteins. RBN: amino-terminal region; RBC: carboxy-terminal region; EID1: E1A-like inhibitor of differentiation; E2F^{TD}: E2F transactivation domain; E2F-DP^{MB}: E2F differentiation-related polypeptide marker box; CDK: cyclin-dependent kinase; PP1: phosphatase 1. *Figure taken from Dick FA et al, Nat Rev Mol Cell Biol 2013*

1.5 pRB and its role in cell cycle

pRb lies at the heart of the regulatory network of cell cycle. In fact, pRb is a key regulator of the G1 to S transition. After passing this checkpoint, cells are irreversibly committed to the next cell division[62]. pRb regulates cell growth at G1/S checkpoint interacting with E2F transcription factor family (Fig. 4)[62- 68]. In cell cycle phases, when cyclin dependent kinases are not active (early G1 phase, quiescence or senescence), pRb is hypophosphorylated and interacts with E2F. In particular, pRB binds the E2F transactivation domain ($E2F^{TD}$) through a highly conserved region of the pocket domain, localized at the interface between A and B cyclin folds[69, 70]. During G1-S transition, cyclin-dependent kinases phosphorylate pRb that loses the ability to interact with E2F. This leads to the expression of numerous genes whose functions are intimately linked with S phase entry and cell cycle progression[71- 73]. The functional relevance of pRb repression of E2F in tumorigenesis has been tested in the mouse by combining $Rb^{+/-}$ with various *E2F* knockout mutations. Knockout of *E2F1*, *E2F2* or *E2F4* delayed pituitary and thyroid tumorigenesis in $Rb^{+/-}$ mice, providing genetic evidence for the role of Rb-mediated E2F repression in Rb mediated tumor suppression[74- 76]. Furthermore, *E2F1* has been shown to be amplified in human erythtroleukaemia cells as well as up-regulated in thyroid carcinogenesis[77, 78].

pRb is capable of repressing cell cycle progression by different mechanisms. In fact, in addition to the binding E2F transcription factors which blocks their ability to activate gene transcription, pRb-E2F complexes can also recruit chromatin-modifying complexes including histone deacetylases (HDACs)[79]. In particular, it has been demonstrated that pRb binds to HDAC1, 2 and 3[80-84]. Over-expression of *HDAC1* has been observed in hormone refractory prostate cancer, showing that changes in *HDAC1* expression levels are implicated in tumorigenesis[85].

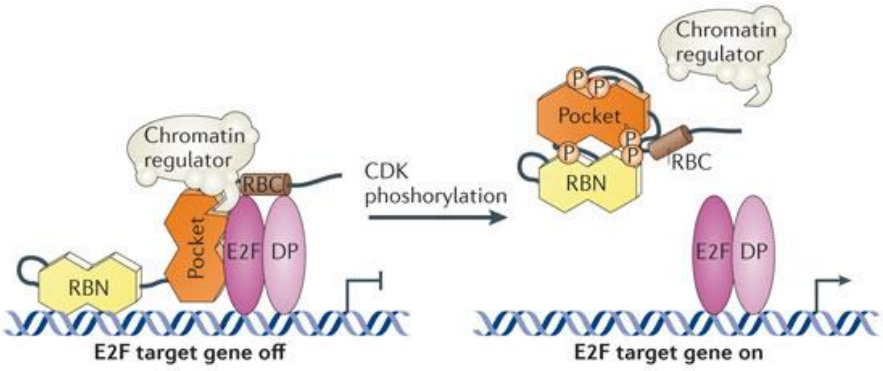

Fig. 4: Scheme of interaction between E2F factors and pRB in cell cycle. During G1 phase, pRB is hypophosphorylated and can bind E2F factors and chromatin regulator resulting in repression of genes required for S phase progression. When in late G1 and in M phases, CDKs phosphorylate high levels of pRB, E2F factors and chromatin regulator are released and the transcription of genes for S phase can take place resulting in the cell cycle advancement. RBN: amino-terminal region; RBC: carboxy-terminal region; E2F: E2F transcription factors; DP: differentiation-related polypeptide subunits (E2F factors are dimeric proteins constituting by E2F and DP); CDK: cyclin-dependent kinase. *Figure taken from Dick FA et al, Nat Rev Mol Cell Biol 2013.*

However, the G1 checkpoint control is not the only function of the RB protein. To date, other activities have been associated to pRB such as control of cellular differentiation during embryogenesis and in adult tissues, apoptosis regulation, maintenance of permanent cell cycle arrest and preservation from chromosomal instability[86, 87, 88].

1.6 RB1 gene mutations and phenotypic correlation

All familial/bilateral RB cases and a significant fraction (15–18%) of unilateral cases carry an *RB1*-predisposing mutation. To date, more than 1,600 different mutations, ranging from single nucleotide changes to large deletions, have been listed in the *RB1* Gene Mutation Database (Lohmann Database)[89]. A large fraction (~40%) of mutations are recurrent and consist in sixteen hot spots, including twelve nonsense, two missense and three splicing mutations (Fig. 5). Most of these frequent mutations correspond to C to T transitions in eleven CGA-arginine codons[90]. Hyper-mutability of these sites probably depends on the methylated status of CpG dinucleotides and the spontaneous deamination of 5- methyl cytosine to thymidine[91]. Remaining mutations are scattered along the 27 exons, the promoter and intronic regions (splice site and deep intronic mutations)[89, 92].

Complete inactivation of the protein is the result of the majority of *RB1* mutations (complete loss of function, amorphic mutations)[90, 93- 98]. These mutations, mostly represented by nonsense and frameshift changes resulting in premature termination codons (PTC), are generally associated with full penetrance[92]. In many disorders, there is a good correspondence between the position of the premature stop codon and phenotypic expression, but in retinoblastoma no such association has been found. On the contrary, a little or no effect on phenotypic expression is showed. A possible reason for this lack of correlation is the action of nonsense-mediated decay, a post-transcriptional mechanism that recognizes the mutant transcripts of *RB1* alleles inducing their degradation[99, 100]. Consequently, when nonsense-mediated decay acts in cells presenting a heterozygous mutation only wild-type transcripts are preserved[72]. Considering this mechanism, we can hypothesize that mutations in the last exons of *RB1* are not oncogenic because the resulting mutant transcripts do not trigger nonsense-mediated decay[101]. The resulting protein will show late C-terminal truncation but it will retain sufficient suppressor activity to prevent tumor development. Moreover, low penetrance has also been observed in families with nonsense mutations in exon 1[92, 102-104]. The inability of the NMD to degrade

mRNAs with 5'-terminal nonsense mutations and the reinitiation of translation downstream from the premature stop codon, could clarify this phenomenon [105, 106].

Another important class of *RB1* oncogenic events is represented by large rearrangements (~15%)[102]. They can include only the *RB1* gene (entire or a portion) or be a part of a larger contiguous deletion involving other genes. In the latter case, the phenotype can include other manifestations such as intellectual disability and dysmorphic features[107, 108]. Contrary to what one can expect, there is a tendency for whole gene deletions to cause fewer tumors [102, 109-112]. In 2014, *Dehainault* and colleagues defined a minimal genomic region associated with low penetrance and identified *MED4* as a gene fundamental for the survival of $RB1^{-/-}$ tumor cells[113].

Families showing incomplete penetrance (some carriers without RB) or low expressivity (fewer tumors, with more unilaterally affected or benign retinomas) are frequently associated with partial inactivation of protein functions[4, 114, 115]. Mutations associated with these phenomena generate "weak" alleles and can be divided in the following categories: 1) mutations in the promoter reducing *RB1* expression; 2) missense and in-frame alterations affecting non-essential sequence motifs; 3) splice site mutations leading to the reduction of normal mRNA splicing[92, 103, 114- 130].

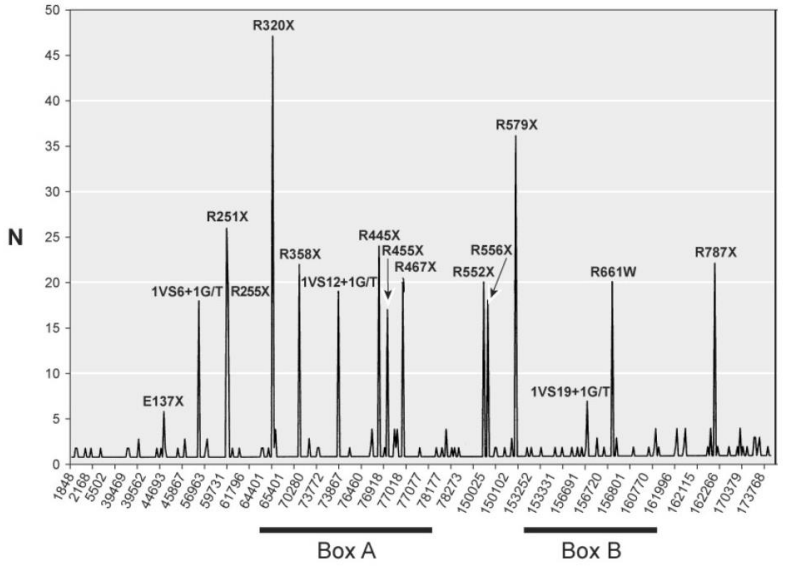

Fig. 5: The most recurrent mutations in hot spot regions of *RB1*. BoxA: pocket A domain; BoxB: pocket B domain. *Figure taken from Valverde JR et al, BMC Genet 2005.*

1.7 Mosaicism

The majority of children with retinoblastoma have newly acquired *RB1* mutations. *De novo* mutations can arise in the parental germ cells (usually the paternal lineage), or post-fertilization, resulting in mosaicism (Fig. 6)[93, 131-133]. Somatic mosaicism can involve the germ cells, if the mutation occurs before the cells committed to the gonads have formed. In this case, tumor predisposition can be transmitted. In diseases like retinoblastoma, showing high rates of *de novo* mutations, mosaicism is expected to occurr. In 1998, a pioneer study conducted in a large cohort documented mosaicism for the first *RB1* mutation in ~10% of families[131]. The authors suggested that, in a pedigree composed by two generations, the higher frequency of bilateral RB observed in the second generation could be associated with the presence of non-penetrant carriers in the first generation, resulting from a mosaic state for the mutation[131]. However, traditional screening techniques employed before the introduction of Next Generation Sequencing (NGS) had not the sufficient level of sensitivity to detect mosaicism, expecially at low level (<15% of mutated alleles), and thus underestimated the incidence of the phenomenon. Accordingly, more recent studies performed by deep sequencing documented a rate of *RB1* mosaicism up to 30%[92, 134, 135].

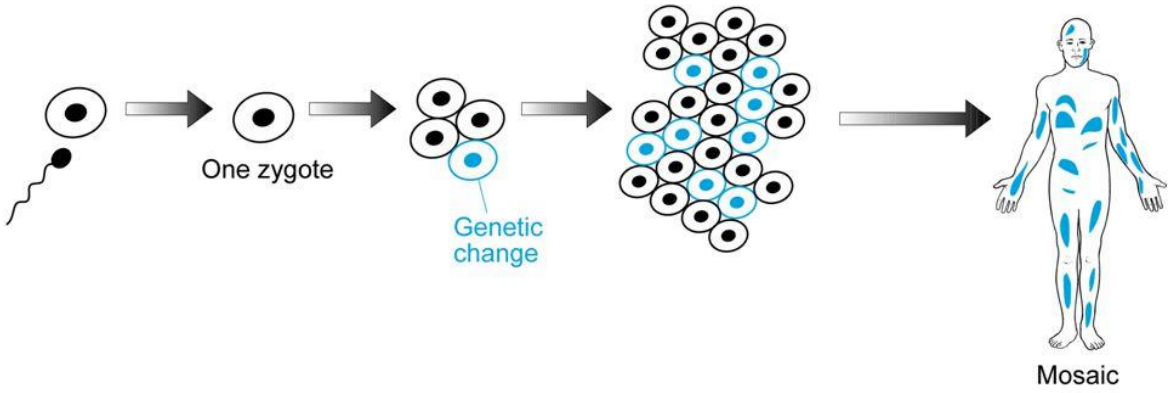

Fig. 6: Representation of mosaicism by post-zygotic event. *Figure taken from web*.

1.8 Imprinting & Parent-of-origin effect

Genomic imprinting is an epigenetic mechanism leading to a parent-of-origin-specific gene expression[136]. In 2009, a landmark study by *Kanber* and colleagues demonstrated that the *RB1* gene is imprinted due to a differentially methylated CpG island (CpG85) localized in intron 2[137] (Fig. 7). CpG 85 is part of a truncated pseudogene (KIAA0649P) that integrated into the *RB1* gene prior to the speciation of extant primates. It is methylated on the maternal chromosome and unmethylated on the paternal chromosome so it can act as a weak promoter for an alternative *RB1* transcript that interferes with the canonical transcript, reducing its expression. As a consequence, *RB1* gene expression is skewed in favor of the maternal allele (about 3 fold) (Fig. 8).

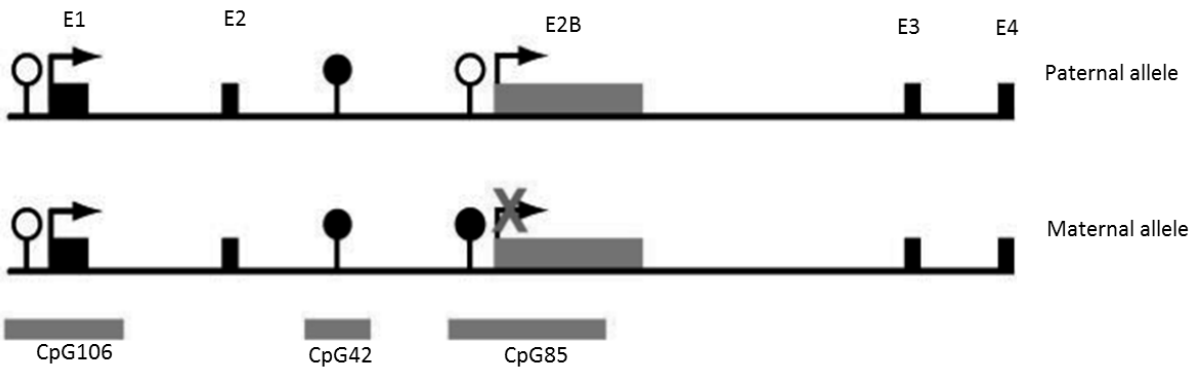

Fig. 7: Schematic representation of *RB1* imprinting mechanism. CpG 106 overlaps the *RB1* promoter and exon 1 and it is biallelically unmethylated. CpG42 places upstream of CpG85 and it is biallelically methylated. CpG85 is differentially methylated (methylated on the maternal allele and unmethylated on the paternal one) and acts as promoter of an alternative transcript localized in exon 3. E1, E2, E3, E4 are canonical exons; E2B is the start exon of the alternative *RB1-2B* transcript. Arrow: transcription start site; open lollipop: unmethylated CpG; filled lollipop: methylated CpG. *Figure taken from Buiting K et al Brief Funct Genomics 2010.*

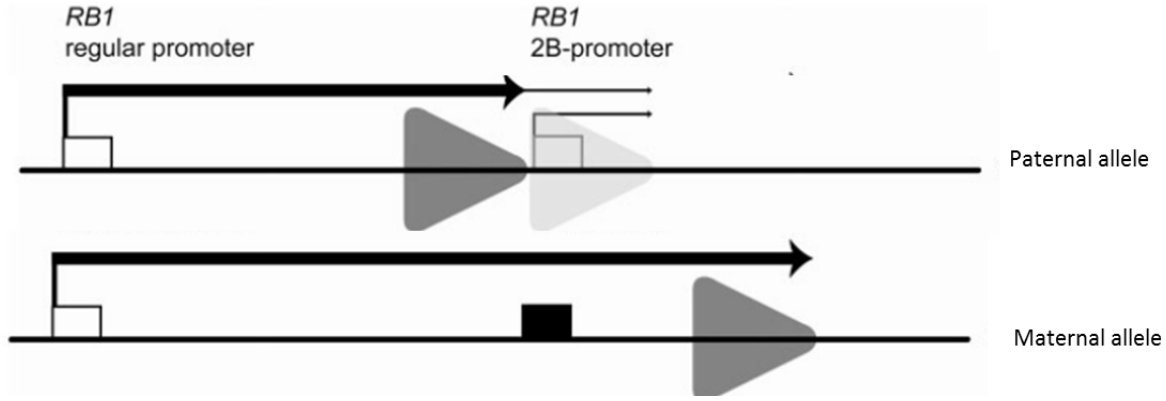

Fig. 8: Schematic representation of co-transcriptional interference. On the maternal allele, the *RB1-2B* promoter is silenced by methylation of CpG85 resulting in normal expression of *RB1* canonical transcript. On the paternal allele, the alternative promoter interferes with the main transcriptional complex. Open rectangle: unmethylated promoter; filled rectangle: methylated promoter; arrows: transcriptional direction; light grey triangle: transcriptional complex of the *RB1-2E* alternative transcript; dark triangle: transcriptional normal complex of the *RB1*. *Figure adapter from Buiting K et al Brief Funct Genomics 2010.*

These findings have led to hypothesize that penetrance and expressivity could be influenced by parent-of-origin-specific *RB1* expression[138]. In fact, this mechanism can be irrelevant for loss-of-function mutations but it can be fundamental in modulating the phenotypic expression associated with hypomorphic mutations (partial loss of function). Maternally inherited mutations may indeed retain sufficient suppressor activity to prevent tumor development and determine reduced penetrance/expressivity (Fig. 9).

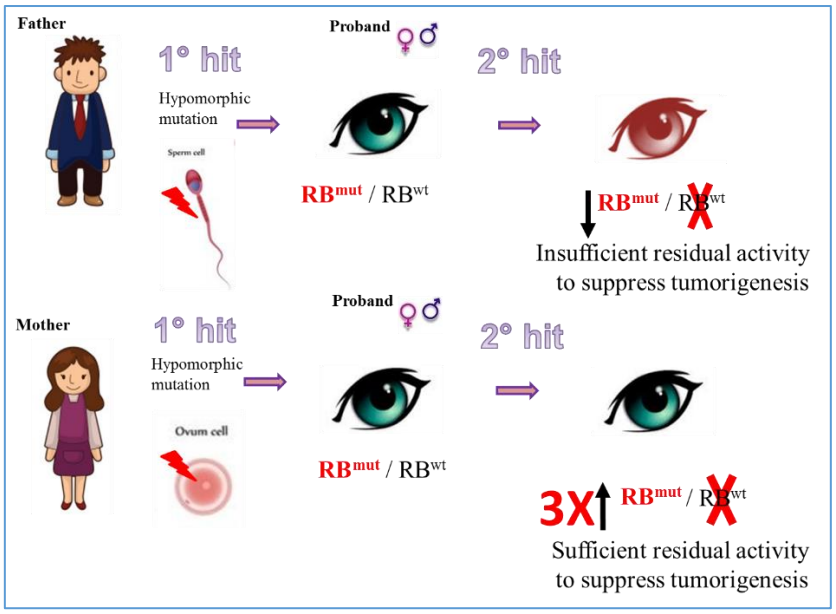

Fig. 9: Schematic representation of the possible effects of the parent-of-origin-specific *RB1* expression due to imprinting. Upper panel: paternal inheritance of a hypomorphic predisposing mutation. A second event occurs in the retinal cells, resulting in complete protein inactivation and full penetrance (knockout). Bottom panel: Maternal inheritance of a hypomorphic predisposing mutation. A second mutation event somatically occurs but the protein derived from the 3-fold expressed hypomorphic allele retains sufficient suppressor activity to prevent tumor development.

2. Rationale and Aim of the study

2. Rationale and Aim of the study

Even though the gentic and molecular mechanisms of retinoblastoma have been extensively investigated many issues still remain unsolved. One important issue is to improve *RB1* mutation detection in order to correctly identify all at risk members and provide optimal genetic counseling and medical surveillance. With regard to this aspect, in the present study, we employed Next Generation Sequencing (NGS) to identify somatic mosaicism in patients in whom traditional analyisis had not revealed the presence of a predisposing mutation. This approach allowed us to detect mosaicism in 6 out of 40 (15%) sporadic RB cases (Results 3.1). Mosaic mutations were confirmed by testing other tissues from affected patients: oral mucosa, urines and eye, when available. In three cases, we detected low-level mosaicism, with important implicantions for genetic couselling and patient' management.

Another issue that has been the subject of an intense debate for many years is the identification of the molecular basis of reduced penetrance/expressivity in retinoblastoma. In a group of six RB families showing reduced penetrance/expressivity, we decided to investigate whether these phenomena could be due to: i) mosaicism for amorphic mutations; or ii) parent-of-origin effect of hypomorphic mutations (Results 3.2). Using targeted high-depth NGS, somatic mosaicism was identified in two families with splice site mutations resulting in a complete loss of protein function. In the remaining four families, we identified paternally inherited hypomorphic mutations in the affected members. Hypomorphic mutations include a splice site deletion resulting in the loss of 37 aminoacids at the N-terminus (one family), a late truncating splice site mutation (one family) and a partially deleterious missense substitution (two families). These results changed the genetic/prenatal counseling and the clinical management of RB families, raising to a 50% the recurrence risk for unaffected carriers, offspring of *RB1*-mutated mothers, and imposing for them the need for second

tumor surveillance in unaffected carriers at risk of developing adult-onset cancer such as osteosarcoma or leiomyosarcoma.

3. Results

3.1 Identification of somatic mosaicism in retinoblastoma patients

Next generation sequencing in sporadic retinoblastoma patients reveals somatic mosaicism

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ARTICLE

Next generation sequencing in sporadic retinoblastoma patients reveals somatic mosaicism

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In about 50% of sporadic cases of retinoblastoma, no constitutive *RB1* mutations are detected by conventional methods. However, recent research suggests that, at least in some of these cases, there is somatic mosaicism with respect to *RB1* normal and mutant alleles. The increased availability of next generation sequencing improves our ability to detect the exact percentage of patients with mosaicism. Using this technology, we re-tested a series of 40 patients with sporadic retinoblastoma: 10 of them had been previously classified as constitutional heterozygotes, whereas in 30 no *RB1* mutations had been found in lymphocytes. In 3 of these 30 patients, we have now identified low-level mosaic variants, varying in frequency between 8 and 24%. In 7 out of the 10 cases previously classified as heterozygous from testing blood cells, we were able to test additional tissues (ocular tissues, urine and/or oral mucosa): in three of them, next generation sequencing has revealed mosaicism. Present results thus confirm that a significant fraction (6/40; 15%) of sporadic retinoblastoma cases are due to postzygotic events and that deep sequencing is an efficient method to unambiguously distinguish mosaics. Re-testing of retinoblastoma patients through next generation sequencing can thus provide new information that may have important implications with respect to genetic counseling and family care.
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INTRODUCTION

Retinoblastoma (RB; OMIM#180200) is an intraocular malignancy that occurs in children, usually before age 5 years, with a reported prevalence of 1 out of 15 000–28 000 live births.[1] RB is responsible for 1% of childhood cancer deaths and 5% of childhood blindness.[2] A two-step inactivation of both alleles of the *RB1* gene is required for tumor initiation, but additional mutational events usually accompany malignancy.[3–5] In hereditary RB, the initial *RB1* mutation (M1) occurs in the germline cells, while the second mutation (M2) occurs in the retinal cells.[6] RB patients transmit their predisposition as an autosomal-dominant trait with high penetrance (>90%).[6] All bilateral RBs (40%) are heritable, while unilateral RBs (60%) are heritable in only a small percentage of cases (7%).[7] Patients with hereditary RB also show an increased risk of developing non-ocular tumors.[8] In non-hereditary RB, mutations of both *RB1* alleles occur in the retinal cells, determining unilateral tumors in children.[6]

The majority of children with RB have newly acquired *RB1* mutations. *De novo* mutations can arise in the parental germ cell (usually in the paternal germ cell), or at some point during embryogenesis, resulting in mosaicism.[9–13] If the alteration occurs during the first embryonic cell divisions, it can be confused with a mutation originated in parental germline cells, but if it occurs later, during the embryonic development, it can be tissue-specific or tissue-limited.[14] One previous study showed that in 10% of RB families the first mutation was in mosaic state, either in the proband or in one of the proband's parents.[11] However, because of the unavailability of key family members, the authors hypothesized that mosaicism could be even more frequent.[11] Underestimation of mosaicism at that time was also due to the limited sensitivity of traditional methods employed for *RB1* mutation analysis (SSCP, Sanger sequencing and Southern blotting). In more recent years, the detection rate of *RB1* gene mutations has been greatly increased by the introduction of highly sensitive allele-specific PCR (AS-PCR).[12] In particular, using this technique, it was possible to identify even low-level *RB1* mosaicisms (frequency <15% of the normal allele). However, this study was limited to only 11 mutational 'hot spots', while >50% of *RB1* mutations are private mutations.[15] The introduction of unbiased next generation (or deep) sequencing technology, reporting exactly how many molecules have been sequenced and the exact percentage of variants, has offered an excellent opportunity to detect mosaicism in different diseases.[16–19] Recently, Chen *et al*,[13] analyzing by deep sequencing (Ion Torrent Personal Genome Machine, Life Technologies, Carlsbad, CA, USA) lymphocyte DNA from 90 RB cases where Sanger sequencing excluded the presence of mutations, were able to identify 30 and 6% low-level *RB1* mosaic mutations in bilateral and unilateral cases, respectively.

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In the present study, using next generation sequencing (454 GS Junior System, Roche, Life Sciences, Indianapolis, IN, USA), we re-analysed a series of 40 sporadic RB cases (11 bilateral and 29 unilateral). Previous analysis by traditional methods (Sanger sequencing and Multiplex Ligation Probe Amplification, MLPA) had shown constitutive *RB1* mutations in apparent heterozygosis in 10 patients (9 bilateral and 1 unilateral), whereas there was no detectable mutation in the remaining 30 patients. In particular, mutations were analysed in tissues other than blood, namely ocular tissues (tumor, muscle, retina and sclera), urine and oral mucosa, whenever available. Present data could have important implications for genetic counseling, because they can alter diagnosis and family care.

MATERIALS AND METHODS

Patient samples

We collected 40 patients with diagnosis of sporadic RB, which was bilateral in 11 cases and unilateral in 29. In the last screening group of our diagnostic service, traditional methods (Sanger sequencing and MLPA) had previously identified 10 heterozygous sequence variants: 9 were in patients with bilateral RB and 1 in a patient with unilateral RB (Table 1). Clinical diagnosis was made by physicians with long-lasting experience in Ophthalmology and Ocular Oncology, belonging to the 'Retinoblastoma Referral Center' of the General Hospital of Siena. All patients underwent genetic counseling and had their blood and tissue samples collected and analysed for *RB1* variants at the Medical Genetics Unit of the University of Siena. Blood samples were collected in EDTA-containing tubes. Urine samples and oral swabs were also collected during genetic couselling. Formalin-fixed paraffin-embedded ocular samples from enucleated RB patients were obtained from the archives of the Pathology Unit of the University of Siena. After surgery, enucleated eyes were immersion-fixed in buffered formalin for 48 h. After fixation, sampling, paraffin embedding and cutting were carried out according to the usual pathological methods. Informed consent was obtained from all adult patients and parents of all affected children.

DNA extraction

Genomic DNA was isolated from EDTA peripheral blood samples using a QIAamp DNA Blood Kit according to the manufacturer's protocol (Qiagen, http://www.qiagen.com). Urine and oral mucosa samples were freshly extracted using the QIAamp DNA Mini kit following the manufacturer's instructions (Qiagen, http://www.qiagen.com). Ocular tissues were identified on H&E-stained sections. Five-micron-thick sections were deparaffinized, rehydrated and stained with Mayer hematoxylin and yellow eosin and then dehydrated with xylene. Slides were observed using an inverted microscope (Zeiss, Telaval 31, Jena, Germany). Cells from normal eye tissues (RB, normal retina, muscle, sclera) were isolated by laser capture microdissection (Arcturus PixCell II, MWG-Biotech, Florence, Italy) as already described.[5] Larger tumor areas were isolated by scraping. Genomic DNA was then extracted from ocular tissues using the QIAamp Mini kit (Qiagen, http://www.qiagen.com).

RB1 analysis by traditional methods

All 40 sporadic RB patients had been previously analysed by Sanger sequencing. Primers and PCR conditions were the same as those described for 454 GS Junior amplicon library preparation (Supplementary Table S1). PCR products were then sequenced using the PE Big Dye Terminator Cycle Sequencing Kit and an ABI Prism 310 genetic analyser (PE Applied Biosystems, Foster City, CA, USA). Sequencer software was used for data analysis.

To identify large rearrangements in the *RB1* gene, MLPA had been previously performed in all 40 patients. Analysis was carried out using the SALSA MLPA kit (P047-B1 RB1) designed by MRC-Holland (Amsterdam, The Netherlands). Experiments were performed as previously described.[20] Coffalyser software (MRC-Holland) was used for data analysis.

RB1 analysis by Roche 454 GS Junior sequencing

Deep sequencing was performed to investigate the presence of *RB1* mosaic variants in 40 sporadic RB cases: 30 'negative' and 10 'positive heterozygous' cases according to traditional methods (Sanger sequencing and MLPA analysis). Given that sporadic unilateral RB is predominantly associated with *RB1* somatic mutations, the optimal procedure would include an initial screening in tumor DNA.[21] However, traditional *RB1* analysis in our unilateral samples (29 cases) did not include tumor DNA screening because of the difficulties in tumor tissue collection in routine molecular diagnosis. For the purposes of this study, we were able to collect five tumor tissues that were analysed by amplicon deep sequencing. The presence of tumor variants in matched lymphocytes of each patient was then analysed by deep sequencing of the specific exons.

To analyse the *RB1* gene (Gene Bank Accession L11910.1; transcript NM_000321.2), we used a strategy based on the locus-specific amplification of genomic DNA, amplifying each amplicon separately, followed by Roche 454 resequencing. Fusion primers are reported in Supplementary Table S1. For DNA samples isolated from peripheral blood and oral mucosa, thermal cycling was performed on an Applied Biosystems 2720 (Life Technologies) using the following cycling profile: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, at the specific annealing temperature for 30 s, at 72 °C for 30 s, followed by a final extension step at 72 °C for 5 min (Supplementary Table S1). As DNA concentration of samples isolated from urine samples or eye tissues was low in some cases, the above described protocol was slightly changed. We elongated annealing and extension time (to 45 s) and the final extension step (to 10 min), increased the number of cycles to 40 and used 1.5 units of polymerase instead of 1 unit. Then 3 µl of amplified PCR products were electrophoretically separated on 1.2% agarose gel. For removal of small DNA fragments, samples were then purified using the AMPure PCR purification system (Agenocourt, Beverly, MA, USA). Amplicons were quantified using the Quant-iT PicoGreen dsDNA reagent (Invitrogen Corporation, Life Technologies). All amplicons were then pooled at an equimolar ratio. Subsequently, the sample pool was diluted to a final concentration of 1×10^7 PCR fragment molecules/ul.The amplicon-PCR-derived fragments were annealed to carrier beads and clonally amplified by emulsion PCR. Subsequently, the emulsions were broken by isopropanol, and the beads carrying the single-stranded DNA templates were enriched, counted and deposited into the PicoTiterPlate for pyrosequencing.[22] Identified variants were submitted to LOVD database (http://rb1-lovd.d-lohmann.de).

To distinguish low-frequency mosaic variants from background errors of 454 platform, deep sequencing was performed in three healthy individuals at the same positions as mosaic variants. To detect potential mosaics among Sanger heterozygous samples, deep sequencing was performed in DNAs from 'known' constitutional heterozygous samples (mutated offspring of familial RB cases). In particular, we selected samples with the same variants of the suspected mosaic or, when not available, samples with variants at least in the same amplicons to maintain sequencing efficiency. Each sample was analysed three times.

454 GS Junior assay validation

To determine the linearity of the deep sequencing assay and the lower limit of detection (LOD), calibration curves for two different variants (c.1363C>T and c.1072C>T) were drawn using DNA isolated from two heterozygous controls (mutated offspring of familial RB cases). Mutated DNAs were serially diluted with wild-type DNA to mimic the presence of variants at levels of 50, 33, 16.5, 8.25, 4.12 and 0%. Each sample at the different dilutions was sequenced three times. EXCEL 2013 (Microsoft, Redmond, WA, USA) was used to analyse data.

454 GS Junior sequencing data analysis

Data analysis was performed using the Roche proprietary software package for the GS Junior system. Image acquisition, image processing and signal processing were performed during the run. Post-run analysis was conducted using the latest version (2.5p1) of GS Amplicon Variant Analyzer (AVA) (http://454.com/downloads/my454/documentation/gs-junior/software-manual/454_Sequencing_Software_Manual_v2.5p1_PartD.pdf). The AVA application computes the alignment of reads from Amplicon libraries obtained on the GS Junior Instrument and identifies differences between the reads and the reference sequence. The AVA software identifies all nucleotide variants and

Table 1 ***RB1*** **variants detected in patients with sporadic retinoblastoma**

Patients' ID	Gender	Current age (years)	UL/BL	Foci	Age of diagnosis (months)	R-E	ABC	Relapses	Therapy	RB1 variant	Forward % (seq n)	Reverse % (seq n)	WM % (seq n)
390	F	35	UL	Uni	6	NA	NA	No	E (RE)	c.1215+1G>A	8.2 (1138)	8.8 (1588)	8.6 (2726)
973/2013	F	31	BL	Multi	36	IVB (RE) VB (LE)	NA	NA	E (LE) RT—FT (RE)	c.596T>G (p.Leu199*)	23.3 (597)	25.2 (934)	24.4 (1531)
39/2013	M	5	UL	Uni	49	VB (RE)	D (RE)	No	IACT (2 cycles M+T) IVM (8 injections) SCC (1 injection)—E (RE)	c.1735C>T (p.Arg579*)	23.0 (724)	26.5 (614)	24.7 (1338)
768	F	4	UL	Uni	29	VB (RE)	D (RE)	No	E (RE)	c.1301_1302dup (p.Gly435Trpfs*23)	38.9 (955)	32.8 (579)	36.6 (1534)
762	F	2	BL	Multi	4	VB (RE) VE (LE)	C (RE) E (LE)	Yes	E (LE) SCT (6 cycles C+Et) IACT (1 cycle M+T) IVM (2 injections)—FT (RE)	c.2212-1G>T	34.7 (1068)	43.8 (491)	37.6 (1559)
795	F	2	BL	Multi	At birth	IIA (RE) IIA (LE)	A (RE) A (LE)	Yes	SCT (2 cycles C+Et+V and 2 cycles C+Et)—FT (RE and LE)	c.1644dup (p.His549Thrfs*6)	47.4 (410)	43.5 (1872)	44.2 (2282)
721	M	2	BL	Multi	1	IV (RE) III (LE)	B (RE) B (LE)	Yes	SCT(6 cycles C+Et)—FT (RE) SCC (1 injections LE)	c.1050-1G>C	48.6 (806)	41.8 (694)	45.5 (1500)
704	M	2	BL	Multi	1	VB (RE) IB (LE)	D (RE) B (LE)	Yes	SCT (1 cycle C+Et)—E (RE) STC (5 cycles C+Et)—FT (LE)	c.1666C>T (p.Arg566*)	44.5 (2684)	46.7 (2576)	45.6 (5260)
779	M	10	BL	Multi	3	II (RE) III (LE)	A (RE) B (LE)	No	SCT (6 cycles C+Et) FT (RE and LE)	c.2359C>T (p.Arg787*)	49.4 (984)	44.1 (719)	47.2 (1703)
714	M	5	BL	Multi	4	IV (RE) IV (LE)	B (RE) B (LE)	Yes	SCT (6 cycles) IACT (1 cycle M+T) (LE)	c.1498+5del	47.8 (5121)	49.5 (7379)	48.8 (12 500)
745	M	15	BL	Multi	8	V (RE) IV (LE)	C (RE) B (LE)	Yes	SCT (6 cycles C+Et and 2 cycles C+Et+V)—E (RE)—FT (LE)	c.1363C>T (p.Arg455*)	48.8 (1405)	48.9 (1095)	48.8 (2500)
777	F	11	BL	Multi	At birth	I (RE) V (LE)	A (RE) D (LE)	Yes	E (LE) SCT (6 cycles C+Et)—FT (RE)	c.1363C>T (p.Arg455*)	48.8 (944)	52.6 (805)	50.5 (1749)
567/2013	M	2	BL	Multi	12	IV (LE) IV (RE)	C (LE) C (RE)	Yes	SCT (7 cycles)—IACT (2 cycles M+T)	c.2211+2dup	56.0 (613)	48.02 (429)	52.7 (1042)

Abbreviations: BL, bilateral; C, carboplatin; E, enucleation; Et, etoposide; FT, focal therapy; IACT, intra-arterial chemotherapy; IVM, intravitreal melphalan; M, melphalan; Multi, multifocal; NA, not available; RT, radiotherapy; SCC, subconjunctival carbolatin; SCT, sistemic chemotherapy; T, topotecan; UL, unilateral; Uni, unifocal; V, vincristine; WM, weighted mean.
Sequencing results are shown as percentage variant of total number of reads.
Reference sequence L11910.1 (Trancript NM_000321.2).

provides read counts and frequencies. Variants are also displayed graphically with a histogram indicating the positions. The default Variant/Consensus parameters include: minimum read percentage of 0.25% (per read direction), minimum read count of two per orientation and appearing in both forward and reverse directions, and dynamic N-mer thresholding for homopolymers. However, to examine a specific sequence position analysis parameters were set to minimum (read count to '1', read percentage to '0', directional support to 'Any' and N-Mer thresholding to 'Fixed'). Moreover, on the basis of our experience, variants with highly unbalanced frequencies (*F/R* strand ratio <0.20) were considered as technical artifacts. Moreover, results in homonucleotide regions were compared with data in normal controls and excluded when present in the latter group. *In silico* analysis of variants' pathogenicity was performed using the interactive biosoftware Alamut v2.3 (Interactive Biosoftware, Rouen, France).

RESULTS

In the present study, 40 patients with a diagnosis of sporadic RB (which was bilateral in 11 cases and unilateral in 29 cases) were re-analysed for *RB1* sequence variants by amplicon next generation sequencing (454 GS Junior System, Roche). In these patients, Sanger sequencing on lymphocytes had previously identified apparent *RB1* heterozygous variants in 10 cases: 9 bilateral and 1 unilateral. MLPA analysis in these samples had not revealed the presence of *RB1* large rearrangements. For amplicon deep sequencing, coverage of the target region (promoter and 27 exons of the *RB1* gene) was 100%. Different amplicons showed different sequencing efficiencies. The average depth and the balance between forward/reverse reads across individual *RB1* amplicons were thus not uniform. In one 454 GS Junior sequencing run, we obtained an average of 2500 reads per sample (minimum 1002, maximum 4716 mapped reads per sample).

454 GS Junior assay validation

To determine linearity of the assay and LOD of *RB1* mosaic variants, two DNA samples with different heterozygous variants were serially diluted with wild-type DNA and analysed by deep sequencing. Supplementary Figure S1 shows the calibration curves and establishes the LOD values as 5.0% (c.1363C>T) and 3.9% (c.1072C>T).

454 GS Junior analysis in mutation-negative cases

Amplicon deep sequencing was performed in five available unilateral tumors. We identified a total of seven truncating variants, including two in apparent homozygosis (Supplementary Table S2). The presence of these variants in matched lymphocytes of each patient was excluded by deep sequencing of the specific amplicons (Supplementary Table S2).

In the remaining 23 unilateral and 2 bilateral patients, next generation sequencing detected 3 cases (samples 390, 973/2013 and 39/2013) with *RB1* variants varying in frequencies from 8.6 to 24.7% (Table 1). These frequencies were confirmed by independent next generation sequencing experiments (Table 2). To rule out the possibility that the variant calls were due to sequencing errors of the 454 Roche platform, data were compared with frequencies obtained in three healthy individuals at the same positions as the mosaic variants (Table 2). Statistical analysis performed using *t*-test indicated that the variants in the three cases were called with high confidence with respect to background (Table 2). To confirm mosaicism, we collected additional tissues from these patients, including eye (tumor, muscle, retina and sclera), oral mucosa and urine, and we analysed the specific amplicon containing the variant by deep sequencing (Supplementary Table S3; Table 3). Different tissues showed variable frequencies of variants, confirming mosaicism (Supplementary Table S3; Table 3).

454 GS Junior analysis in heterozygous cases

To detect possible mosaicism among the 10 patients (9 bilateral and 1 unilateral) interpreted as heterozygotes after Sanger sequencing on lymphocytes, next generation sequencing was performed. To investigate whether detected variant frequencies were statistically different from those of a 'known' constitutional heterozygote, data were compared with frequencies obtained in mutated offspring of familial RB cases with the same variant of the mosaic variant or, when not available, a different variant present at least in the same amplicon of the suspected mosaic (Table 4). Unfortunately, for variants in exons 13 (sample 768) and 23 (sample 779), 'known' heterozygous controls were not available. For variants c.1666C>T in exon 17 of patient 704 and c.2212-1G>T in exon 22 of patient 762, *t*-test demonstrated that the differences were statistically significant (Table 4). Suspected mosaicism of both samples was then confirmed by indipendent experiments of next generation sequencing in additional tissues (Supplementary Table S3; Table 5). Analysis in additional tissues also confirmed that sample 768, showing a read percentage of 36.6% in blood DNA, was a mosaic case (Supplementary Table S3; Table 5). Four other cases showed a range of frequency values compatible with an heterozygous sample (Supplementary Table S3; Table 5).

DISCUSSION

In disorders such as RB, associated with a high rate of *de novo* mutations, mosaicism is not expected as a rare event.[23] Accordingly, since the late seventies, an increased number of patients with mosaicism of the *RB1* gene has been reported.[10–13] The reported incidence of mosaicism in RB has become increasingly accurate with the advancement of technologies employed for *RB1* variant analysis. In particular, next generation sequencing technology, based on single-molecule counting, allows to detect even mosaic variants with low frequencies.[16] In the present study, we re-tested by deep sequencing 40 sporadic RB cases (11 bilateral and 29 unilateral) in order to investigate the presence of mosaicism. In these samples, traditional methods (Sanger sequencing and MLPA) had previously identified 10 *RB1* sequence variants (9 bilateral and 1 unilateral cases). Next generation sequencing was performed in the blood and in the additional available tissues (ocular tissues, urine and/or oral mucosa).

The present study detected a significant percentage (10%) of patients with sporadic RB and no apparent germline mutations in

Table 2 *t*-Test of variant frequencies for three suspected low-level mosaic RB cases against background noises from runs of three healthy controls

RB1 variants (ID of patient)	*Blood % of RB cases (CI 95%)*			*Blood % of healthy controls (CI 95%)*			*t-Test*
	1	*2*	*3*	*1*	*2*	*3*	
c.1215+1G>A (390)	8.6 (7.6–9.7)	8.5 (7.8–9.2)	9.4 (8.8–10.0)	0.1 (0.0–0.2)	0.1 (0.0–0.3)	0.0 (–)	$P<0.0001$
c.596T>G (973/2013)	24.4 (22.1–26.7)	23.3 (21.4–25.2)	25.4 (22.8–28.0)	0.7 (0.3–1.1)	0.5 (0.2–0.8)	0.7 (0.3–1.1)	$P<0.0001$
c.1735C>T (39/2013)	24.7 (22.4–27.0)	27.4 (25.1–29.7)	26.5 (24.3–28.7)	0.1 (0.0–0.2)	0.1 (0.0–0.2)	0.2 (0.0–0.4)	$P<0.0001$

Abbreviation: CI, confidence interval. Variant frequencies, expressed in percentages, represent the weighted mean values calculated on the number of forward and reverse reads for each sample.

Table 3 Frequencies of *RB1* variants in additional tissues of Sanger mutation-negative cases

Patient ID	Eye tissues						Oral mucosa			Urine		
	Retina			Sclera								
	1 (seq n)	2 (seq n)	WM (CI 95%)	1 (seq n)	2 (seq n)	WM (CI 95%)	1 (seq n)	2 (seq n)	WM (CI 95%)	1 (seq n)	2 (seq n)	WM (CI 95%)
390	—	—	—	—	—	—	12.7 (1611)	11.2 (1680)	11.9 (10.8–13.0)	11.4 (1532)	13.5 (1549)	12.4 (11.2–13.6)
973/2013	—	—	—	—	—	—	16.8 (1914)	18.8 (2800)	18.0 (16.9–19.1)	23.8 (1584)	25.6 (1074)	24.5 (22.9–26.1)
39/2013	6.2 (3064)	4.9 (1904)	5.7 (5.1–6.3)	3.4 (2663)	4.1 (4177)	3.8 (3.4–4.3)	1.0 (1050)	1.2 (1551)	1.2 (0.8–1.6)	6.8 (1081)	7.8 (2397)	7.5 (6.6–8.4)

Abbreviations: CI, confidence interval; WM, weighted mean. Variant frequencies, expressed in percentages, represent the weighted mean values calculated on the number of forward and reverse reads. Each amplicon has been sequenced twice (1, 2). Highlighted in grey, the weighted mean values calculated on the total of reads number of the two experiments.

Table 4 *t*-Test of variant frequencies of RB cases against frequencies of heterozygous controls

Amplicon	Blood % of RB cases (CI 95%)				Blood % of heterozygous controls (CI 95%)				t-Test
	Variant (patient ID)	1	2	3	Variant (control ID)	1	2	3	
11	c.1050-1G>C (721)	45.5 (43.0–48.1)	50.1 (48.0–52.2)	49.7 (47.8–51.6)	c.1072C>T (1)	50.6 (48.9–52.4)	51.1 (49.5–52.7)	51.1 (49.6–52.6)	$P>0.05$*
14	c.1363C>T (745)	48.8 (46.8–50.8)	49.9 (47.5–52.3)	49.9 (46.9–51.0)	c.1363C>T (2)	50.3 (48.2–52.4)	50.2 (48.3–52.2)	51.4 (49.5–53.3)	$P>0.05$
14	c.1363C>T (777)	50.5 (48.2–52.8)	48.1 (46.1–50.1)	50.9 (49.3–52.3)	c.1363C>T (3)	50.3 (48.2–52.4)	50.2 (48.3–52.2)	51.4 (49.5–53.3)	$P>0.05$
15–16	c.1498+5del (714)	48.8 (47.9–49.7)	45.3 (42.4–48.3)	45.8 (43.1–48.6)	c.1399C>T (4)	49.9 (47.0–52.8)	49.7 (47–52.4)	48.9 (46.2–51.6)	$P>0.05$
17	c.1644dup (795)	44.2 (42.2–46.3)	49.3 (46.2–52.4)	46.6 (44.2–49.1)	c.1666C>T (5)	48.0 (45.9–50.1)	50.5 (48.4–52.4)	48.6 (46.2–51.0)	$P>0.05$
17	c.1666C>T (704)	45.6 (44.3–47.0)	45.9 (43.3–48.5)	45.9 (43.4–48.5)	c.1666C>T (5)	48.0 (45.9–50.1)	50.5 (48.4–52.4)	48.6 (46.2–51.0)	$P=0.0480$*
21	c.2211+2dup (567/2013)	52.7 (49.6–55.8)	51.8 (49.0–54.6)	48.3 (46.0–50.6)	c.2173_2174insT (6)	51.7 (49.5–53.9)	49.7 (47.8–51.6)	48.7 (46.8–50.6)	$P>0.05$
22	c.2212-1G>T (762)	37.6 (35.2–40.1)	34.0 (31.7–36.3)	32.7 (29.9–35.4)	c.2325+1G>A (7)	45.4 (43.6–47.3)	48.9 (46.9–50.9)	49.8 (47.7–51.9)	$P=0.0026$

Abbreviation: CI, confidence interval. Variant frequencies, expressed in percentages, represent the weighted mean values calculated on the number of forward and reverse reads for each sample. Each amplicon has been sequenced three times (1, 2, 3). The Fisher Snedecor *F*-test was performed to assess the equality of variance. If variances were not equal, the Satterthwaite's approximate *t*-test was used (*). Highlighted in grey, mosaic cases.

Table 5 Frequency of *RB1* variants in different tissues of Sanger heterozygous cases

Patient ID	Tumor			Muscle			Eye tissues			Retina			Sclera			Oral mucosa			Urine		
	1	2	WM %	1	2	WM %	1	2	WM %	1	2	WM %	1	2	WM %	1	2	WM %	1	2	WM %
	%(seq n)	%(seq n)	(CI 95%)	%(seq n)	%(seq n)	(CI 95%)	%(seq n)	%(seq n)	(CI 95%)	%(seq n)	%(seq n)	(CI 95%)	%(seq n)	%(seq n)	(CI 95%)	%(seq n)	%(seq n)	(CI 95%)	%(seq n)	%(seq n)	(CI 95%)
768	91.9 (3473)	91.7 (1728)	91.8[a] (91.1–92.3)	—	—	—	10.1 (3024)	11.3 (3796)	10.8 (10.1–11.5)	20.7 (2502)	17.3 (1477)	19.5 (18.3–20.7)	4.0 (3730)	4.2 (6090)	4.1 (3.7–4.5)	17.7 (2904)	19.9 (5924)	19.2 (18.4–20.0)			
762	100.0 (1314)	100.0 (1199)	100.0 (—)	18.1 (1368)	24.4 (1921)	21.8 (20.4–23.2)	—	—	—	—	—	—	22.8 (1321)	33.1 (3966)	30.5 (29.3–31.7)	27.1 (1521)	32.9 (3293)	31.1 (29.8–32.4)			
704	—	—	—	—	—		—	—		—	—	—	38.1 (1523)	35.9 (1518)	37.0 (35.3–38.7)	23.3 (1716)	27.3 (1374)	25.1 (23.6–26.3)			
721	—	—	—	—	—	—	—	—	—	—	—	—	48.3 (1050)	48.2 (1404)	48.2 (46.2–50.2)	46.9 (1208)	49.3 (2564)	48.5 (46.9–50.1)			
779	—	—	—	—	—	—	—	—	—	—	—	—	49.1 (1952)	51.2 (1899)	50.1 (48.5–51.7)	53.1 (1780)	50.1 (1845)	51.6 (50.0–53.2)			
745	46.5 (5449)	48.0 (5195)	47.2 (46.3–48.2)	—	—	—	—	—	—	—	—	—	49.2 (1127)	51.4 (1940)	50.6 (48.8–52.4)	45.4 (1714)	50.2 (3998)	48.7 (47.4–50.0)			
777	93.9 (1871)	95.0 (3330)	94.6[a] (94.0–95.2)	48.2 (1929)	48.6 (1023)	48.3 (46.5–50.1)	—	—	—	—	—	—	45.3 (1742)	50.6 (2925)	48.6 (47.2–50.0)	49.2 (1121)	48.7 (2865)	48.8 (47.3–50.4)			

Abbreviations: CI, confidence interval; WM, weighted mean. Variant frequencies, expressed in percentages, represent the weighted mean values calculated on the number of forward and reverse reads. Each amplicon has been sequenced twice (1, 2). Highlighted in dark grey, the weighted mean values calculated on the total of reads number of the two experiments. Highlighted in light grey, mosaic cases.
[a]The fraction of normal allele detected in tumor probably depends on contamination with normal cells during scraping.

RB1 that are actually cases of mosaicism showing low variant frequency (8–24%) in the blood. These results are in accordance with a previous study reporting that the lowest proportion of the *RB1* mutant alleles detectable unambiguously by Sanger sequencing ranges between 15 and 30%, depending on the specific change and sequence context.[13] Unfortunately, among unilateral samples, we had the availability of only five tumor tissues for mutation analysis. This is a limit compared with the previous study by Chen *et al*,[13] but it can represent a not infrequent situation in a diagnostic setting. The limited availability of tumor tissues was partially due to the fact that enucleation has decreased in favor of conservative treatments, including the recent superselective ophthalmic artery infusion chemotherapy promoted by Italian radiologists and ophthalmologists.[24] The added value of deep sequencing with respect to Sanger analysis is that it allows for more sensitive and accurate detection of mosaic variants even without hints from the tumor. In our series, thanks to the employment of next generation sequencing, detection rate changed from 3 to 10% in unilateral cases and from 82 to 91% in bilateral cases.

The failure to identify *RB1* mutations in the other samples, in particular the remaining bilateral case, could be due to the presence of mosaic sequence variants present at lower frequencies with respect to the LODs of our assay (Supplementary Figure S1) or to the presence of variants limited to tissues that were not tested in the present study.[13,25] Alternatively, deep intronic splice mutations in regions not included in the amplicon library might be responsible for failed identification.[26,27] Finally, mosaic large rearrangements of *RB1* were not investigated by our approach.

When the tumor tissue is not available, the identification of low-frequency mosaic variants in DNA isolated from the blood of patients is an important finding for genetic counseling. The failure to identify the low-level mosaic mutations by traditional methods would have led to give empirical risks based on tumor presentation (ie, unifocal or multifocal) to the family (Figure 1, left). The identification of the mutation instead is sufficient to propose testing in future offspring and make early identification of tumors without costly and invasive (under general anesthesia) eye examinations in those at-risk family members who have not inherited the mutation (Figure 1, right). Concerning the recurrence risk of the couple with the affected child, it is close to zero and thus lower than that attributable to a *de novo* germline mutation (Figure 1).

Present results show for the first time that the use of next generation sequencing combined with analysis in multiple tissues can reveal a significant fraction (3/7; 43%) of patients with high-level mosaicism in the blood who have previously been interpreted as cases with constitutional germline mutations. In fact, using deep sequencing, we were able to demonstrate that three cases (samples 768, 762 and 704) interpreted on the basis of Sanger electropherograms as heterozygotes were indeed mosaic cases (read frequencies of 36.6, 37.6 and 45.6%). This observation was confirmed by the analysis in other available tissues in which deep sequencing detected the same variants at variable percentages (Table 5). These results are in accordance with a recent paper showing that amplicon deep sequencing is more accurate for the quantification of mosaic variants and that Sanger sequencing gives estimations of higher percentages of mutated alleles.[25] We therefore concluded that all these cases derived from deleterious postzygotic events instead of germline mutations. Depending on the time of occurrence during embryonic development, mosaicism is expressed at variable percentages in different tissues. When blood – that is, the tissue that is generally collected for DNA analysis – is the tissue that eventually shows a variant frequency

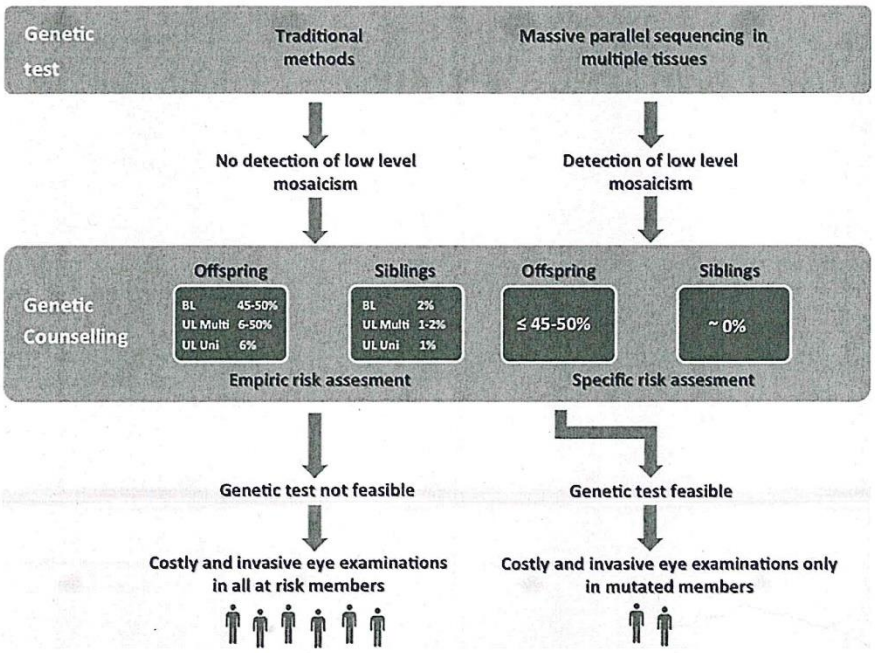

Figure 1 Implications of detection of low-frequency mosaic variants in sporadic retinoblastoma patients. Screening of the *RB1* gene performed by next generation sequencing in multiple tissues allows the detection of low-frequency mosaic variants (right), while this is not possible by traditional sequencing (left). This has important implications in genetic counseling. When an *RB1* mutation is not detected in DNA from leukocytes of the proband, empirical risks are given for siblings and offspring depending on tumor presentation (bilateral, unilateral multifocal, unilateral unifocal). Genetic test is not feasible and frequent eye examination under anesthesia are performed in all at-risk members. When a low-frequency mosaic variant is detected, specific recurrence risks are established for siblings and offspring. Genetic test is performed only in offspring and frequent eye examination are performed only in members who have inherited mutation. BL, bilateral; UL, unilateral; Multi, multifocal; Uni, unifocal.

close to 50%, misinterpretation with a constitutional variant may easily occur.

Following these results, genetic test in siblings is not proposed. Moreover, the recurrence risk to offspring can be <45–50% depending on the level of germline involvement in mosaicism. Quantitative analysis in sperm may be helpful.[12] However, as the germline cells may not be stable over time, the best approach would be to perform the test in close proximity to child conception.[11,28–30]

Reaching the average depth (~2500×) required for the identification of low-level mosaicism leads to an increase in costs (~800 Euros/sample) that become comparable to the costs of traditional analysis. However, deep sequencing significantly reduces working time and molecular diagnosis is achieved in 5 days instead of ~30-40 days necessary for conventional analysis in our country.

In conclusion, we identified a significant fraction (6/40; 15%) of mosaic patients among RB sporadic cases. Concerning clinical presentation, mosaic cases included the same number of unilateral and bilateral cases. These findings have to be taken into serious consideration in genetic counseling, where mosaicism has important implications for both recurrence risk assessment and family care. Moreover, this study confirms the higher sensitivity of deep sequencing technology in comparison with traditional methods for the detection and quantification of low-frequency mosaic variants. Finally, this paper highlights the importance of collecting other tissues in addition to blood, because they can be relevant to confirm somatic mosacism suspected by next generation sequencing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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3.2 Identification of molecular mechanisms explaining low penetrance in retinoblastoma families

Parent-of-origin effect of hypomorphic mutations and somatic mosaicism can explain low penetrance in retinoblastoma

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Unpublished Data

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Parent-of-origin effect of hypomorphic mutations and somatic mosaicism explain low penetrance in retinoblastoma.

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Abstract

Retinoblastoma is the most common eye cancer in children. Numerous families have been described displaying reduced penetrance and expressivity. An extensive molecular characterization of seven families led us to identify the two main mechanisms underlying these phenomena: i) mosaicism of amorphic mutations; and ii) parent-of-origin-effect of hypomorphic mutations. Somatic mosaicism for *RB1* splicing mutations (c.1960+5G>C and c.2106+2T>C) leading to a complete loss of function was demonstrated by high-depth NGS in two families. In both cases, the healthy carrier parent (one with retinoma) showed a mutation frequency lower than that expected for a heterozygous individual, indicating a 56-60% mosaicism level. Parent-of-origin-effect is a mechanism by which the phenotypic impact of an allele depends on the parental origin. An imprinting mechanism justifying a ~3 fold excess of the *RB1* maternal canonical transcript has been previously described. As a consequence, hypomorphic mutations, if maternally inherited, may retain sufficient suppressor activity to prevent tumor onset. In five low-penetrant families we identified paternally inherited hypomorphic mutations in the affected members, namely a deletion resulting in the loss of 37 aminoacids at the N-terminus (c.608-16_608del), an exonic substitution with a "leaky" splicing effect (c.1331A>G), a partially deleterious substitution (p.Arg661Trp) and a truncating C-terminal mutation (c.2663+2T>C). The identification of these mechanisms changes the genetic/prenatal counseling and the clinical management of families, raising to a 50% the recurrence risk for unaffected carriers, offspring of *RB1*-mutated mothers, and imposing the need for second tumor surveillance in unaffected carriers at risk of developing adult-onset cancer such as osteosarcoma or leiomyosarcoma.

INTRODUCTION

Retinoblastoma (RB; OMIM #180200) is the most common pediatric intraocular tumor, affecting about 1 in 15,000 to 20,000 live births (Broaddus et al. 2009). It is caused by biallelic inactivation of *RB1*, the first characterized tumor suppressor gene, located at 13q14.2 (Friend et al. 1986; Knudson 1971). The most frequent form is the non hereditary one (60%), with both inactivating events occurring in cells of the developing retina. In this form, the tumor is confined to a single eye (unilateral) and develops after the first year of life. The hereditary form (40%) is due to a *RB1* germline predisposing mutation and subsequent somatic inactivation of the other allele, mostly occurring through mitotic recombination/nondisjunction or large deletions (Cavenee et al. 1983). In this form, the mean age of diagnosis is anticipated (within the first year of life) and cancer develops as multiple foci (multifocal), usually involving both eyes (bilateral).

Hereditary RB is transmitted according to an autosomal dominant pattern; the penetrance is incomplete and generally reported as equal to 90%. However, it is common to observe families in which a significant fraction of mutation carriers remain unaffected or develop only unilateral tumors or benign lesions called retinomas (Harbour 2001), a phenomenon that has always posed a question about the underlying mechanisms. Mutations reducing *RB1* expression or partially inactivating pRB activity have been associated to milder phenotypic expression (Bremner et al. 1997; Cowell et al. 1996; Eloy et al. 2016; Gamez-Pozo et al. 2007; Genuardi et al. 2001; Harbour 2001; Hung et al. 2011; Klutz et al. 2002; Kratzke et al. 1994; Lefevre et al. 2002; Lohmann et al. 1994; Otterson et al. 1997; Sakai et al. 1991; Sampieri et al. 2006; Scheffer et al. 2000; Schubert et al. 1997). However, the description of low-penetrant families bearing classical “null” mutations and the observation that penetrance/expressivity can vary significantly between and within families with identical mutations has led to speculate that other mechanisms may be determinant (Abouzeid et al. 2009; Dalamon et al. 2004; Onadim et al. 1992; Sampieri et al. 2006; Sanchez-Sanchez et al. 2007).

As early as the eighties, mosaicism has been established as an important cause of phenotypic variability (Hall 1988). Especially in disorders such as RB, in which a high proportion of cases represents new mutations, this mechanism is expected to occur and to influence phenotypic expression (Carlson and Desnick 1979). In 1998 Sippel and coworkers investigated this phenomenon in a large cohort and documented mosaicism for the initial *RB1* mutation in ~10% of families (Sippel et al. 1998). Subsequently, the employment of more sensitive techniques has allowed the detection of even low-level mosaicism and the identification of mosaic RB cases has increased up to 30%, but the impact of this phenomenon on phenotypic expression has been poorly investigated (Amitrano et al. 2015; Chen et al. 2014; Rushlow et al. 2009).

Clinical penetrance can also be influenced by the gender of the transmitting parent (parent-of-origin effect), as demonstrated in different diseases such as myoclonus dystonia (Muller et al. 2002) or paraganglioma (Badenhop et al. 2001). The best characterized mechanism underlying this phenomenon is genomic imprinting (Lawson et al. 2013). An association between parent-of-origin dependent allelic expression and phenotype was already observed in 2002 for a splicing *RB1* mutation segregating in two families (Klutz et al. 2002). In 2009, a ~3 fold excess of the *RB1* maternal canonical transcript due to an imprinting mechanism was demonstrated(Buiting et al. 2010). As a consequence, maternally inherited hypomorphic mutations may retain sufficient suppressor activity to prevent tumor development. In accordance, Eloy and collaborators demonstrated a parent-of-origin effect in low penetrance pedigrees segregating the common c.1981C>T/p.Arg661Trp mutation (Eloy et al. 2016). The impact of a parent-of-origin mechanism on phenotypic expression for other *RB1* mutations has not been established.

Herein, we investigated mosaicism and parent-of-origin effect as the two main mechanisms capable to explain reduced penetrance/expressivity in familial RB. Somatic mosaicism for truncating splice site mutations (c.1960+5G>C and c.2106+2T>C) producing only the mutated transcript was

identified in two families. A parent-of-origin effect was demonstrated in five families harboring hypomorphic *RB1* mutations: two already reported exonic substitutions (c.1981C>T and c.1331A>G) and two new splice site mutations (c.608-16_608del and c.2663+2T>C).

MATERIALS AND METHODS

Families

Diagnosis of RB was established at the Retinoblastoma Referral Center of Siena (Ophtalmology Department, AOUS) and in external Institutes (Istituto Gaslini of Genova and Pio Istituto Oftalmico of Milano). Families underwent genetic counseling and blood samples were collected in EDTA-containing tubes and sent to the Unit of Medical Genetics of Siena for *RB1* mutational analysis (Sanger sequencing and MLPA). When a germline mutation was found in the proband, molecular testing was extended to relatives. To assess penetrance/expressivity, the diseased-eye-ratio (*der*) was calculated (Lohmann et al. 1994). Additional blood samples from probands and their relatives were collected in PAXgene tubes for RNA isolation. Informed consent was obtained from all adult patients and parents of affected children.

DNA/RNA isolation

Genomic DNA was extracted from EDTA peripheral blood samples using MagCore HF16 (Diatech Lab Line). RNA was isolated from whole blood stabilized in PAXgene Blood RNA tubes (PreAnalytiX®, Qiagen) following manufacturer's instructions. RNA quality was evaluated by NanoDrop 2000 Spectrophotometer (Thermo Scientific).

High-depth NGS

To investigate mosaicism for *RB1* mutations (GeneBank Accession L11910.1; transcript NM_000321.2), the specific exonic/intronic sequences were analysed by Next Generation Sequencing (NGS) at high-depth (at least 1000X allowing to detect a minimum of 5% of mosaicism) using Roche 454 GS Junior platform as previously described(Amitrano et al. 2015). Target regions were amplified using fusion primers reported in Supplementary Table 1. Variants i*n silico* analysis was carried out using the Interactive Biosoftware Alamut v2.3 (Interactive Biosoftware, Version 2.7)

(Houdayer 2011). Relating to the p.Arg661Trp substitution, the above described protocol was also employed to quantify mutant/wild-type allelic expression. cDNA, retrotranscribed from patient's RNA, was amplified using primers reported in Supplementary Table 1.

Minigene assays

A portion of *RB1*, containing the exon close to or harboring the variant of interest and part of the upstream and downstream, was amplified from patient's DNA (oligonucleotides and PCR conditions are available upon request). Concerning the c.1331A>G allele, exon 13 and part of the flanking introns were amplified from human genomic DNA and the specific mutation was introduced by site-directed mutagenesis using the QuikChange II Site Directed Mutagenesis kit (Stratagene). We employed as backbone the β-globin-pCDNA3.1 vector (Forzan et al. 2010) and obtained the hybrid minigene constructs as previously described (Fallerini et al. 2016). For expression analysis, we selected one clone containing the wild-type allele, one clone with each variant and the empty vector. Minigene expression was carried out on Y79 cells (ATCC® HTB18™), a human retinoblastoma cell line. Cells were cultured in suspension in RPMI 1640 medium (Gibco Thermo Fischer Scientific) supplemented with 20% fetal bovine serum. After twenty-four hours of serum-deprivation, cells were transfected with Lipofectamine® 2000 according to the manufacturer's protocol. After 24 hours, total RNA was extracted using the TRIzol kit (Thermo Fischer Scientific) and retrotranscribed. The cDNA was amplified using primers located on β-globin exon 2 and 3 to avoid eventual interference from the endogenous gene and PCR fragments were analyzed as previously reported (Fallerini et al. 2016).

RNA analysis

RNA was retrotranscribed into cDNA using Qiagen RT-PCR kit (QuantiTect® Reverse Transcription Kit) and 1µg of total RNA per reaction. Amplification was conducted on a Thermal Cycler 2720 (Applied Biosystems) using primers reported in Supplementary Table 1. Thermal cycling was the

following: 95°C for 5 min, followed by 35 cycles at 95°C for 30s, at the specific annealing temperature for 30s, at 72°C for 30s, followed by a final extension at 72°C for 5 min. PCR products were separated by electrophoresis on a 3% agarose gel. When multiple bands were obtained, cDNA fragments were separately sequenced after extraction by Zyomoclean Gel DNA Recovery kit (Zymo research). Relating to the p.Arg661Trp substitution, total sample was purified using AMPure PCR purification system (Agenocourt). DNAs were sequenced by Sanger sequencing using the PE Big Dye Terminator Cycle Sequencing Kit on an ABI Prism 3130 analyser (Applied Biosystems). Data were analysed using the Sequencher version 4.9 software.

Real Time qRT-PCR

Primers were specifically designed to quantify mutant and wild-type transcripts (Supplementary Table 2). *GUSB* was selected as housekeeping gene. Quantitative PCR was carried out in single-plex reactions in a 96-well optical plate with FastStart SYBR Green Master Mix (Roche) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Experiments were performed in triplicate in a final volume of 20ul with 25ng of cDNA and 25nM of each primer, following the SYBR Green protocol (Roche). Standard thermal cycling conditions were: 2 min at 50°C and 10 min at 95°C; 40 cycles at 95 °C for 15s and 60°C for 1 min; dissociation stage 95°C for 15s, 60°C for 20s and 95°C for 15s. Data were analyzed using the comparative Ct method (Livak 1997).

RESULTS

Herein, we investigated the molecular mechanisms underlying reduced penetrance/expressivity in seven RB families (five novel and two already reported) (Fig. 1) (Tab. 1) (Genuardi et al. 2001; Sampieri et al. 2006). In these families, traditional sequencing had previously shown the presence of four intronic variants (c.608-16_608del, c.1960+5G>C, c.2106+2T>C and c.2663+2T>C) and two exonic substitutions (c.1331A>G and c.1981C>T) (Fig. 1) (Genuardi et al. 2001; Sampieri et al. 2006). Analysis of RNA directly isolated from patient's blood samples and/or minigene assays in an RB cell line (Y79) allowed to characterize the consequences of splicing mutations and to investigate a possible "leaky" effect (Fig. 2 and 4). High-depth NGS and wild-type/mutant allelic expression analysis allowed to characterize the molecular mechanisms explaining phenotypic presentations: I) somatic mosaicism of amorphic mutations; and II) parent-of-orgin-effect of hypomorphic mutations.

Table 1. Clinical presentations of mutated

Family #	Family member	Gender (F/M)	Age at counseling	Retinal Lesion (RB/RN)	UL/BL	Age of diagnosis	Other tumors (age of diagnosis)	DER
1	IV1	M	2y	yes (RB)	UL	2y	-	0.5
	III2	M	30y	no	-	-	-	
	II2	F	47y	no	-	-	-	
	II4	F	50y	no	-	-	-	
	III4	M	32y	no	-	-	-	
	IV2	M	6y	yes (RB)	BL	NA	-	
2	II2	M	3y	yes (RB)	BL	5m	-	1.0
	I2	F	44y	no	-	-	-	
3	III1	M	12y	yes (RB)	BL	8m	-	1.0
	II2	M	38y	-	-	-	Osteosarcoma (14y)	
4	IV5	F	55y	yes (RB)	UL	48m	-	0.67
	IV4	M	†30m	yes (RB)	BL	24m	-	
	III3	M	†81y	no	-	-	Leiomyosarcoma (80y)	
	II2	F	†NA	no	-	-	-	
	II1	M	†NA	no	-	-	-	
	III1	F	†5y	Yes (RB)	BL	NA	-	
	III2	F	†75y	no	-	-	-	
	IV1	M	58y	no	-	-	-	
	V1	M	31y	yes (RB)	UL	60m	-	
5	II2	M	19y	yes (RB)	BL	4m	-	1.5
	I2	M	55y	yes (RN)	UL	36y	-	
6	II1	M	42y	yes (RB)	BL	3m	-	1.67
	II3	F	39y	yes (RB)	BL	3m	-	
	I1	M	69y	yes (RN)	UL	69y	-	

Probands' samples were recorded in the internal biobank with the following codes: #4675/16 (family 1), #298/2014 (family 2), #198 (family 3), #3812/16 (family 4), #15 (family 5) and #3000/15 (family 6). Abbreviations: RB, retinoblastoma; RN, retinoma; UL, unilateral; BL, bilateral; DER, diseased eye ratio; y, years; m, months; NA, not available; †, deceased.

Clinical presentations

Family 1. The proband is the first child of healthy non consanguineous Chinese parents (Fig. 1) (Table 1). From the paternal side, it was reported a 2nd degree cousin affected by bilateral RB (IV2) diagnosed at 11 months of age. Proband, at the age of two years, was referred to an ophthalmologist because of onset of anisocoria in his right eye. Brain and spinal MRI revealed an expansive partially calcified lesion suggestive for RB extending through the papilla to the proximal portion of the ipsilateral optic nerve, without signs of dissemination. The child underwent chemotherapy and successive enucleation. Histological examination confirmed RB diagnosis. After six months, brain and spinal MRI screening did not show any residual disease or dissemination. An ophthalmological screening with fundus examination in both parents resulted normal.

Family 2. The proband was born from the second pregnancy of healthy non consanguineous Greek parents (Fig. 1) (Table 1). The first pregnancy was interrupted at 20 weeks of gestation for oligohydramnios. At the age of 2 months, parents noticed strabismus in the left eye of the child and soon afterwards the diagnosis of bilateral RB was established. Family history was unremarkable.

Family 3 . The proband is the first child of non consanguineous parents (Fig. 1) (Table 1). The father (II2) presented osteosarcoma of the right leg that was surgically resected at the age of 14 years. Fundus examination, performed in the father after RB diagnosis in the son, was referred as normal. At the age of 8 months the proband was referred to an ophthalmologist for the observation of leukocoria in the right eye. Brain MRI revealed a mass of 1.6cm x 1.1cm x7 mm suggestive for intrabulbar RB. Ophthalmoscopic evaluation revealed a focal lesion spanning more than 50% of the retinal surface and four additional foci in the nasal retina. In the left eye, a paramacular lesion of the

retina of one-disc diameter in size was observed. The child underwent focal and systemic chemotherapy.

Family 4. The female proband is the second child of a non consanguineous couple (Fig. 1) (Table 1). She had the enucleation of the right eye for unilateral RB at the age of 4 years. A bilateral RB was diagnosed in the brother (IV4) at the age of 2 years and 4 months. Histological examination reported an undifferentiated RB of 12x17mm rich of eosinophils involving the optic nerve and the meninges. Chemotherapy and radiotherapy followed enucleation. A single tumour was then identified in the right eye, treated by several photocoagulation sessions. The patient died 5 months later for a recurrence of the disease in the left orbital frontal region. The father died for a leiomyosarcoma at the age of 81 years. A paternal second cousin (V1) developed unilateral RB at the age of 5 years. A cousin of the proband's father (III1) died at the age of 5 years for RB.

Family 5. This family, already reported in one of our previous studies, was initially classified as a sporadic case in which the proband had a diagnosis of bilateral RB at the age of 4 months (Fig. 1)(Table 1) (Sampieri et al. 2006). RB started in the right eye and required chemotherapy and successive enucleation. Histological examination showed an endofitic and esofitic mass, well-differentiated, which did neither infiltrate the anterior chamber nor the optic nerve. A following involvement of the left eye required chemotherapy, laser therapy, radiotherapy and enucleation. After RB diagnosis in the proband, fundus examination in the father (I2) showed the presence of a retinoma that underwent spontaneous calcification in the left eye, leading to a reclassification of this case as a familial RB.

Family 6. This family came to our attention to define the recurrence risk for RB in II2 subject's newborn. His two siblings both presented with bilateral RB starting from 3 months of age. The disease

required enucleation of both eyes in the older brother, enucleation of the right eye and radiotherapy of the controlateral eye in the younger sister. Anamnestic history was silent for RB in other family members. An ophthalmological screening with fundus examination performed in the father revealed the presence of a retinoma in the left eye.

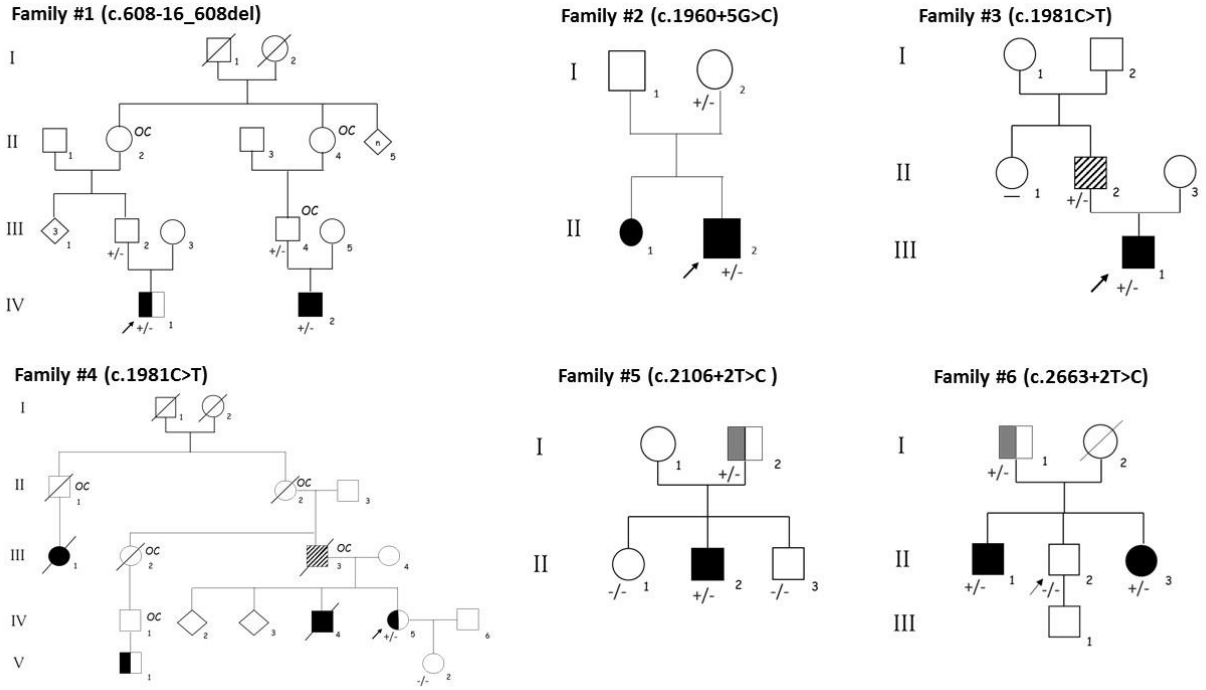


Fig. 1 Pedigrees of RB families with reduced penetrance/expressivity. Blackened symbols indicate patients with bilateral retinoblastoma; half-blackened symbols indicate patients with unilateral retinoblastoma; half-grey symbols indicate subjects with retinoma; crosshatched symbols indicate subjects with other *RB1*-related neoplasms: osteosarcoma in family 3 (subject II2) and leiomyosarcoma in family 4 (subject III3); OC: obligated carriers; dashed symbols: deceased subjects.

Molecular analysis

Minigene assays

To characterize the splicing effects of *RB1* variants and investigate a possible "leaky" effect, a hybrid minigene approach was employed. Our results showed that all the four intronic variants resulted in aberrant splicing, without the formation of the wild-type transcript, proving that they exert a severe effect on mRNA maturation (Fig. 2A, B, C and D). The corresponding wild-type constructs generated instead the expected splicing products with the exception of the plasmid containing exon 19: in this case splicing was less efficient, showing in addition to the correctly spliced transcript, some degree of intronic retention (with a longer isoform retaining 125 nucleotides of intron 19) (Fig. 2B). Nevertheless, no functional transcripts containing exon 19 were expressed from the mutated construct and two bands were obatained: a fainter upper one corresponding to the same *mis-spliced* transcript present in the wild type and a lower most abundant band showing complete skipping of exon 19 with retention of the same portion of intron 19 found in the longer transcript. Concerning the other variants, minigene assay revealed that the c.608-16_608del causes the skipping of exon 7 (Fig. 2A). Expression of the c.2106+2T>C substitution in the β-globin minigene led to the usage of an upstream alternative 5' splice site resulting in the expression of a single transcript containing exon 20 lacking the final 34 nucleotides, thus resulting in a frameshift (Fig. 2C). As for the c.2663+2T>C variant, expression analysis confirmed an aberrant splicing pattern, consisting of both exon 25 skipping and intronic retention, in the absence of the normally spliced transcript (Fig. 2D).

Minigene assay was also employed to evaluate the splicing effect of an exonic substitution (c.1331A>G) previously reported in a low penetrant RB family showing a clear parent-of-origin effect with a higher probability to manifest the tumor in case of paternal inheritance (Genuardi et al. 2001). As shown in Figure 2E, expression of the mutant minigene yielded two bands: sequencing of

the lower RT-PCR product confirmed skipping of exon 13, whereas the upper fainter band corresponds to the wild-type transcript, demonstrating a “leaky” effect (hypomorphic mutation).

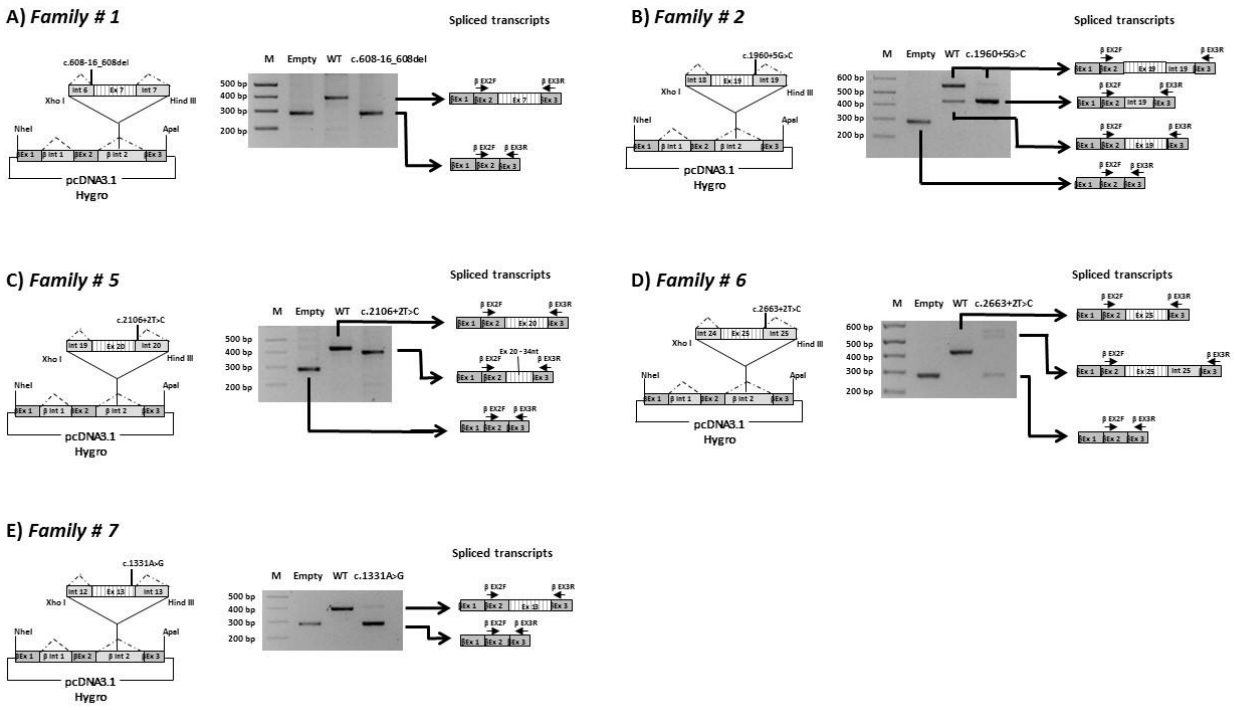


Fig. 2 Minigene assay results. RT-PCR analysis of the *RB1* minigene constructs expressed in Y79 cells using vector specific primers. A schematic representation of the hybrid minigene constructs is depicted on the left; the dotted lines indicate normal splicing. Each construct contains the exon close to, or harboring the variant of interest and part of the upstream and downstream introns of *RB1* gene. In Fig. 2A the construct contains the exon 7 of the *RB1* gene; c.608-16_608del, construct containing the c.608-16_608del variant. In Fig. 2B, the construct contains exon 19 of the *RB1* gene. Legends as above, c.1960+5G>C, construct containing the c.1960+5G>C variant; In Fig. 2C, the construct contains exon 20 of the *RB1* gene. c.2106+2T>C, construct containing the c.2106+2T>C variant. In Fig. 2D, the construct contains exon 25 of the *RB1* gene. c.2663+2T>C, construct containing the c.2663+2T>C variant. In Fig. 2E, the construct contains exon 13 of the *RB1* gene. c.1331A>G, construct containing the c.1331A>G variant. Legend: M, 1kb plus molecular marker (Invitrogen); Empty, empty β-globin-pCDNA3.1 vector; WT, wild type construct

High-depth NGS

On the basis of pedigree characteristics, we tested the hypothesis that reduced penetrance/expressivity in families 2, 3, 5 and 6 were due to somatic mosaicism. We therefore employed high-depth NGS to analyze the percentage of mutated molecules in DNAs isolated from probands and carrier parents. In families 3 and 6 we detected about the same percentage of mutated molecules compatible with a heterozygous state (data not shown). In the other two families we found a ~50% of mutated molecules in the affected proband in comparison with a lower percentage in the unaffected mother (~28%; family 2) and in the father with retinoma (~34%; family 5), supporting the conclusion that somatic mosaicism is responsible for reduced penetrance/expressivity (Fig. 3A and B).

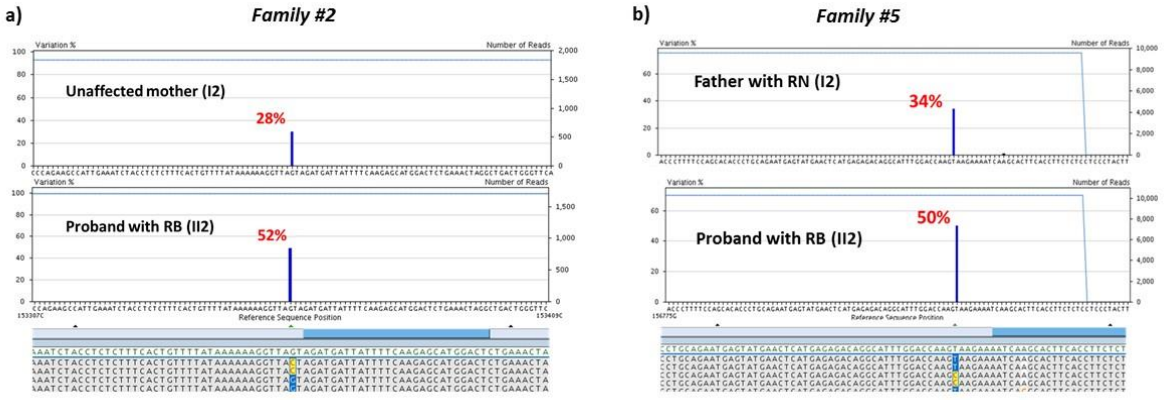


Fig. 3 High-depth NGS in families 2 and 5. Sequencing results are showed as percentage variant of total number of reads. a) In family 2, the c.1960+5G>C mutation was detected at a percentage of ~50% in the proband with retinoblastoma and ~28% in the unaffected mother. b) In family 5, the c.2106+2T>C mutation was detected at a percentage of ~50% in the proband with retinoblastoma and ~ 34% in the father with retinoma.

Transcript analysis: quantification of wild-type/mutant allelic expression

Based on pedigrees, a parent-of-origin-effect was hypothesized to explain reduced penetrance/expressivity in the previously studied families (3 and 6) and in the remaining families (1 and 4) (Tab. 1). Transcript analysis with quantification of wt/mutant allele ratio was thus performed on mRNA directly isolated from peripheral blood of available individuals to look for an association with clinical presentations.

RT-PCR for the splicing mutations c.608-16_608del (family 1) and c.2663+2T>C (family 6) confirmed results obtained by minigene assay: in-frame skipping of exon 7 in the first case and frameshift skipping of exon 25 in the second case (Fig. 4A and B, upper panels) (Supplementary Fig. 1). For the c.2663+2T>C mutation, RT-PCR did not identify the additional aberrant transcript with intronic retention detected by minigene assay probably due to product instability. At protein level, the aberrant transcript with exon 7 skipping is predicted to encode a product lacking 37 amino acids at the N-terminus (Fig. 4a, middle panel). On the other hand, exon 25 skipping leads to a frameshift and ultimately to a premature stop codon (aa 841), predicted to result in a truncated protein lacking the carboxy-terminus (Fig. 4b, middle panel). For both mutations Real Time qPCR expression analysis showed a higher expression of the abnormally spliced products in the unaffected carrier fathers along with an inversion of the ratio between the two differentially spliced isoforms respect to the affected offspring (Fig. 4, lower panels).

The p.Arg661Trp substitution was identified in two families (3 and 4) (Fig. 1). In family 4, we had the possibility to investigate mRNA isolated from proband blood sample and quantify wt/mutant allelic expression using high-depth NGS. We found that mutant molecules are ~23%, in accordance with an expected lower expression of a paternally inherited mutation (Supplementary Figure 2).

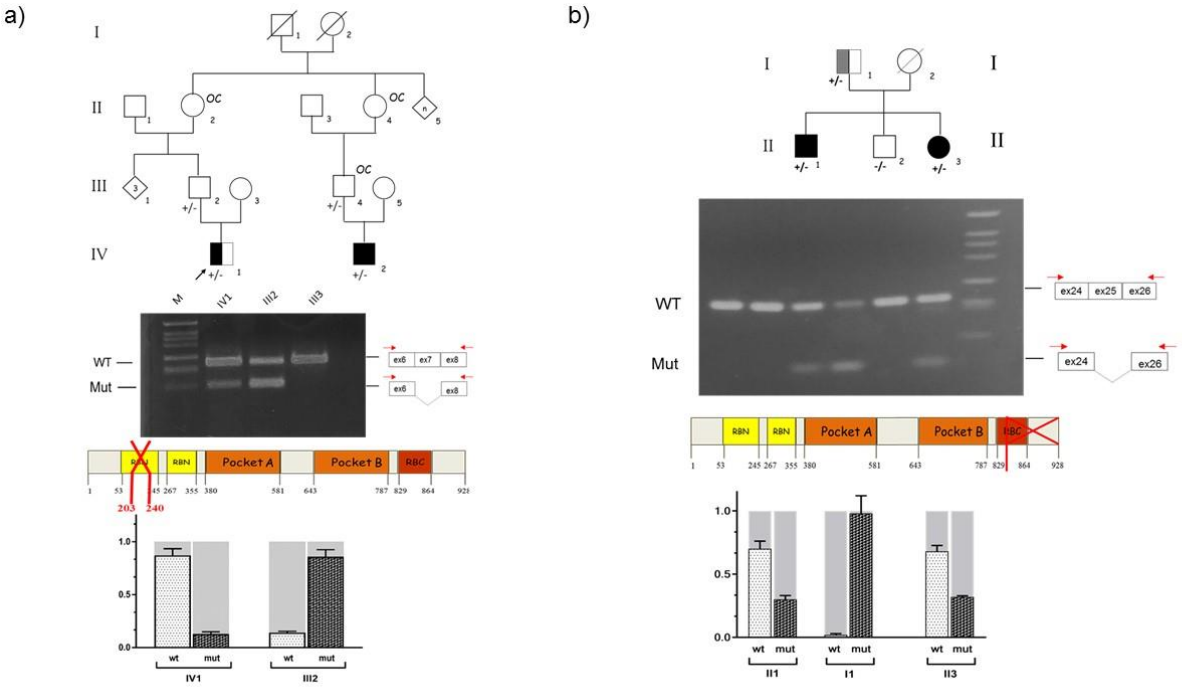
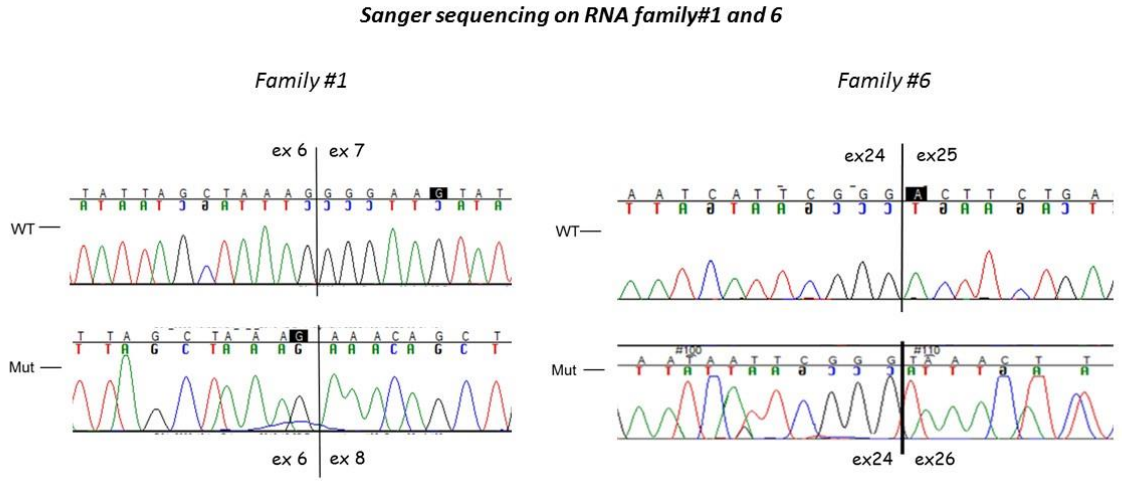
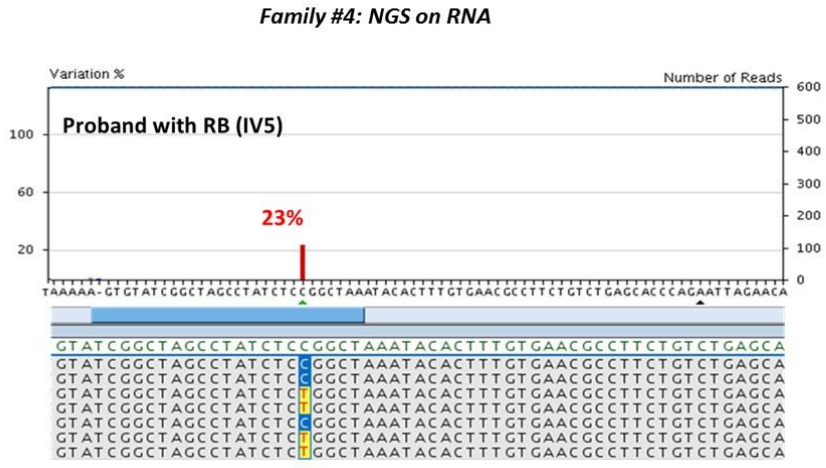


Fig. 4 mRNA wt/mutant allelic quantification. a) Characterization of the effect of the splicing deletion c.608-16_608del identified in family 1. RT-PCR using primers spanning exons 5-8 showed two products in both the affected proband and the unaffected carrier father: the expected normal-sized transcript (263bp) and a shorter one of 151bp (upper panel). The latter product was not observed in the mother not bearing the mutation. Direct sequencing of the shorter product showed the complete skipping of exon 7. At protein level, aberrant transcript with exon 7 skipping is predicted to encode a product lacking 37 amino acids at the amino terminus (middle panel). Real time PCR relative quantification, normalized respect to the total transcript (shaded in grey), showed an inverted ratio between wt and mutant isoform in the affected child (87% vs 13%) respect to the father (14% vs 86%) (lower panel). b) Characterization of the effect of the c.2663+2T>C mutation identified in family 6. RT-PCR was carried out using primers spanning exons 23-27. An additional shorter product respect to the normal-sized one was observed in mutated family members (I1, II1 and II3) (upper panel). Sanger sequencing of the abnormal transcript showed the complete exclusion of exon 25, resulting in a frameshift that insert a stop codon at amino acid 841 (middle panel). By real time qPCR analysis, the two affected individuals showed a higher (70%) wt allelic expression respect to the mutant one (30%), while the father displayed almost exclusively (~98%) the mutant transcript (lower panel). WT, normal-sized wild-type product; Mut, abnormally spliced product; Ctrl1 and Ctrl2, control samples.



Supplementary Fig. 1 Sanger sequencing of abnormally spliced product in families 1 and 6. Sequence chromatograms of aberrant isoforms identified analyzing mRNA of mutated individuals IV1 and III2 of family 1 (left) and II1, I1 and II3 of family 6 (right).



Supplementary Fig. 2 Quantification of allelic expression for the p.Arg661Trp mutation identified in family 4. High-depth NGS on mRNA isolated from blood sample of patient III5 showed that mutant molecules are ~23%, in accordance with an expected lower expression of a paternally inherited mutation.

DISCUSSION

The identification of the molecular bases of penetrance and expressivity represents a key point to better understand disease mechanisms and provide optimal genetic counseling. Several studies suggest that penetrance and expressivity of hereditary RB can be influenced by the nature of the predisposing germline mutation (Lohmann et al. 1994). However, different evidences have suggested that this factor alone cannot be sufficient and that other mechanisms are involved. Importantly, *MED4* and *MDM2* genes have been identified as RB modifiers (Castera et al. 2010; Dehainault et al. 2014). More recently, a parent-of-origin effect due to an imprinting mechanism has been demonstrated to impact on phenotypic expression associated with the common p.Arg661Trp mutation (Eloy et al. 2016). Herein, an extensive molecular characterization of seven families with reduced penetrance/expressivity led us to identify the two main mechanisms underlying these phenomena: i) mosaicism of amorphic mutations; and ii) parent-of-origin-effect of hypomorphic mutations.

Based on pedigrees analysis, we hypothesized that healthy mutation carriers could be mosaic for the predisposing *RB1* mutation in families 2, 3, 5 and 6. According to this model, the reduced tumor susceptibility would be associated to a decreased pool of retinal cells that can be targeted by a second inactivating event. In families 2 and 5, by high-depth NGS we demonstrated that healthy carrier parents showed *RB1* mutation frequencies that were less than the 50% expected for a heterozygote, indicating a mosaic condition and supporting the conclusion that this mechanism is responsible for the observed clinical presentations. The identified mutations (c.2106+2T>C and c.1960+5G>C) were already reported in RB patients (Price et al. 2014; Sampieri et al. 2006; Tsai et al. 2004). By hybrid minigene assay we showed that both mutations provoke an aberrant splicing with a severe effect on mRNA maturation since no wild-type transcript could be generated, suggesting a complete loss of function effect. In agreement, a previous study reported that the c.1960+5G>C mutation gives rise to a truncated protein without detection of normal pRb (Tsai et al. 2004). The introduction of NGS has greatly improved the detection of mosaicism and has demonstrated that a

high fraction of *RB1* mutations arises by post-zygotic events (Amitrano et al. 2015; Chen et al. 2014). However, the phenotypic impact of mosaicism in RB remains to be determined. Only few low-penetrant families with identified or presumed mosaicism have been reported so far (Rushlow et al. 2009; Sippel et al. 1998). These pedigrees show the more common ‘‘null’’ alleles, which are expected to completely abolish repressor activity. Reduced penetrance/expressivity can be thus observed also in the context of a predisposing amorphic mutation and, in this case, mosaicism probably represents the underlying mechanism. On the other side, mosaicism has not been reported in association with weak alleles, suggesting that this combination is less likely to result in RB.

The remaining five low-penetrant pedigrees were compatible with the hypothesis of a parent-of-origin effect combined with hypomorphic *RB1* mutations (Genuardi et al. 2001). According to this model, maternal inheritance is associated with a higher probability of being unaffected or developing only benign retinomas. This model is in agreement with the previous demonstration that *RB1* gene is preferentially expressed from the maternal allele due to an imprinting mechanism and consistent with the hypothesis that maternally inherited hypomorphic mutations may retain sufficient suppressor activity to prevent tumor development (Buiting et al. 2010). In this study, mutations include the well-known p.Arg661Trp substitution (families 3 and 4), one exonic variant with splicing impact (family 7) and two splice site changes whose effect at the transcript level has never been previously investigated (families 1 and 6). The p.Arg661Trp mutation is known to partially inactivate pRb and a correlation between the gender of the transmitting carrier and penetrance has been recently demonstrated (Eloy et al. 2016; Fang et al. 2002). As to the c.1331A>G exonic substitution, minigene assay confirmed aberrant splicing and showed that a fraction of normal transcript was still produced by the mutant allele, justifying the milder phenotypic impact (Genuardi et al. 2001). Trans-regulatory factors can change the ratio between wild-type and aberrant isoforms over time and in different tissues, resulting in a modulation of the “leaky” effect and contributing to clinical variability.

Concerning intronic variants, our results demonstrated that both changes result in aberrant splicing without evidence of leaky effect. In accordance with the hypothesis of hypomorphic alleles, the c.608-16_608del mutation results in the in-frame skipping of exon 7, leading to the loss of 37 aa at the N-terminus. Previous studies on humans and mice agreed that loss of this domain generates partially penetrant phenotypes on tumor incidence (Dryja et al. 1993; Goodrich 2003; Jakubowska et al. 2001; Lefevre et al. 2002; Otterson et al. 1997; Riley et al. 1997; Sanchez et al. 2000). More recently, Borysov and colleagues provided a molecular explanation for these findings showing that this protein portion is important for growth-suppressive functions, but its absence only partially impairs the ability of pRB to inhibit DNA replication and block G1-to-S cell cycle transit (Borysov et al. 2015). The c.2663+2T>C mutation generates an abnormal transcript that, lacking exon 25, is predicted to truncate the protein at the carboxy-terminus. Late truncating mutations have been previously associated to low penetrant RB and have been demonstrated to partially impair pRB functions such as nuclear localization and repression (Bremner et al. 1997). In families 1 and 6, quantitative analysis showed an inversion of the expression ratio between the two differentially spliced isoforms in the unaffected carrier fathers compared to the affected offspring: the higher expression of the mutated transcript in healthy fathers is in accordance with maternal inheritance.

The identification of mechanisms underlying reduced penetrance and variable expressivity changes the genetic/prenatal counseling and the clinical management of RB families. In sporadic cases such as families 2 and 5, parents are at increased risk to have other affected children due to a varying degree of mosaicism. Already in 2000, the risk for siblings of RB patients was suspected to be underestimated due to parental mosaicism (Smith et al. 2000). NGS now allows to detect even low-level mosaicism and to provide a better estimate of the impact of this phenomenon. A very recent study provided a recurrence risk estimation due to parental mosaicism 266-533 fold higher, as compared to the general population (Dehainault et al. 2014). Because of a parent-of-origin effect, unaffected fathers, who are born from of mothers with RB, can harbor the mutation with a 50% risk

of passing it down to their children who can thus be affected. Interestingly, in families segregating the p.Arg661Trp mutation, we observed extraocular *RB1*-related cancers such as osteosarcoma or leiomyosarcoma in adult carriers who had not developed RB.

In conclusion, this study indicates that mosaicism of amorphic mutations and parent-of-origin effect of hypomorphic mutations are the main mechanisms accounting for reduced penetrance/expressivity observed in RB. Thus, the identification of these mechanisms has important implications for genetic counseling, allowing to provide a better estimation of the recurrence risk, a more accurate prognostic assessment, prenatal diagnosis options and optimal medical surveillance in all at-risk individuals.

Author's roles

V. Imperatore has performed the experiments and has drafted the manuscript. A. Renieri and F. Ariani have made substantial contributions to conception and design of the study and reviewed the manuscript. AM. Pinto has made important contributions in interpretation of molecular results and clinical data collection. E.Gelli, E.Trevisson, V. Morbidoni, E. Frullanti have contributed to the experiments and data acquisition. T. Hadijstilianou, S. De Francesco and E. Gusson are ophthalmologists that have made RB diagnosis. P. Toti has conducted histopathological studies in available eye samples. V. Capra and A. Accogli are neurosurgeons who have dealt with patients treatment. G. Roversi and MA. Mencarelli have conducted the genetic counselling to RB families. All authors have given the approval of the final version of the manuscript to be published. They ensure that all aspects of the work are investigated and resolved with accuracy and integrity.

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Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Ethical Approval

All procedures performed in studies involving human participants were in accordance with ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

This article does not contain any studies with animals performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Legends to Figures

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Fig. 3 High-depth NGS in families 2 and 5. Sequencing results are showed as percentage variant of total number of reads. a) In family 2, the c.1960+5G>C mutation was detected at a percentage of ~50% in the proband with retinoblastoma and ~28% in the unaffected mother. b) In family 5, the c.2106+2T>C mutation was detected at a percentage of ~50% in the proband with retinoblastoma and ~ 34% in the father with retinoma.

Fig. 4 mRNA wt/mutant allelic quantification. a) Characterization of the effect of the splicing deletion c.608-16_608del identified in family 1. RT-PCR using primers spanning exons 5-8 showed two products in both the affected proband and the unaffected carrier father: the expected normal-sized transcript (263bp) and a shorter one of 151bp (upper panel). The latter product was not observed in the mother not bearing the mutation. Direct sequencing of the shorter product showed the complete skipping of exon 7. At protein level, aberrant transcript with exon 7 skipping is predicted to encode a product lacking 37 amino acids at the amino terminus (middle panel). Real time PCR relative quantification, normalized respect to the total transcript (shaded in grey), showed an inverted ratio between wt and mutant isoform in the affected child (87% vs 13%) respect to the father (14% vs 86%) (lower panel). b) Characterization of the effect of the c.2663+2T>C mutation identified in family 6. RT-PCR was carried out using primers spanning exons 23-27. An additional shorter product respect to the normal-sized one was observed in mutated family members (I1, II1 and II3) (upper panel). Sanger sequencing of the abnormal transcript showed the complete exclusion of exon 25, resulting in a frameshift that insert a stop codon at amino acid 841 (middle panel). By real time qPCR analysis, the two affected individuals showed a higher (70%) wt allelic expression respect to the mutant one (30%), while the father displayed almost exclusively (~98%) the mutant transcript (lower panel). WT, normal-sized wild-type product; Mut, abnormally spliced product; Ctrl1 and Ctrl2, control samples.

Supplementary Fig. 1 Sanger sequencing of abnormally spliced product in families 1 and 6. Sequence chromatograms of aberrant isoforms identified analyzing mRNA of mutated individuals IV1 and III2 of family 1 (left) and II1, I1 and II3 of family 6 (right).

Supplementary Fig. 2 Quantification of allelic expression for the p.Arg661Trp mutation identified in family 4. High-depth NGS on mRNA isolated from blood sample of patient III5 showed that mutant molecules are ~23%, in accordance with an expected lower expression of a paternally inherited mutation.

Supplementary Table 1

Family#	Mutation	Primer Fw	Primer Rv	
1	c.608-16_608del	5'-CCAGCAGTTCGATATCTACT-3'	5'-TGCTATCCGTGCACTCCTG-3'	
2	c.1960+5G>C	5'-CGTATCGCCTCCCTCGCGCCATCAGACGAGTGCGTTGTACAACCTTGAAGTGTATG-3'	5'-CTATGCGCCTTGCCAGCCCGCTCAGACGAGTGCGTCTGAGACACAGAGATATTAAG-3'	mid1
3, 4	c.1981C>T p.Arg661Trp	5'-CGTATCGCCTCCCTCGCGCCATCAGACGAGTGCGTCTGCAGCAGATATGTATCTT-3'	5'-CTATGCGCCTTGCCAGCCCGCTCAGACGAGTGCGTCACGTTTGAATGTCTCCTGA-3'	mid1
		5'-CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACACTGCAGCAGATATGTATCTT-3'	5'-CTATGCGCCTTGCCAGCCCGCTCAGACGCTCGACACACGTTTGAATGTCTCCTGA-3'	mid2
5	c.2106+2T>C	5'-CGTATCGCCTCCCTCGCGCCATCAGACGAGTGCGTAAAAGAGTGGTAGAAAAGAGG-3'	5'-CTATGCGCCTTGCCAGCCCGCTCAGACGAGTGCGTAGTTAACAAGTAAGTAGGGAG-3'	mid1
6	c.2663+2T>C	5'-CGTATCGCCTCCCTCGCGCCATCAGACGAGTGCGTGACTCCAAGATCAAGAATCTT-3'	5'-CTATGCGCCTTGCCAGCCCGCTCAGACGAGTGCGTTTCGAGTAGAAGTCATTTCTG-3'	mid1
		5'-CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACAGACTCCAAGATCAAGAATCTT-3'	5'-CTATGCGCCTTGCCAGCCCGCTCAGACGCTCGACATTCGAGTAGAAGTCATTTCTG-3'	mid2

Blue: tail; red: key; green: mid; black: primer sequence.

ID	Mid Sequence
Mid1	ACGAGTGCGT
Mid2	ACGCTCGACA

Supplementary Table 1: *RB1* primers designed on DNA and cDNA for the 454 Roche techonology.

Supplementary Table 2

Family#	Mutation	Primer Fw	Primer Rv	
1	c.608-16_608del	5'-AGCTAAAGGGGAAGTATTACAA-3'	5'-TGCTATCCGTGCACTCCTG-3'	WT allele
		5'-AGCTAAAGAAACAGCTGTTATA-3'	5'-TCCAAGAGAATTCATAAAAGGTA-3'	Mutated allele
6	c.2663+2T>C	5'-CATTCGGGACTTCTGAGAAG-3'	5'-TCGAGTAGAAGTCATTTCTGC-3'	WT allele
		5'-TGGTGAATCATTCGGGTAAAC-3	5'-ATGAATCCAGAGGTGTACACA-3'	Mutated allele

Supplementary Table 2: *RB1* primers designed on cDNA in order to perform real-time quantitative RT-PCR.

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4. Conclusions & Future Perspectives

4. Conclusions & Future Perspectives

Retinoblastoma represents the most common eye tumor in children. In the past, the study of retinoblastoma has provided important clues for our understanding the molecular basis of cancer, leading to the development of a model that is still valid (*Knudson* "two-hit model"). From the seventies onwards, other significant progresses have been made and many events accompanying retinoblastoma progession have been also characterized. However, many issues still remain unresolved. The present study was focused on: 1) the identification of the molecular basis of retinoblastoma cases without an identified predisposing mutation; and 2) the characterization of factors influencing its phenotypic variability.

In the first part of our study, to better characterize the events determining tumor onset, we re-analyzed a group of patients with sporadic retinoblastoma employing *Next Generation Sequencing* (NGS) technique, following a protocol that allows the identification of mosaicism, even at low-level. This approach led to identify six cases in mosaic condition, demonstrating that a high fraction (15%) of *RB1* mutations arises by post-zygotic events. These findings have important implications in genetic counseling. Indeed, the identification of *RB1* low-level mosaic mutations allowed to perform the genetic test in family members and provide more accurate recurrence risks compared to the empirical risk estimated on the basis tumor presentation (bilateral, unilateral multifocal, unilateral unifocal). As a consequence, appropriate medical surveillance has been provided and frequent eye examination under anesthesia have been performed only in members who have inherited the predisponsing mutation.

In the second part of this work, we moved forward and we decided to investigate if mosaicism could be responsible for variable phenotypic expression observed in retinoblastoma. We thus selected

6 families showing reduced penetrance/expressivity and we analysed *RB1* mutation frequencies by targeted high-depth NGS. In two cases, bearing amorphic *RB1* mutations (complete loss of function), we identified somatic mosaicism, supporting the conclusion that this mechanism was responsible for the observed clinical presentations. In the remaining families with hypomorphic *RB1* mutations, we identified a *parent-of-origin-effect* due to imprinting as the mechanism underlying phenotype variability. Also in this case, results can change the genetic/prenatal counseling and the clinical management of RB families. Indeed, unaffected carriers, offspring of *RB1*-mutated mothers, have 50% recurrence risk. Moreover, our study alerts on the possibility of adult-onset cancer development (osteosarcoma or leiomyosarcoma) in unaffected carriers, suggesting the need for second tumor surveillance.

As future perspectives, by using NGS, we plan to investigate mosaicism in a larger group of low-penetrant families and to test *RB1* mutation frequency in additional tissues such as oral mucosa or urines to identify mosaicisms not detectable in blood cells. Relating to the *parent-of-origin-effect,* we plan to verify its correlation with phenotypic expression also in cases with *de novo RB1* mutations, investigating the parental origin of the mutated allele.

5. References

5. References

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Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC)

Dear sir,
I have been asked to review the PhD thesis of Dr Valentina Imperatore:

Parent inheritance of *RB1* hypomorphic mutations and somatic mosaicism can explain low penetrance in retinoblastoma

I have read the thesis with great interest. In the first part Dr Imperatore provides a review of the epidemiological, clinical, molecular and genetic aspects of Retinoblastoma. The review clearly depicts how far knowledge of this rare cancer have progressed since Knudson proposed the classical two hit model, thanks to the availability of well characterized patients series, combined with the use of newer techniques of gene and genome analysis.

The experimental part includes both published work on the improved detection of mosaicism through NGS, in which the participation of Dr Imperatore is clearly stated, as well as more recent and still unpublished work aimed to clarify the mechanisms underlying the variable expressivity of the disease. In the latter, the name of Dr. Imperatore in first position highlight her major role among several Authors.

The clinical and molecular findings in families who showed evidence of partial penetrance are finely presented, by intermixing the description of hard molecular data– e.g. somatic/germline mosaicism, transcript analysis of putative hypomorphic mutations, and imprinting effects - with prediction of their functional effects, so to guide the reader to an easy understanding of the subtleties of the proposed interpretation.

I noticed a number of few minor typos that will be easy to amend.

In conclusion, this Thesis gives convincing proof that dr. Imperatore is able not only to produce the data but to master the complex matter as well, a competence that will become increasingly needed in the era of genomic medicine.

Therefore, I have no hesitation in approving the Thesis as suitable to be presented to the examining Committee of the Doctorate.

Yours faithfully,

Torino, March 15, 2017

Mario De Marchi

Full Professor (until October 31, 2016) of Medical Genetics at the University of Torino, Department of Clinical and Biological Sciences

UNIVERSITÀ CATTOLICA del Sacro Cuore

Istituto di Medicina Genomica

Il Direttore

Roma, 16 marzo 2017

Spett. Segreteria
Dottorato di Ricerca GenOMeC
Università degli Studi di Siena

Valutazione Tesi di Dottorato presentata dalla Dr.ssa Valentina Imperatore

Ho ricevuto dalla Dr.ssa Valentina Imperatore la tesi dal titolo "Parent inheritance of hypomorphic RB1 mutations and somatic mosaicism can explain low penetrance in retinoblastoma" presentata per il conseguimento del titolo di Dottore di Ricerca in Genetica, Oncologia e Medicina Clinica.

La tesi affronta una tematica importante, i meccanismi sottostanti la variabilità fenotipica, per le patologie genetiche e i tumori ereditari in generale, utilizzando come modello il prototipo dei tumori ereditari, il retinoblastoma, di cui presso il centro di Siena è disponibile un'ampia casistica. Il quesito scientifico è quindi importante, il disegno sperimentale è valido, e le metodologie impiegate sono congrue. I risultati hanno evidenziato in maniera chiara due diversi meccanismi alla base della variabilità di espressione (intesa come ridotta penetranza) nel retinoblastoma.

Il lavoro della tesi è stato oggetto di una pubblicazione scientifica, ed un secondo paper è stato recentemente sottomesso.

Nel complesso il giudizio è senz'altro positivo e suggerisco quindi l'ammissione della Dr.ssa Valentina Imperatore alla discussione finale della tesi da parte del collegio dei docenti.

Distinti saluti

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