University of Siena

Ph.D. in Medical Genetics

CONSTITUTIVE AND SOMATIC GENETIC EVENTS IN RETINOMA AND RETINOBLASTOMA

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Thesis suitable for the title of "Doctor Europaeus"

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1. Introduction

1.1 Retinoblastoma

The disease

Retinoblastoma (RB, OMIM#180200) is a malignant tumor of the developing retina. While it is very rare in adults, retinoblastoma is the most common intraocular malignancy in children; affecting about 1:14,000 to 1:22,000 live births[1].Worldwide, retinoblastoma is responsible for 1% of childhood cancer deaths, and 5% of childhood blindness[2].

Diagnosis is based on clinical signs and symptoms which are usually present before the age of five years. Most often, the first clinical sign is a white pupillary reflex (leukocoria) (Fig. 1). Strabismus, is the second most common sign and may accompany or precede leukocoria. Retinoblastoma occur in both hereditary (40%) and non-hereditary (60%) forms; the latter by autosomal dominant transmission with high penetrance (90%). Patients with unilateral retinoblastoma, affecting only one eye, usually have a non-inherited, "sporadic" form of the disease (85%), whereas the remaining 15% of cases are inherited (familial retinoblastoma). The word "sporadic" only means that no family history is evident; the majority of both bilateral and unilateral retinoblastoma patients have sporadic disease. Although sporadic in 90% of cases, bilateral retinoblastoma (where both eyes are affected) is always hereditable to offspring, and patients are at risk to pass the autosomal dominant acquired trait to their children. Generally, diagnosis of bilateral retinoblastoma is made earlier than diagnosis of unilateral retinoblastoma; where median age at diagnosis is 11 and 22 months, respectively. Patients with familial or bilateral retinoblastoma have an increased risk of specific neoplasms outside of the eye (second tumors) including osteogenic sarcoma, soft tissue sarcoma, and malignant melanoma[3]. Although readily curable by several means, including focal cryotherapy, laser surgery, and radiotherapy or chemotherapy with follow-up focal therapy when detected early, the most common cure for retinoblastoma is enucleation; removal of the entire eye[4]. Even then, if the tumor has escaped from the eye, usually along the optic nerve, it is fatal in 50-85% of cases[4].

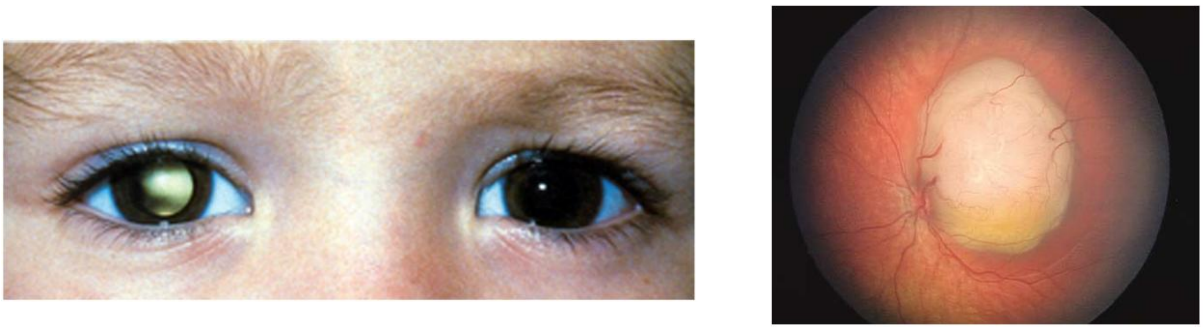


Fig.1 Leukocoria, most common warning sign of retinoblastoma (left). Entophytic tumor growth pattern, group B retinoblastoma (classification ABC) (right).

From disease to gene

The importance of hereditary factors in the etiology of retinoblastoma has been recognized since the 19th century. Familial aggregation of retinoblastoma was noted as early as 1821 (Kaelin, 1955). After improvement in the treatment, more patients reached adult age and had children with retinoblastoma displaying an autosomal dominant mode of inheritance. Initially, all cases were regarded as hereditary, but later it was recognized that in a significant proportion of patients with sporadic retinoblastoma, the etiology is nonhereditary [5]. In 1971, Alfred Knudson realized that individuals with hereditary bilateral retinoblastoma were diagnosed at a younger age than those children with unilateral, mostly non-hereditable, disease[6]. Statistical analysis indicated that as few as two mutational "hits" were rate limiting for the development of retinoblastoma tumors. The occurrence of the first mutation (M1) in the germline and all the developing retinal cells gives retinoblastoma tumors a "head start" in hereditary cases (only M2 must arise in a retinal cell), compared to nonhereditary tumors where both M1 and M2 must arise in a single retinal cell. Later, Comings refined this idea to suggest that these two mutational events can be loss of both alleles of a tumor suppressor gene[7]. The retinoblastoma locus was mapped to chromosome 13q14 by linkage studies [8, 9] and deletion analysis[10]. The observation that the normal allele was often lost (M2) and the mutant retinoblastoma locus (M1) was duplicated in many retinoblastoma tumors represented the proof that the *RB1* gene is a tumor suppressor gene[11, 12]. This loss of heterozygosity (LOH) is evident in 60% of both hereditary and nonhereditary retinoblastoma tumors, manifest by such non-disjunction and duplication of the whole mutant chromosome, or mitotic recombination[13]. *Dryja et al.* located a molecular clone at the retinoblastoma gene locus that was totally deleted from one retinoblastoma tumor. This clone was used to isolate a cDNA that showed sequence conservation among different species[14]. Internal mutations in *RB1* in retinoblastoma tumors and patients confirmed the identity of the gene[15]. The normal allele provides the required functions of *RB1* in the constitutional cells, including the non-malignant cells of the retina, of persons with germline *RB1* gene mutations. The process of LOH is probably occurring in all tissues, yet only in specific tissues pRB loss of function has malignant consequences. The contrast between widespread expression of *RB1* and tissue-specific tumorigenicity could be due either to compensation by other pRB related proteins[16], or efficient clearance of certain $RB1^{-/-}$ cell types by apoptosis[17 18 19].

1.2 Retinoblastoma: the gene and the protein

The *RB1* gene (GeneBank accession number L11910) consists of 27 exons that are scattered over 183 kb of genomic sequence on chromosome 13q14 [20] (Fig. 2). At its 5'-end, the *RB1* gene has a CpG-island, which is normally unmethylated. The promoter region contains binding motifs for transcription factors such as Sp1, ATF and E2F but not TATA or CAAT elements. The gene is transcribed into a 4.7 kb mRNA with no convincing evidences of alternative splicing[21]. The open reading frame, which starts in exon 1 and is terminated in exon 27, spans over 2.7 kb and is followed by 2 kb untranslated region[22]. Comparison of the human and mouse *RB1* genes shows 80% homology in the first 235 bp upstream the initiating methionine, 89% homology in the coding sequence, and 77% homology in the 3' untranslated region. The human and mouse proteins show 91% identity suggesting that the function of this protein is highly conserved. The *RB1* gene encodes pRB which is a 928 amino acid nuclear phosphoprotein that migrates at 110 kD in SDS-PAGE when hypophosphorylated. pRb is part of a small family of nuclear proteins that includes p107 and p130 (encoded by *RBL1* and *RBL2* genes respectively). These three proteins share sequence similarity and act on cell cycle regulation. Functional domains of the coding region of pRB were delineated using deletion mutants analysis. The protein is divided in four regions, the N-terminus, the A and B domains (separated by a spacer region), and the C-terminus (Fig. 3). The A/B regions are conserved throughout the family, and depending on the phosphorylation status at multiple serine and threonine residues in other regions of the protein, the A/B pocket can bind to E2Fs, HDACs and other nuclear proteins that contain the LxCxE peptide motif. This domain is necessary for biological functions such as the regulation of growth and differentiation and biochemical activities including transcriptional regulation and interaction with viral and cellular proteins[23]. The A and B domains require each other to form a functional repressor motif [24]. Structural X-ray crystallography revealed that the A domain forms a supportive scaffold for the B domain which ensures proper folding and stability[25]. Missense mutations affecting the A/B interface are tumorigenic suggesting that its structural integrity is important for pRB function. The C-terminal is less characterized but is critical for growth suppression, and it contains a nuclear localization signal (NLS) and cyclin binding motif ([R/K]xL) that are important for phosphorylation of pRB [26 27, 28]. The C-terminal domain has an intrinsic non-specific DNA-binding activity and binds to the oncoproteins c-abl and MDM2[29]. Mutations occurring in the C-terminal domain determine low-penetrance retinoblastoma suggesting the biological relevance of this domain[30]. The N-terminus is also not structurally well characterized; although deletion mutants are found in human retinoblastoma. The N-terminus, like the C-

terminus, also appears to bind the pocket[31]. However, this interaction may potentiate activity since several pocket functions are impaired by N-terminal mutations [26, 32, 33]. Thus, the N-terminus may promote an active pRB conformational state. In addition to this putative regulatory function, the N-terminal domain may have other roles given that it binds several proteins[34-36].

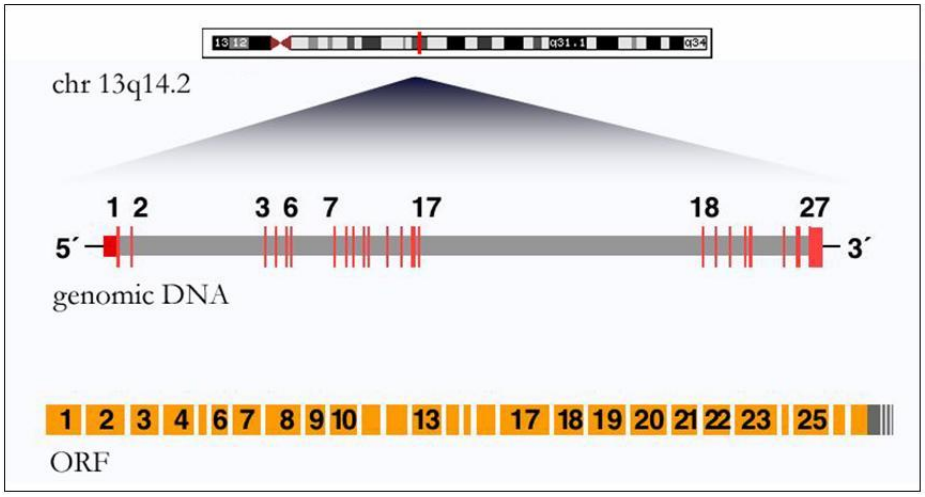


Fig.2 *RB1* gene structure. *RB1* gene consist of 27 exons scattered over 183 kb.

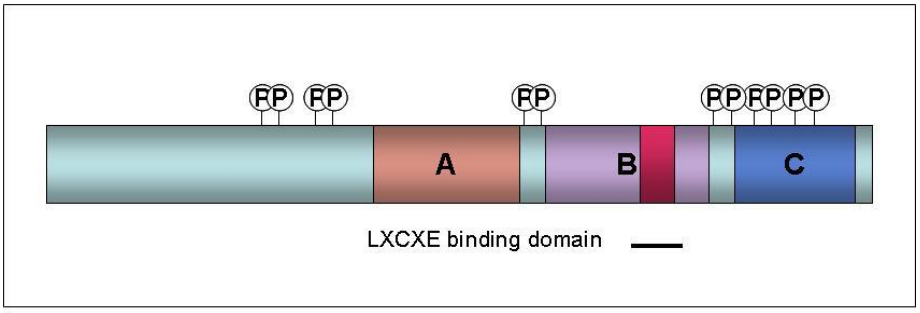


Fig.3 pRB protein structure. The protein is phpsphorylated in several serine and threonine residues.

pRb and cell cycle.

After its discovery, pRb was identified as a binding partner for mitogenic oncoproteins expressed by DNA tumor viruses including SV40 large T antigen[37], adenovirus E1A[38], and papillomavirus E7 proteins[39]. In addition, pRb was discovered to be phosphorylated in synchrony with the cell cycle[40-42]. This data suggested that pRB may be a general cell cycle regulator. pRB tumor suppressor function was originally thought to be largely due to its capacity to arrest cells in G1 by inhibiting the activity of E2F transcription factors. It is now believed that RB has many cellular roles in addition to serving as a G1 checkpoint including control of cellular differentiation during embryogenesis and in adult tissues, regulation of apoptotic cell death, maintenance of permanent cell cycle arrest and preservation of

chromosomal instability [43, 44]. Recent studies have also demonstrated that stabilization of the p27 cell cycle inhibitor (which is encoded by *CDKN1B*) by pRB, through the interaction with the anaphase-promoting complex/cyclosome (APC/C), is an important part of pRB's capacity to control the G1-S transition [45, 46]. pRB is now viewed as a transcriptional co-factor that can bind to and either antagonize or potentiate the function of numerous transcription factors [47, 48]. Furthermore, pRB is also an adaptor protein that recruits chromatin remodeling enzymes to control the expression of specific target genes and to modify chromatin structure at a chromosome-wide level [49, 50].

\The ability of pRB to bind E2Fs, silence E2F-dependent transcription, and restrain cell cycle progression is regulated by phosphorylation which occurs through the action of the cyclin-dependent kinases (CDKs). Cyclin-dependent kinases can phosphorylate pRB leading to the inhibition of pRB/E2F complex formation[51], as well as pRB-mediated cell cycle arrest activity[52]. Up to 16 possible CDK phosphorylation sites exist on pRB, and multiple CDKs can phosphorylate pRB with some site specificity [52, 53]. Other proline-directed kinases like p38 stress activated kinase 1 [54, 55], ERK1/2 [56], and possibly Raf-1 [57] can also phosphorylate pRB. Whereas some phosphorylation sites appear to be critical for negative regulation of pRB [52, 58], the effects of phosphorylation at individual sites varies depending on the phosphorylation status of other sites[58-60]. Phosphorylation of pRB, as a result of appropriate mitogenic stimulation and activation of CDKs, blocks pRB/E2F complex formation resulting in derepression and activation of E2F-dependent gene expression. This E2F-dependent gene expression facilitates continued cell cycle progression. pRB protein has also been implicated in other aspects of cell cycle control. For example, pRB can inhibit S phase progression by attenuating cyclin A/Cdk2 activity resulting in disruption of PCNA function and DNA replication [61, 62]. Also pRB protein is required for efficient activation of the G1/S cell cycle checkpoint in response to DNA damage[63], and is required to maintain cell cycle exit in quiescent and senescent cells[64]. Loss of pRB can cause G2/M cell cycle defects through deregulation of the E2F target gene Mad2, a mitotic checkpoint gene[65].

pRB protein can also regulate the cell cycle through mechanisms that are independent of E2F. For example, pRB can interact with SKP2 which is the substrate-recognition subunit of the ubiquitin protein ligase, SCFSKP2. Binding of pRB to Skp2 prevents degradation of the CDK inhibitor $p27^{KIP1}$, a normal target for SCFSKP2-mediated protein degradation[45]. Stabilization of $p27^{KIP1}$ inhibits the CDK activity that is required for normal cell cycle progression. Also, at least in some cell types, pRB can bind the transcription factor Mitf and cooperate with Mitf in activating transcription of the CDK inhibitor $p21^{CIP1}$ [66].

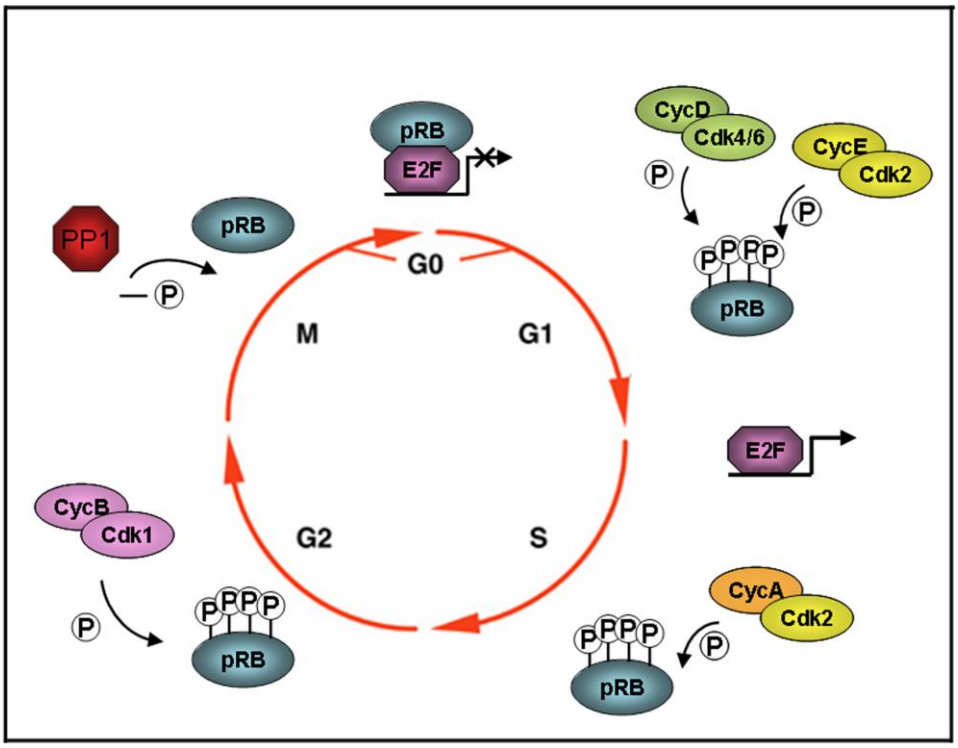


Fig.4. **pRB regulates cell cycle progression, differentiation and apoptosis**. Hypophosphorylated pRB represses genes necessary for S-phase entrance by repressing transcriptional activation by E2F, causing cell cycle arrest, and activates genes necessary for differentiation by binding cell type-specific transcription factors. Phosphorylation of pRB stimulates exit from G0, and in late G1 phosphorylation by cyclin D-Cdk4/6 and cyclin E-Cdk2 kinases releases E2F, allowing cell cycle progression. Continual phosphorylation of pRB occurs by cyclin A-Cdk2 and cyclin B-Cdk1 complexes in S and G2 phases. pRB is activated in late mitosis when PP1 phosphatase dephosphorylates it.

1.3 Role of pRB in cancer initiation and progression

The pRB pathway plays an important role in controlling proliferation and is frequently perturbed in human tumors. Although the developing retina is at nearly 100% risk of forming retinoblastoma when both *RB1* alleles are mutated, only a few other types of tumors, such as sarcoma and melanoma, are also initiated by loss of pRB [3, 67]. However, expression and loss of heterozygosity studies indicate that loss of pRB function is associated with the progression of human cancers, in addition to its role in cancer initiation [68].

The degree of differentiation of human cancers is often used to designate the pathological grade of an individual tumor, with the most differentiated being the lowest grade and the least differentiated being the highest grade. The presence of pRB in tumor cells could, in theory, promote their differentiation and thereby restrict their proliferative potential, which might explain why pRB must be lost during the progression from a well differentiated to a

poorly differentiated tumor. Altered pRB expression is observed to be correlated with higher grade, poorly differentiated gastrointestinal cancers [69].

Furthermore, human cancers demonstrate a high degree of genomic instability; mutations in *RB1* might participate in this phenotype by inducing defects during DNA replication and abnormal chromosomal segregation in mitosis [70-72], which could result in abnormal expression of other cancer genes, enabling tumor progression. Wild type pRB activity might normally slow down the proliferation of tumor cells by acting as a checkpoint not only at the G1-S transition but also during S phase and the G2-M transition, and loss of this checkpoint could contribute to the ability of a tumor cell to proliferate in the presence of genomic abnormalities. In addition, pRB might maintain genomic stability by regulating the expression of, and associating with, chromatin remodeling complexes, thereby regulating chromatin structure, in particular at centromeres and telomeres[73-76]. Loss of pRB can lead to chromosome segregation defects through the misregulation of genes that are important for processes such centrosome duplication and DNA replication[77-79]. Rb-deficient cells fail to properly arrest in G1 upon DNA damage and might replicate mutated DNA, leading to the accumulation of mutations[71, 75, 80].

Beside inducing gross chromosomal changes, loss of pRB function might also alter epigenetic definition of the genome through misregulation of chromatin remodeling enzymes and DNA modifiers [81]. Both chromatin remodeling and DNA methylation have been linked to tumorigenesis [82, 83]. These epigenetic changes might result in abnormal inactivation of tumor suppressors and activation of oncogenes without the physical mutation of the genes.

Strong evidence identifies RB-E2F activity and associated chromatin-regulating complex as a key regulator of senescence in cell culture [84-86]. Cellular senescence acts to suppress tumors *in vivo* in response to oncogenic stress. The RB family controls the length of telomeres and mediates the cellular signals to induce senescence in cells with shortened telomeres[87-89]. Therefore, loss of pRB function allows a tumor cell to bypass the cell cycle arrest associated with both replication and oncogene-induced senescence during cancer progression. The senescent cell cycle arrest depends on the presence of functional pRB [90] and is in part mediated by the RB-SKP2 (S-phase kinase-associated protein 2)-p27 pathway. Through this pathway, the presence of pRB leads to downregulation of SKP2 and therefore stabilization of p27, which inhibits cyclin-E-associated kinase activity [91].

Mouse models

The cloning of the human *RB1* gene led to a race for a knockout mouse model of retinoblastoma. Given the clinical phenotype of human retinoblastoma, it was predicted that mice heterozygous for a null allele of Rb1 would develop retinoblastoma at some frequency. Remarkably, back-to-back 1992 articles by three independent groups demonstrated that this was not the case [17-19]. Mice homozygous for inactivating *Rb1* mutations died between embryonic day 14 and 15. Surprisingly, heterozygotes did not display susceptibility to retinoblastoma at any measurable frequency, although they did exhibit susceptibility to pituitary tumors. It was hypothesized that $Rb^{+/-}$ mice do not develop retinoblastoma because of compensation by its family members p107 and p130, or because additional hits in other pathways are required. Generation of compound mouse mutants has addressed this hypothesis and illuminated the molecular requirements for retinoblastoma progression. Efforts by multiple groups culminated recently in the development of the St. Jude Retinoblastoma (SJ-RBL) mouse, a conditional triple mutant for Rb, p107, and p53 in retinal progenitor cells[92]. These mice rapidly develop invasive retinoblastoma exhibiting histologic features of human retinoblastoma. These studies also highlighted the importance of p53 inactivation in retinoblastoma progression, a finding that has since been validated in human retinoblastoma cells[93]. Furthermore, this intriguing finding suggests additional "hits" may be a prerequisite for retinoblastoma progression.

1.4 Genetics of Retinoblastoma

RB1 gene mutations

RB1 mutations are spread throughout the gene including the promoter, most exons, and splicing regions of introns. More than 50% of *RB1* mutations have been found only once and the majority of germline mutations originate in the proband. Molecular identification of *RB1* mutations allows accurate determination of retinoblastoma risk in relatives. Heterozygous carriers of oncogenic *RB1* gene mutations show variable phenotypic expression. Patients may develop tumors in both eyes or in one eye only (variable expressivity), while some carriers show no retinoblastoma at all (incomplete penetrance). According to the two-hit hypothesis, variation of phenotypic expression is to not surprising because the development of an individual tumor focus depends on the chance occurrence of a second somatic mutation. However, a quantitative analysis of phenotypic variation in families with retinoblastoma shows that stochastic effects can account for only a part of the observed differences. Penetrance and expressivity of hereditary retinoblastoma can vary with the nature of the predisposing mutation[94].

The majority of germline mutations that have been identified in patients with hereditary retinoblastoma are nonsense or frameshift mutations. These mutations are located in exons 1–25 of the *RB1* gene. With rare exceptions, nonsense or frameshift mutations in internal exons (2–25) are associated with bilateral retinoblastoma [94]. Genotype-phenotype variations associated with distinct phenotypic expression, depending on the location of the premature stop codon, have not been observed in hereditary retinoblastoma, and the site of the internal premature stop codon within the *RB1* gene seems to have little or no effect on phenotypic expression. This is may be due to the fact that transcripts of *RB1* alleles with internal premature stop codons are recognized by nonsense mediated decay; a posttranscriptional surveillance mechanism that causes degradation of mutant transcripts[95]. Consequently, in cells heterozygous for a mutation that triggers nonsense mediated decay, only transcripts of the normal allele remain. Interestingly, no mutation has been identified in the two terminal exons of the *RB1* gene (exons 26 and 27), although this region contains two CGA codons which are potential hot spots of nonsense mutations [96]. However, according to what is known from other genes, premature stop codons in these regions will not trigger nonsense mediated decay [97]. This suggests that proteins resulting from these mutant *RB1* alleles, pRBs with late C-terminal truncation, may have sufficient tumor suppressive activity to prevent development of retinoblastoma.

Carriers of *RB1* alleles with nonsense or frameshift mutations in internal exons generally develop bilateral retinoblastoma. Occasionally, such a mutation is identified in a patient with isolated unilateral retinoblastoma or in a unilaterally affected parent of a child with bilateral retinoblastoma [98, 99]. However, in some of these patients the mutation is present in a mosaic state. Mosaicism is not infrequent in patients with retinoblastoma and has been detected in 10% and 3.7% of retinoblastoma families and isolated unilateral cases, respectively [98, 99]. Because of the implication for genetic counseling, it is important that these patients are not missed.

A second important class of oncogenic alterations of the *RB1* gene are point mutations in intronic or exonic sequences that cause aberrant splicing. Splice mutations are associated with milder phenotypic expression [100, 101]. A reasonable explanation is that some splice mutations do not result in premature termination codons and thus retain some functions of the normal *RB1* gene. Most splice mutations in families with milder phenotypic expression results in premature termination codons, while less than half of the splice mutations families with full penetrance for bilateral disease or individual with sporadic bilateral retinoblastoma causes premature termination[102]. However, this is only valid for point mutations that alter invariable splice signals. Mutations that affect splice signals in exons, or in less conserved intronic splice signals, are more likely to be associated with unilateral retinoblastoma or incomplete penetrance. Another class of splice mutations associated with atypical mild phenotypic expression are deep intronic mutations[103]. In a few monogenic disorders, the association between milder phenotypes and certain mutations was linked to leakiness (i.e., processing of a fraction of the mutant transcript into a normally-spliced mRNA) and consequently, a higher dosage of normal transcript compared to cells that are heterozygous for a null mutation [104-106]. It has been observed that the effect of splice mutations can show substantial quantitative differences in different tissues [107] and therefore, it is conceivable that leakiness of misplicing is more pronounced in the cells that are relevant for initiation of retinoblastoma.

Mutations that do not result in complete loss of function are alterations of promoter sequences. In fact, such mutations have been identified in several families with incomplete penetrance and milder expressivity of retinoblastoma. Missense and small in-frame–length alterations do not result in premature termination codons; therefore, the transcripts expressed from these alleles are not recognized by posttranscriptional surveillance mechanisms. However, it is important to investigate the effect of supposed missense and in-frame mutations on the RNA level. It has been shown that mutations in exons may result in premature termination because of altered splicing[108]. Such data are not available for most

reported missense and inframe mutations in the *RB1* gene. Despite the uncertainty about the effect of individual mutations, the phenotypic expression that is associated with missense and inframe mutations is often well distinguished from that of alleles with premature termination codons. Many missense and in-frame mutations are associated with incomplete penetrance or milder expressivity. The amino acids that are substituted, deleted, or inserted as a consequence of these mutations are most often part of the A/B pocket domain of pRB. A few in-frame mutations have been identified outside the regions that encode for the A/B pocket including deletions of exon 4 [109] and exons 24–25 [30]. Functional studies have shown that mutant pRB expressed from alleles that are associated with incomplete penetrance show only a partial loss of normal function [30, 110].

Gross rearrangement screening is mandatory in *RB1* analysis as on average 13% of the mutational spectrum consists of large deletions[111]. In early studies that used Southern blot analysis for mutation detection, gross rearrangements were identified in peripheral blood DNA of about 12.5–16% of patients with bilateral or familial retinoblastoma [112, 113]. Recently, PCR-based methods have facilitated the identification of gross *RB1* alterations, and thereby increasing the number of reported mutations considerably [111, 114]. Genotype–phenotype analyses showed that, on the whole, carriers of gross deletions develop fewer retinoblastomas compared to patients who are heterozygous for other types of *RB1* null mutations. Specifically, carriers of cytogenetic and submicroscopic whole gene deletions often have unilateral tumors only. By contrast, almost all patients with gross deletions with one breakpoint in *RB1* have bilateral retinoblastoma[115].

Retinoblastoma and associated malformations

Cytogenetic deletions involving *RB1* in 13q14 are known to be related to a contiguous gene syndrome that is characterized by retinoblastoma, developmental abnormalities and peculiar facial dysmorphisms (13q- deletion syndrome) [116-118]. In 1983, Motegi suggested for the first time that the 13q deletion could be associated with retinoblastoma and common facial abnormalities. Later, other authors reported several cases with the 13q14 deletion, defining in detail the clinical features of the syndrome [119-121]. In particular, the 13q14 deletion syndrome is characterized by peculiar facial traits such as anteverted ear lobes, high and broad forehead, and prominent philtrum together with retinoblastoma and mental retardation. To identify patients affected by this syndrome, a cytogenetic analysis should be performed in carriers of whole *RB1* gene deletions.

1.5 Somatic events in Retinoblastoma

Tumor progression has been explained by the hypothesis of clonal evolution, which suggests that genomic instability generates new cellular populations, some of which have selective advantage that could be associated with their genetic changes[122, 123]. Therefore, it is possible to establish a correlation between specific genetic alterations and malignant progression. In 1999 Gallie et al. introduced a model for the development of retinoblastoma in which they assumed that mutations in both *RB1* alleles in a retinal progenitor cell of the developing retina are required but not sufficient for the exponential expansion of retinoblastoma[124]. This assumption was supported by the observation that all retinoblastoma have mutations of both *RB1* alleles, but they always display additional recurrent genomic deletions/duplications. These data suggest that additional somatic events could be important not only to tumor progression, but also for retinoblastoma initiation. Cytogenetic analysis and comparative genomic hybridization (CGH) studies have revealed recurrent genomic alterations in retinoblastoma, the most frequent being gains on chromosome 6p and 1q and losses of 16q [125-128]. In 2001, Herzog et al. investigated chromosomal abnormalities in tumor samples of children with unilateral isolated retinoblastoma in which somatic mutations in both *RB1* alleles had been previously identified. They found a marked difference in the incidence and the extent of chromosomal imbalances between tumors from children with a young age and those with an old age at operation [129]. In 2003, Lillington et al. analyzed 49 primary retinoblastoma tumors by CGH and they performed clinical/histological correlations[130]. Interestingly, they found that loss of 13q and 16 was significantly associated with tumors displaying adverse histo-prognostic factors, whereas -16q was associated with tumors without malignant features. In addition, they found -13q and -5q abnormalities associated with metastasis. The technique array-based Comparative Genomic Hybridization (array CGH) allows analysis of chromosomal imbalances with an extremely high resolution [131, 132]. This technique has contributed to the study and understanding of underlying genetic changes that correspond to disease progression in tumors. Association of DNA copy number aberrations with prognosis has been found for a variety of tumor types, including prostate cancer, breast cancer, gastric cancer and lymphoma [133-137]. Recently, Zielinski et al. used array CGH to detect chromosomal imbalances in retinoblastoma[138]. Compared to previous studies, four recurrently altered regions could be identified through analysis of minimally overlapping regions on 1q22, 1q32.1q32.2, 2p24.1, and 6p21.33-p21.31. Furthermore, two previously unreported high level amplifications at 1p34.2 and 1p33 each were detected in a single tumor. Key results of the six human studies are summarized in Fig. 6 (from Corson and Gallie,

2007). The characterization of these genomic imbalances have led to identify candidate oncogenes and tumor suppressors that may have wide importance in cancer biology: *MDM4* and *KIF14* at 1q32, *MYCN* and *DDX1* at 2p24, *E2F3* and *DEK* at 6p22, and *CDH11* and *RBL2* at 16q [139]. On the basis of the frequencies of these genomic changes, a multi-step model for RB progression has been proposed [140]. In this model, in contrast to the two-hit hypothesis, loss of both *RB1* alleles (M1-M2) is not sufficient to initiate tumorigenesis. Then the most common genomic changes in retinoblastoma (M3-Mn) are likely crucial for tumor progression.

Recently, the first aCGH study of murine retinal tumors, caused by retinal-specific *Rb1* knockout in a *Rbl2*$^{-/-}$ (p130$^{-/-}$) background was presented[141]. These results overlap with the human findings: recurrent gains of mouse chromosome 1 (which includes large regions of synteny to human 1q, 2q, 6 and 8) and 12 (with synteny to human 2p, 7 and 14q).

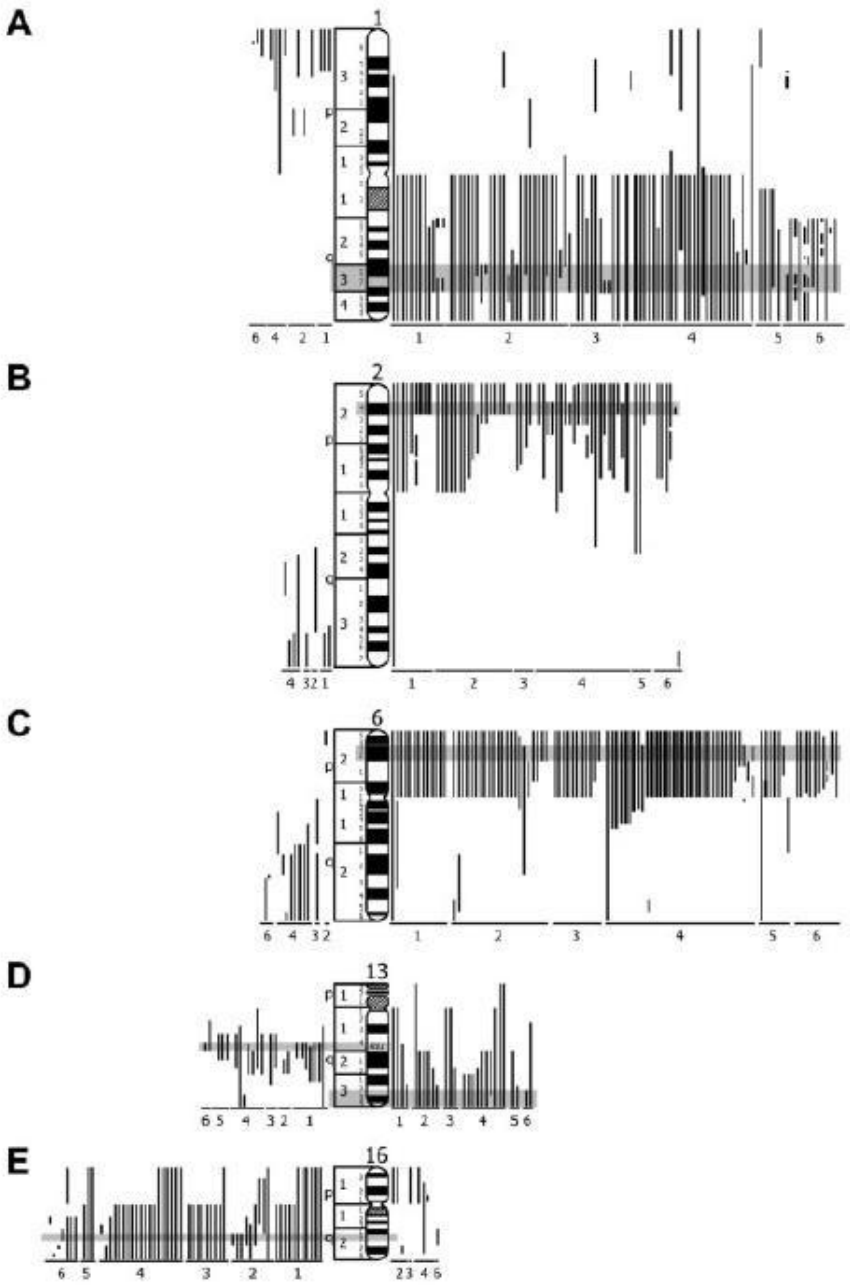


Fig.5 (Corson and Gallie, 2007) Recurrent chromosome aberrations in retinoblastoma detected by CGH and aCGH. A) Chromosome 1; B) chromosome 2; C) Chromosome 6; D) Chromosome 13; E) Chromosome 16. each vertical line represents a single tumor; lines to the right of the chromosome ideogram represent gains, lines to the left represent losses. Gray boxes identify the minimal regions of gain or loss. Numbers below figure indicate references: 1. Mairal et al., 2000; 2. Chen et al., 2001; 3. Herzog et al., 2001; 4. Lillington et al., 2003; 5. van der Wal et al., 2003; 6. Zielinski et al., 2005.

1.6 Retinoma

Retinoma (RN) is a benign retinal lesion considered the precursor of retinoblastoma [142]. Distinct from a retinoblastoma which is typically opaque white, retinoma appears as a translucent grey retinal mass, and is frequently associated with calcification and retinal pigment epithelial hyperplasia. Histopathology of retinoma includes foci of photoreceptor differentiation (fleurettes), momomorphic round nuclei, abundant fibrillar eosinophilic stroma, and absence of mitotic activity. A subject carrying a constitutive *RB1* mutation can manifest either retinoma, retinoblastoma or, more exceptionally, a combination of both manifestations in different eyes or in the same eye as two separate foci. The reported proportion of retinoma among the retinoblastoma population ranges from 1.8% to 10% [143-145]. Recently, after a retrospective review of archived paraffin-embedded sections of eyes enucleated for retinoblastoma, Dimaras et al. identified areas displaying features of retinoma in 15.6% of cases[142]. The mean age at diagnosis for retinoma is 15 years. The higher mean age at diagnosis of retinoma, with respect to retinoblastoma, is most likely due to a bias in ascertainment; since the diagnosis of retinoma is subsequent to that of retinoblastoma in a relative.

Retinomas have also been called "spontaneous regressions" because they resemble retinoblastoma that have involuted after radiotherapy and chemotherapy [146-149]. However, Gallie and colleagues proposed, rather, that they represent a stage in the retinoblastoma development [144, 146-150]. Malignant transformation of retinoma into retinoblastoma occurs rarely and the probability decreases with the age of the patient [143, 144]. There are cases of retinoma that stay benign for the lifetime of an individual [143, 145, 150-153].

Recently, it has been demonstrated that the two mutational events inactivating *RB1* gene are already present in retinomas [142]. The study by Dimaras et al. in retinoma clarified that the 'two hits' in *RB1* (M1-M2) do not inevitably cause a malignant phenotype, whereas they always lead to genomic instability [142]. At some point, this instability can lead to further genomic rearrangements (M3-Mn) that result in tumor progression [124, 142]. They also demonstrated that in retinoma the senescence-associated proteins $p16^{INK4a}$ and p130 are upregulated after loss of *RB1*, while in the adjacent retinoblastoma their expression is reduced (Dimaras et al., 2008). The authors proposed that the high levels of $p16^{INK4a}$, perhaps in response to genomic instability induced by pRB loss, may be sufficient to block the cell cycle through p130, even in absence of pRB [142].

However, studies aimed at clarifying retinoma/retinoblastoma relationship are rarely performed. This is mainly due to the fact that retinoma tissue is difficult to obtain since the affected eyes generally function and require no therapy. To date, genome wide studies in retinoma have never been performed.

2. Rationale, aim and outline of the study

Retinoblastoma research has been fundamental in uncovering the role of tumor suppressor genes in human cancer, and this discovery lead to Alfred Knudson's 'two-hit' model of cancer initiation. According to this model, two mutational events (M1-M2) in the *RB1* gene represent the first rate-limiting step in tumor development. Furthermore, recent studies have also identified recurrent genomic rearrangements in RB tissues that may represent M3-Mn events required for cancer progression.

The characterization of constitutive and somatic events, present in both retinoblastoma and benign lesions called retinoma, were the focus of this study. We achieved this goal using several approaches: 1) the set up and use of a clinically and molecularly, well-defined patient database; 2) the identification of the germline *RB1* mutations in the cohort of patients; 3) the characterization of three patients with retinoblastoma and mental retardation microdeletion syndrome using array CGH; 4) the identification of the genomic differences between retinoma and retinoblastoma lesions using both quantitative Real Time PCR and genome wide array-CGH technology; 5) the characterization of recurrent genomic imbalances in retinoblastoma tumor using array CGH. Each point has been developed as follow:

1) *Set up of a retinoblastoma database*

The availability of a large number of retinoblastoma samples was essential in setting up the retinoblastoma database used for this study. A cell line bank, along with genomic and tumor DNA from retinoblastoma patients, has been present in the Medical Genetics Unit of Siena since 1998. Recognizing that this large collection of patient samples represents a very important tool for the scientific community, we placed this bank of samples in a University of Siena on-line catalogue database available upon request to all researchers working on retinoblastoma (see section **Result 3.1**).

2) *Identification of RB1 mutations*

About half of retinoblastoma patients have a hereditary predisposition to retinoblastoma because they are heterozygous for an oncogenic mutation in *RB1* gene. It is important to identify these patients and to determine their specific predisposed mutation so appropriate risk assessment and clinical management can be provided to relatives and offspring. However, genetic analysis of patients with retinoblastoma is not a trivial task. The nature and location of *RB1* gene mutation is not only heterogeneous, but there are only few sites of recurrent mutation. Using a combination of different strategies, we searched for germline mutations in the *RB1* gene of our cohort of retinoblastoma patients; identifying both point mutations and gross rearrangements that were suspected to cause the disease. First,

traditional techniques were employed for *RB1* analysis which included both SSCP and polymorphic markers. However, these analyses showed low sensitivity and were prone to miss large rearrangements. To combat these issues, our lab developed a protocol that involved a combination of denaturing high performance liquid chromatography (DHPLC) and multiplex ligation probe-dependent amplification (MLPA) techniques which proved to be both faster and more sensitive to the presence of *RB1* mutation (see section **Result 3.2**).

3) *Characterization of patients with 13q deletion*

Cytogenetic deletions involving *RB1* in 13q14 region are related to a contiguous gene syndrome characterized by retinoblastoma, developmental abnormalities and peculiar facial dysmorphisms. Using array CGH, we characterized two patients with 13q deletion syndrome and studied another case, where only a maternally inherited 7q deletion was present, probably not related to the disease (see section **Result 3.3**).

4) *Identification of genomic differences between retinoma and retinoblastoma*

Retinomas are benign retinal tumors that rarely progress to malignancy, and are found clinically in 2% of people carrying a mutant *RB1* allele. Since retinoma can occur in individuals who carry one mutant *RB1* allele, and since most retinoblastomas contain additional genomic changes after loss of both copies of *RB1*, we decided to investigate the genomic differences between these two lesions using the DNA extracted from normal retina, retinoma and retinoblastoma tissues found in the right eye of two patients. Using qPCR, we examined four candidate genes involved in retinoblastoma pathogenesis (*MDM4*, *MYCN*, *E2F3* and *CDH11*) for copy number variations. In addition, we also analyzed the same tissues by array-CGH to extend the analysis to the genome-wide level (see section **Result 3.4** and **Result 3.5**).

5) *Characterization of genomic rearrangements in retinoblastoma*

Cytogenetic and CGH studies have shown that M1-M2 mutational events are necessary for initiation of retinoblastoma, but they are not the only genomic changes that occur in the disease. Pooled data across six studies revealed recurrent gain on 6p (54%), 1q (53%) and 2p (34%), along with losses of 16q (32%), suggesting that these regions may harbor oncogenes and tumor suppressors genes. However, the vast majority of these data are from low-resolution studies. For this reason, we employed high-resolution oligonucleotide-based array CGH to pinpoint minimal regions and identify new candidate genes important for tumor initiation in 18 tumors from patients affected by retinoblastoma (see section **Result 3.5**).

The characterization of genomic imbalances was made possible in this study through the use of the array CGH method which allowed us to identify M3-Mn events in retinoblastoma tumors regardless of the fact that DNA extracted from paraffin embedded tissues was used. Furthermore, the availability of two eye samples containing both retinoma and retinoblastoma lesions were essential to this study and enabled us to determine the genetic events responsible for malignant tumorigenesis. This research on a range of tissues from normal retina, to the benign retinoma, until the dramatic retinoblastoma initiation, allowed us to establish a molecular basis for future studies aimed towards understanding tumor initiation.

3. Results

3.1 On-line RB database

Unpublished data.

A bank of retinoblastoma DNA and cell lines has been active in the Medical Genetics Unit of Siena since 1998. During the course of my PhD, I have been involved in the creation of an on-line database, accessible through the University of Siena website, which contains all retinoblastoma patient data collected in the Medical Genetics Unit of Siena bank. The database is now publicly available and represents a precious resource for all researchers working on retinoblastoma. The general home page of the website is available at http://www.biobank.unisi.it (Fig. 6). This introductory page gives access to five independent databases: 1-Microdeletion/duplications Syndrome; 2-Autosomal Mental Retardation; 3-X-Linked Mental Retardation; 4-Rett syndrome; 5-Retinoblastoma.

Fig. 6. General home page of the bank. Here, users can find links to the three distinct databases: 1-Microdeletion/duplication Syndromes; 2-Autosomal MR; 3-XLMR; 3- RTT; 4-Retinoblastoma. Moreover, this page gives access to general information useful for site navigation and use.

Methods

The retinoblastoma database is maintained and updated on the server of the University of Siena. The database was created using the Access software (Microsoft Office; http://www.microsoft.com) for data management. The web-site is written in VBScript and takes advantage of a Microsoft IIS (Internet Information Server) with ASP (Active Server Pages) technology.

How to use the database

By accessing the retinoblastoma section, users can see a list of all patients available with additional specific information. Through genetic counseling by the Medical Genetic Unit and the collaboration with both Dr. T. Hadjistilianou (Dept. of Ophthalmology and Neurosurgery, University of Siena) and Prof. A. Acquaviva (Pediatric Unit, University of Siena), 326 samples have now been collected from retinoblastoma patients and healthy relatives. At present, the website contains 116 DNA samples from patients affected by retinoblastoma. For all patients, we collected a detailed list of clinical information in order to disclose a possible contiguous gene syndrome in 13q14. This information included data concerning familial history, involvement of one or two eyes, age at diagnosis, tumor dimension and location, number of foci, degree of differentiation, events of relapses, presence of primary second tumors and the presence of additional abnormalities. We collected 22 eye tissues from enucleated retinoblastoma patients (8 with a characterized germline *RB1* mutation) through the collaboration with Prof. Paolo Toti (Department of Human Pathology and Oncology of the University of Siena). Among the 22 tissues, two samples show areas of retinoma adjacent to retinoblastoma. In the first case (*Case#206*), there was clinical evidence that the retinoma later progressed into retinoblastoma, necessitating removal of the eye. A retrospective review of available archived paraffin-embedded sections of eyes enucleated for retinoblastoma, revealed areas with features of retinoma in another sample (*Case#297).*

The database is organized on two levels: a "public" level freely available to all researchers and an "administrator" level accessible only to website administrators through the use of a password (Fig. 7). This level contains personal data and clinical information of patients and their relatives.

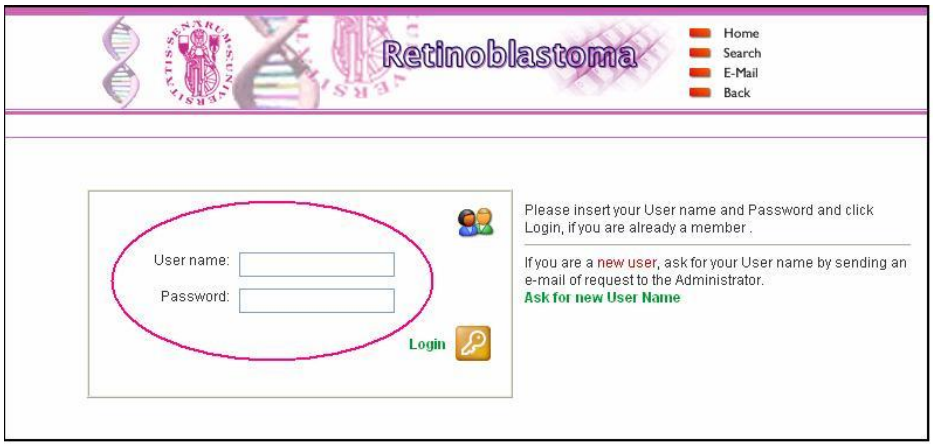
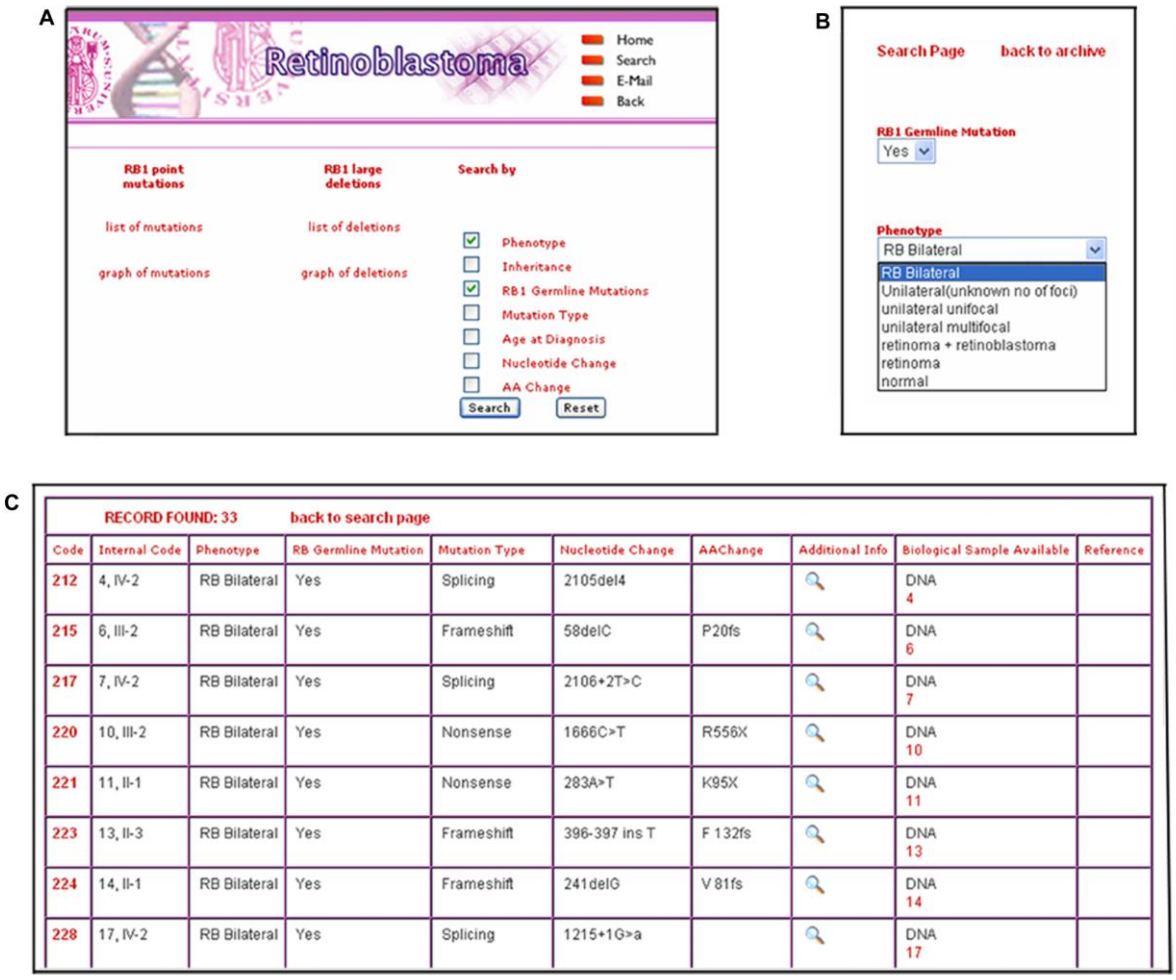


Fig. 7. Login page. A username and a password guarantee a regulated access to information. Only administrators can insert new records, modify information and access to patients personal data and clinical details.

In both cases, the main page consists of a table indicating all inserted samples with the following information: a) **INTERNAL CODE**: internal codes of bank samples. b) **CODE**: a code composed of a progressive number identifying each single family followed by patient pedigree position (i.e. 3-IV-5). c) **PHENOTYPE**: retinoblastoma bilateral, unilateral (unknown n° of foci), unilateral unifocal, unilateral multifocal, retinoma + retinoblastoma, retinoma and normal. d) **INHERITANCE**: sporadic, familial or unknown. e) **MUTATION TYPE**: missense, nonsense, frameshift, splicing, large deletions, no mutations, analysis in progress and unknown. f) **NUCLEOTIDE CHANGE**. g) **AMINOACID CHANGE**. h) **ADDITIONAL INFO**: In this page, external users can visualize information about the age at diagnosis and the presence of second primary tumors. Here, bank curators can also visualize personal data about patients. i) **BIOLOGICAL SAMPLE AVAILABLE**: lymphoblastoid cell lines, leukocytes in DMSO medium, plasma, DNA, fibroblasts, tumoral tissues. j) **REFERENCE**: PubMed references of articles including the specific patient. The website is interactive with a user-friendly graphical interface. Researchers can rapidly verify if there are the patients they need by exploiting the **"Search by"** option available directly on the main page. This option allows users to select patients by: i) **retinoblastoma phenotype**; ii) **inheritance**; iii) **mutation type**; iv) **nucleotide change**; v) **aminoacid change**. Users can choose to search using one single options or a combination of two or more options. As a result of the search, they will visualize a table with all retinoblastoma patients fulfilling the requested features (Fig. 8). Finally, on the main page, users can find links to the following pages: **list and graph of *RB1* point mutations** (a table with all point mutations and a dynamic graph showing the position of the mutations on the gene and their relative frequency), **list and graph of *RB1* large deletions** (a table with all gross rearrangements and a dynamic graph showing the extension of the deletions and their relative frequency).



Code	Internal Code	Phenotype	RB Germline Mutation	Mutation Type	Nucleotide Change	AAChange	Additional Info	Biological Sample Available	Reference
212	4, IV-2	RB Bilateral	Yes	Splicing	2105del4			DNA 4	
215	6, III-2	RB Bilateral	Yes	Frameshift	58delC	P20fs		DNA 6	
217	7, IV-2	RB Bilateral	Yes	Splicing	2106+2T>C			DNA 7	
220	10, III-2	RB Bilateral	Yes	Nonsense	1666C>T	R556X		DNA 10	
221	11, II-1	RB Bilateral	Yes	Nonsense	283A>T	K95X		DNA 11	
223	13, II-3	RB Bilateral	Yes	Frameshift	396-397 ins T	F 132fs		DNA 13	
224	14, II-1	RB Bilateral	Yes	Frameshift	241delG	V 81fs		DNA 14	
228	17, IV-2	RB Bilateral	Yes	Splicing	1215+1G>a			DNA 17	

Fig. 8. Search of specific RB samples in the database. a) Users can search RB patients with a specific feature or combining two or more features through the "Search by" option available on the main page of the website. b) The "Search" option gives access to a page in which users can indicate the specific phenotype, for example RB bilateral and the presence or absence of a *RB1* mutation. c) As a result, users visualize a table containing all the fields present in the general table and listing all bank patients with bilateral RB and with a *RB1* mutation identified.

3.2 Identification of RB1 mutations

3.2.1 Mutational screening of the RB1 gene in Italian patients with retinoblastoma reveals 11 novel mutations.

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ORIGINAL ARTICLE

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Mutational screening of the *RB1* gene in Italian patients with retinoblastoma reveals 11 novel mutations

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Abstract Retinoblastoma (RB, OMIM#180200) is the most common intraocular tumour in infancy and early childhood. Constituent mutations in the *RB1* gene predispose individuals to RB development. We performed a mutational screening of the *RB1* gene in Italian patients affected by RB referred to the Medical Genetics of the University of Siena. In 35 unrelated patients, we identified germline *RB1* mutations in 6 out of 9 familial cases (66%) and in 7 out of 26 with no family history of RB (27%). Using the single-strand conformational polymorphism (SSCP) technique, 11 novel mutations were detected, including 3 nonsense, 5 frameshift and 4 splice-site mutations. Only two of these mutations (1 splice site and 1 missense) were previously reported. The mutation spectrum reflects the published literature, encompassing predominately nonsense or frameshift and splicing mutations. *RB1* germline mutation was detected in 37% of our cases. Gross rearrangements outside the investigated region, altered DNA methylation, or mutations in non-coding regions, may be the cause of disease in the remainder of the patients. Some cases, e.g. a case of incomplete penetrance, or variable expressivity ranging from retinoma to multiple tumours, are discussed in detail. In addition, a case of pre-conception genetic counselling resolved by rescue of banked cordonal blood of the affected deceased child is described.

Keywords *RB1* gene · Italian population · Retinoblastoma · Mutation screening

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Introduction

Retinoblastoma (RB; OMIM #180200) is a childhood malignant eye tumour originating from progenitors of retinal sensory cells, with an incidence of 1 case in every 15,000–28,000 live births (Gallie et al. 1999). Inactivation of both alleles of the *RB1* gene (GenBank accession no. L11910), within chromosome band 13q14.2, is the cause of tumour development. Approximately 40% of RB cases are hereditary, with bilateral and/or multifocal tumours, while the other 60% are sporadic cases presenting with unilateral disease (Vogel 1979).

In most sporadic RB cases, two mutational events have occurred in somatic cells, neither of which is present in DNA from constitutional cells (Schimizu et al. 1994; Lohmann et al. 1997; Klutz et al. 1999). However, nearly 15% of unilaterally affected patients have a germline *RB1* mutation, representing a 45% risk for their offspring (Alonso et al. 2001). In the familial and bilateral form of RB, the first mutational event is a germline mutation, either transmitted from an affected parent as an autosomal dominant trait with high penetrance (90%), or acquired during gametogenesis or gestation (Knudson 1971; Comings 1973). The inactivation of the second *RB1* allele is somatic and results in multiple, and often bilateral, tumours.

The presence of *RB1* germline mutations also confers an increased risk of developing second primary tumours, with a cumulative incidence of 22% by the age of 25 years (Moll et al. 2001). In fact, loss of function of the *RB1* gene is associated not only with RB but also with a variety of other tumours, such as osteosarcoma, small cell lung carcinoma, bladder tumour, and breast carcinoma (Wadayama et al. 1994; Lee et al. 1988; Hansen et al. 1985).

The identification of a broad spectrum of nearly 500 distinct somatic or germline mutations resulting in *RB1* gene inactivation in RB, is the basic information required to develop rapid procedures for mutational analysis as well as to understand the molecular mechanisms underlying different degrees of penetrance or expressivity of the disease (Lohmann 1999; Alonso et al. 2001; Richter et al. 2003; Tsai et al. 2004; Houdayer et al. 2004; Harbour 2001). Here, we report our experiences of genetic counselling and molecular diagnosis at our centre during the last 5 years in a cohort of 35 patients.

Materials and methods

Patients

This study includes 35 unrelated cases of RB with different clinical presentations: 7 familial bilateral, 2 familial unilateral, 13 sporadic bilateral and 13 sporadic unilateral cases. Blood samples were obtained from patients and available parents after informed consent. DNA was extracted from peripheral blood using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) or by the phenol/chloroform method (Sambrook et al. 1989).

Molecular analysis

Genomic DNA was amplified by polymerase chain reaction (PCR). Primers and PCR conditions used for single exons and promoter analysis were as described previously (Hogg et al. 1992; Scheffer et al. 2000; Houdayer et al. 2004).

Single-strand conformational polymorphism (SSCP) was performed on a Genephor apparatus (Pharmacia Amersham, Little Braunschweig, Germany) using a GeneGel Excel 12.5/24 Kit (Pharmacia Amersham). PCR products were mixed with an equal volume of formamide, denatured by heating at 95°C for 5 min followed by immediate chilling on ice. Fragments showing altered mobility relative to controls were directly sequenced. Amplicons were purified and sequenced in both directions using the BigDye Terminator Cycle Sequencing V1.1 Ready Reaction Kit (Applied Biosystems, Foster City, CA) with incorporation of PCR oligonucleotides as extension primers and following electrophoresis in an ABIPrism 310 Genetic Analyzer. The analysis was performed using the Collection and Sequence Analysis software package (Applied Biosystems). The results were compared with the reference *RB1* genomic sequence (GenBank accession no. L11910.1). The putative consequences of the DNA variations were deduced from *RB1* cDNA (NCBI accession NM_000321.1) and protein (NCBI accession NP_000312) sequences.

Negative cases were analysed by real-time quantitative PCR. The TaqMan probe and primers were designed using Primers Express software (Applied Biosystems). The *RB1* probe is complementary to a segment located in exon 17. *RB1* exon 17 forward primer: 5′-AACACATT TGTCTTTCCCATGGATTCT-3′; *RB1* exon 17 reverse primer: 5′-GCCTTCTGCTTTGATAAAACTTTCGA-3′; *RB1* exon 17 TaqMan probe: 5′-AATGTGCTTAAT TTAAAAGCC-3′. The *RB1* probe contained a fluorophore 5′ FAM as a reporter. The size of the amplicon was 91 bp. An RNAaseP kit was used as an internal reference (Applied Biosystems). In order to validate the presence of the identified deletion, a microsatellite analysis was performed using two intragenic polymorphic markers located in intron 2 (Rbi2/ D13S153), and in intron 20 [RBi20/CTTT(T) repeat] of the *RB1* gene, respectively (Alonso et al. 2001). The aplotype of each tested sample was assessed by gel electrophoresis.

Results

During the last 5 years, 35 Italian RB patients have been referred to our centre for genetic counselling and molecular diagnosis. Concerning inheritance, 9 were familial cases, (2 unilateral and 7 bilateral), and 26 were sporadic cases, (13 unilateral and 13 bilateral). The whole coding region (27 exons) and nearby intronic regions were screened by PCR-mediated SSCP analysis followed by direct DNA sequencing. In 12 cases, a causative point mutation was identified. The negative cases were then analysed by the recently developed real-time quantitative PCR method and one deletion was identified. This deletion was confirmed by microsatellite analysis (Rbi2 and RBi20 markers). In total, we identified a causative *RB1* mutation in 13 out of 35 patients (mutation rate of 37.1%). In particular, 7 patients have

Table 1 Identified mutations. Nucleotide numbering for genomic DNA and cDNA are according to GenBank accession numbers L11910.1 and NM_000321.1, respectively. Amino acid numbering refers to the reference sequence for pRB protein, NP_000312. *S* Sporadic, *F* familial, *U* unilateral, *B* bilateral, *mf* multifocal, *uf* unifocal

ID	Location	DNA variation	AA change	Phenotype		Present in relatives	References
12	Exon 1	g.2117_2118delC c.58delC	p.P20fs	F	U uf	Father, healthy sister	New
50	Intron 1	c.137 + 1G > A g.21997G > A	Splicing site 5′ intron 1	S	B	No	New
30	Exon 2	g.5527_5528delG c.241delG	p.V81fs	S	B	Foetus	New
23	Exon 3	g.39464A > T c.283A > T	p.K95X	S	B	No	New
29	Exon 4	g.41941_41942insT c.396_397insT	p.F132fs	F	B	No	New
52	Exon 11	g.65392_65393delA c.1078_1079delA	p.S360fs	S	B	No	New
39	Intron 12	g.70330G > A c.1215 + 1G > A	Splice site 5′ intron 12	F	B	Father	Lohmann et al. (1996)
21	Exon 17	g.78250C > T c.1666C > T	p.R556X	F	B	No	Cowell et al. (1994)
8	Intron 20	g.156837_156840delAAGT c.2105del4	Splice site 5′ intron 20	S	B	No	New
15	Intron 20	g.156840T > C c.2106 + 2T > C	Splice site 5′ intron 20	F	B	Father with retinoma	New
90	Exon 22	g.162021G > T c.2236 G > T	p.E746X	S	B	No	New
107	Exon 24	g.170374_170377delTCTT c.2492_2495delTCTT	p.I831fs	S	B	No	New
48		Del Rbi2		F	U mf	Mother	New

bilateral sporadic RB, and 6 patients had familial RB, 2 unilateral and 4 bilateral. These results are summarised in Table 1.

Eleven were novel mutations. Only two mutations were previously reported: one is a G to A substitution at the first intronic base of the exon 12 donor splice site (g.70330G > A) and the other is a nonsense mutation in exon 17 due to a single C to T substitution (g.78250C > T) (Lohmann et al. 1996; Cowell et al. 1994). Of the 11 novel mutations, 3 were nonsense (p.K95X, p.R556X and p.E746X), 5 were frameshifts, including 4 deletions (g.2117_2118delC, g.5527_5528delG, g.65392_65393delA, g.170374_170377delTCTT) and 1 insertion (g.41941_41942insT), and 4 were splice site mutations. Among splice site changes, three were point mutations (g.21997G > A, g.70330G > A, g.156840T > C) whereas one was a deletion of four bases (g.156837_156840delAAGT) involving the donor splice site of exon 20. Five cases of particular interest are discussed in detail.

A case of non-penetrance

The female proband was diagnosed with unilateral RB at the age of 14 months during a screening program (Fig. 1a). Her father was enucleated at the age of 15 months for a suspected RB. Mutational analysis of DNA from peripheral blood documented the frameshift mutation c.58delC. The same mutation was also found in her father. Extension of the analysis to other at risk family members revealed the presence of the same point mutation in the healthy older sister, aged 11; she therefore represents a case of incomplete penetrance.

A case of retinoma

The male proband underwent right eye enucleation at the age of 4 months (Fig. 1b). After 1 year of chemotherapy for controlateral disease, he underwent enucleation of the left eye. Monolateral retinoma was found in the father after diagnosis in the child. Molecular analysis of the *RB1* gene revealed the same splicing mutation in the proband and in his father (c.2106 + 2T > C). This is a case of variable expression of the disease.

A case of adenocarcinoma of ethmoid sinus

A left-eye RB was diagnosed early in the proband, who was treated with radiotherapy during the first months of life (Fig. 1c). She underwent right-eye enucleation at the age of 3.5 years followed by radiotherapy and chemotherapy for a controlateral tumour. At the age of 22 years, the patient developed a left ethmoidal adenocarcinoma identified by nasal biopsy. The neoplasia extended to the ethmoid, nasal fossa, left maxillary sinus, medial and inferior orbital paries and rhinopharynx. Mutation analysis of the patient and her relatives

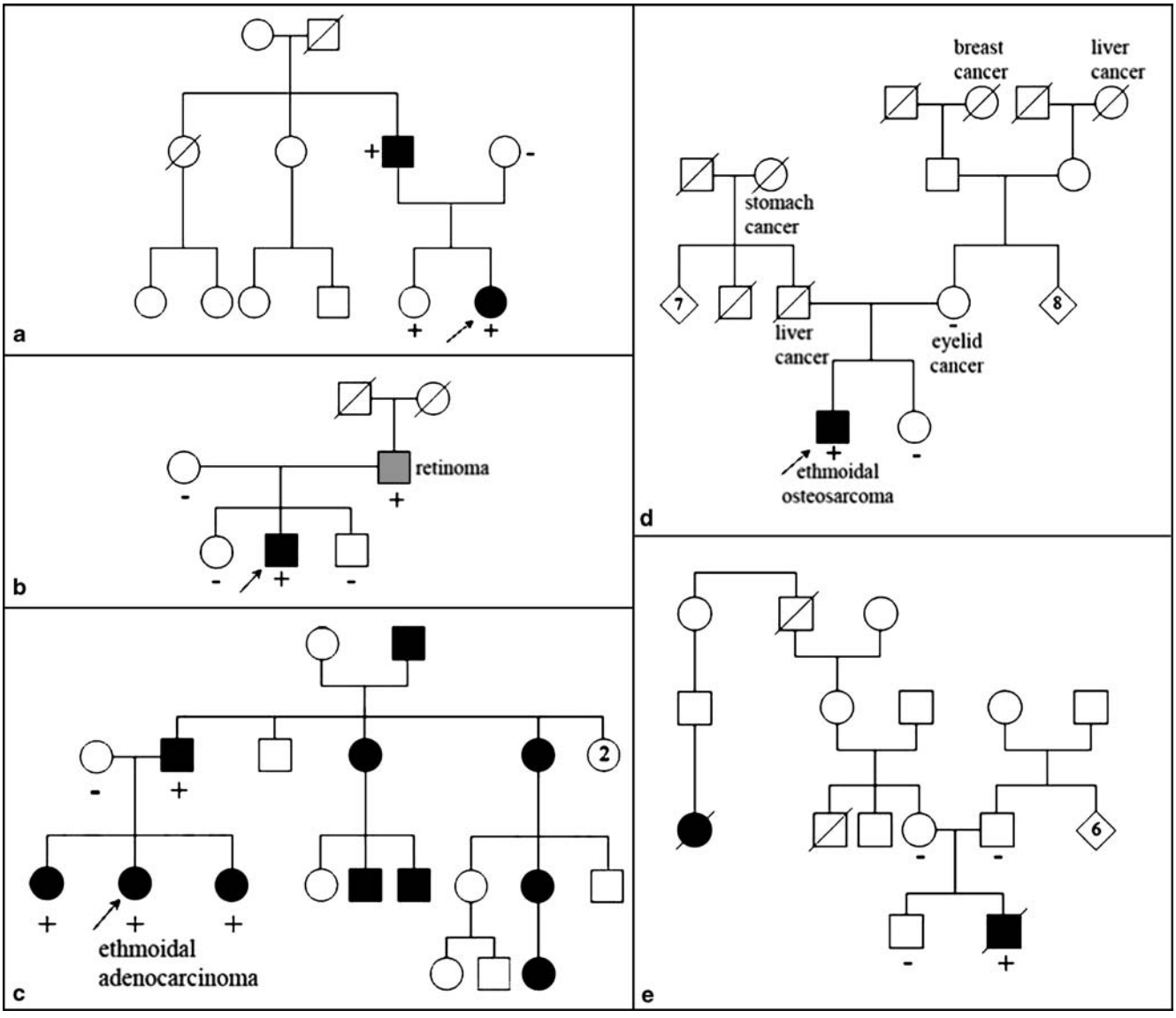

Fig. 1a–e Pedigrees of five families with retinoblastoma (RB). *Black symbols* Patients with at least RB, *grey symbol* patient with retinoma, + presence of mutation. **a** Case of non-penetrance (case 12 in Table 1). **b** Case of retinoma (case 15 in Table 1). **c** Case of coexistence of RB and adenocarcinoma (case 39 in Table 1). **d** Case of RB associated with osteosarcoma (case 50 in Table 1). **e** Case of pre-conception genetic counselling (case 107 in Table 1)

identified a mutation in the donor site of intron 12, c.1215 + 1G > A. The same mutation was identified in several other relatives affected by isolated RB.

A case of ethmoidal osteosarcoma

The proband developed RB at the age of 6 months on the right eye, and a controlateral tumour at the age of 2 years (Fig. 1d). He underwent bilateral eye enucleation followed by radiotherapy only on the right eye. At the age of 24 years the patient showed right ethmoidal osteosarcoma. The patient is alive and healthy at 28 years old. Molecular analysis revealed the point mutation c.137 + 1G > A (or IVS1 + 1G > A). The mutation was absent in the mother and in the sister. The mutation is probably "de novo", although the father was not investigated (died at the age of 45 of post-HCV cirrhosis cancer).

A case of pre-conception genetic counselling

A couple asked for pre-conception genetic counselling as their second-born child died of RB 2 years earlier (Fig. 1e). The couple described that the child presented at 3 years of age, with bilateral and multifocal RB. Despite left enucleation followed by brachytherapy and

Table 2 Frequency and distribution of small mutations in the coding sequence of the *RB1* gene reported from different countries. *SSCP* Single-strand conformational polymorphism, *DHPLC* denaturing high performance liquid chromatography, *FISH* fluorescent in situ hybridisation, *DGGE* denaturing gradient gel electrophoresis, *S* sporadic, *F* familial, *U* unilateral, *B* bilateral

Origin of patient population	Reference	Screening method(s)	No. of Patients	Phenotype	Detection rate in F and BS (%)	No. of US patients with RB1 mutations	Frequency of detection (%)	Nonsense /frameshift mutations (%)	Missense and in frame mutations (%)	Splice Site/intron mutations (%)	Gene deletions (%)
Japan	Sugano et al. 2004	SSCP, DHPLC, FISH	51	11 FB 4 FU 16 SB 20 SU	61	1	39	70	5	15	10
United Kingdom	Liu et al. 1995	SSCP	98	98 B	48	ND[a]	48	74	8	17	ND
France	Blanquet et al. 1995	DGGE	232	25 F 151 SB 56SU	27	7	20	76	13	11	ND
Germany	Lohmann et al. 1996	SSCP, HAD, sequencing	71	B or F	72	ND	72	88	0	11	ND
Spain, Colombia and Cuba	Alonso et al. 2005	Sequencing, microsatellite markers	107	11 FB 4 FU 49 SB 43 SU	67	9	50	58	11	23	8
China	Choy et al. 2002	SSCP	42	14 SB 28 SU	50	1	19	63	0	37	ND
India	Kiran et al. 2003	Sequencing, SSCP	20	20 B	65	ND	65	69	0	31	ND
Argentina	Dalamon et al. 2004	Sequencing	21	6 FB 7 SB 8 SU	80	1	24	80	20	0	ND
Italy	This study	SSCP, sequencing, real-time PCR	35	7 FB 2FU 13 SB 13 SU	59	0	37	62	0	31	7

[a]Not done

chemotherapy, the child died thereafter with progressive disease. Cordonal blood collected at birth was recovered from a cordonal blood bank. The DNA extracted revealed the presence of a frameshift mutation, c.2492_2495delTCTT. The compatibility both of maternity and paternity in respect to the recovered DNA was confirmed by the analysis of 12 polymorphic loci. The mutation was not present in either parent although unilateral RB was reported in a far relative of the maternal branch. The couple was reassured of a very low recurrence risk in the case of future pregnancy and a prenatal test was offered considering the possibility of gonadal mosaicism. In this case, recovery of a biological sample from the dead child and identification of the causative *RB1* mutation allowed the correct recurrence risk assessment and prenatal test to be offered.

Discussion

Our study identified a mutation in the *RB1* gene in 13 out of 35 patients (37%). The mutation detection rate in either familial or bilateral RB was 59% (13/22). Comparison of data obtained in the present study with the frequency and type of *RB1* mutations reported in the literature is shown in Table 2. While the frequency of mutations detected varies over a wide range, the pattern of mutations is very similar in various populations, the most predominant categories being nonsense and frameshift mutations (Table 2). An interesting aspect of this work is the high rate of novel mutations found (11 out of 13 mutations) (Table 1). A possible explanation is the different geographical provenance of our cohort of patients, since this is the first report of *RB1* mutational screening in an Italian population. Further studies on a wider group of Italian patients will be necessary to assess whether this represents a casual or real observation.

Heterozygous carriers of a nonsense or frameshift mutation almost invariably develop multiple RB foci in both eyes (Lohmann 1999). However, in some families a significant proportion of carriers remain unaffected (reduced penetrance), and many affected individuals have only unilateral RB or benign retinocytomas (reduced expressivity) (Harbour 2001).

Immunological or other host-mediated factors were early hypotheses proposed for incomplete penetrance. It is now clear that most mild cases are associated with mutations leading to a reduction in the quantity or quality of cellular pRB activity. Most families showing incomplete penetrance have distinct *RB1* mutations such as missense and in-frame mutations that do not result in premature termination codons (Sakai et al. 1991; Onadim et al. 1992; Lohmann et al. 1994; Bremner et al. 1997; Otterson et al. 1997; Ahmad et al. 1999; Lohmann and Gallie 2004). However, the case of incomplete penetrance described in Fig. 1a bears a frameshift mutation. This data lead us to speculate that other factors besides the type of *RB1* mutation may determine the penetrance of the disease. In a recent study, it has been demonstrated that diversity in the level of expression of the disease-associated allele could be a potential moderator of phenotypic variation among individuals (Murakami et al. 2004).

Another feature of the disease is that tumours sometimes apparently regress spontaneously, leaving characteristic scars on the retina. An alternative explanation is that scars represent retinomas—a more benign form of the disease (Onadim et al. 1992). Retinoma is clinically defined as a grey translucent mass extending into the vitreous from the retina, associated with calcified foci and pigment–epithelium hyperplasia. In some cases, calcified vitreous seeding has been reported. Retinoma and RB have the same genetic implications; in fact the same mutations can cause both conditions depending on the maturation status of the retinoblasts. When *RB1* loss occurs in relatively mature retinoblasts it results in benign hyperplastic nodules or retinomas; when the same events arise in immature retinoblasts, the result is malignant RBs (Gallie et al. 1982). Previous studies indicate a retinoma frequency of 8.5% among RB patients and their families. We have identified a case of retinoma in a cohort of 19 individuals bearing an *RB1* mutation (5.2%). The difference may be due to the restricted number of cases analysed.

Homozygous loss of *RB1* gene function is necessary and sufficient for the clinical manifestation of RB. The increased risk of second cancers observed in patients with germline *RB1* mutations arises because the number of sequential ''hits'' required for clonal expansion of a malignancy would have been reduced by one (Kaye and Harbour 2004).

Germinal mutations in the *RB1* gene are also implicated in increased mortality associated with other tumours. As previously reported, additional tumours develop in 4.4% of cases during the first 10 years of follow-up, in 18.3% after 20 years, and in 26.1% after 30 years (Roarty et al. 1988). Early-onset second cancers, such as midline intracranial primitive neuroectodermal tumours, generally arise months to years after the diagnosis of hereditary RB. Osteosarcomas and soft-tissue sarcomas usually arise during adolescence, whereas melanomas tend to occur in patients in their 20s or older (Wong et al. 1997). The case reported in Fig. 1d had RB followed by osteosarcoma. It is well known that patients with RB during early childhood are at risk of developing osteosarcoma during adolescence. It is reported that osteosarcoma occurred 1.2 years earlier inside than outside the radiation field in patients who underwent external beam irradiation with a mean of 12.2 ± 5.7 and 13.4 ± 3.3 years, respectively (Chauveinc et al. 2001). In our case, an ethmoidal osteosarcoma arose in the proband at 24 years although it was within the irradiation field.

The case reported in Fig. 1c was diagnosed with RB followed by the development of ethmoidal adenocarcinoma at the age of 22. Adenocarcinoma of the ethmoid sinus is rare, representing only 4–8% of malignancy of

the paranasal sinuses. Only one case was reported in 1980, a patient with a papillary adenocarcinoma of the ethmoid sinus arising 30 years after a high-dose radiotherapy for bilateral RB (Rowe et al. 1980). In our case, we know that the proband was treated in the past with chemotherapy and radiotherapy. Previous studies reported the presence of similar numbers of second primary tumours both inside and outside the irradiation fields, suggesting that irradiation is not the major risk factor for all additional tumours (Moll et al. 2001). The adenocarcinoma and osteosarcoma in the cases reported in Fig. 1c and d, respectively, both arose within the irradiation field. These data focus attention on the importance of irradiation as a cofactor in addition to the *RB1* mutation in the development of second primary tumours.

The case described in Fig. 1e shows how essential the availability of any type of patient tissue is when analysis is requested after the patient's death by family members interested in risk assessment. In the reported case, the availability of the cordonal blood of the affected dead child allowed us to ascertain the presence of a constitutive pathogenic *RB1* mutation in this patient. Subsequent analysis of the parental DNA led us to assume a "de novo" origin of the mutation. All these data allowed us to accurately estimate the recurrence risk for the couple in case of future pregnancies. The couple was reassured of a very low recurrence risk. Moreover, the identification of the causative mutation allows us to offer a prenatal test to avoid the effect of gonadal mosaicism. Finally, this experience strengthens the importance of collecting biological samples from each subject in order to ascertain the cause of the genetic disease even post-mortem and in order to provide the family with accurate recurrence risk.

The mutation rate reported in our study is quite similar to those found by other groups using the same methodological approach (Table 2). The most frequently used screening method in these studies is the SSCP technique, whose sensitivity in general may not be high enough to detect all changes. More efficient techniques such as DHPLC or direct sequencing may provide a better estimate of the frequency of point mutations in the patient population studied. However, two groups that performed the analysis by direct sequencing did not have a higher mutation detection rate (Table 2). A higher detection rate has been reported using a combination of approaches (Richter et al. 2003; Houdayer et al. 2004). Beside the techniques used for detection analysis, other explanations for our high rate of negative cases can be given. In our cohort of negative patients, 13 were unilateral sporadic and so they may bear either two somatic mutations or have an *RB1* mutation in a mosaic state. The absence of detectable mutation could also be explained by the presence of rearrangements not visible by the real-time assay used in this study. In fact, our probe designed towards exon 17 reveals only whole-gene deletions and partial deletions including exon 17. Other phenomena, such as epigenetic alterations or a possible inactivation of *RB1* through mutation of non-coding sequences, may also be invoked to explain the cause of disease in the remainder of the patients, especially in our six bilateral sporadic and three familial cases.

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3.2.2 Sensitive multistep molecular screening for *RB1* mutation detection

Unpublished data.

In retinoblastoma, highly heterogeneous inactivating mutations are distributed along the entire length of the gene which suggests that no single technology will be fully sensitive or efficient in their detection. Many techniques for gene mutation detection can be either labor-intensive, expensive (DGGE, FAMA, direct sequencing) or have low sensitivity (SSCP), so to obtain efficient and high throughput coverage of the *RB1* mutational spectrum we decided to use a combination of two approaches: a semi-automated Denaturing High-Performance Liquid Chromatography (DHPLC) method for *RB1* point mutations screening and a Multiplex Ligation-dependent Probe Amplification (MLPA) analysis for deletions/duplications detection.

DHPLC is a technique largely used for the screening of point mutations. It is a semi-automated methods that does not require post-PCR manipulation. The DHPLC methods depends upon the detection of PCR heteroduplex using ion-pairing reverse phase chromatography (IPRPC) in a thermal gradient[154] . Its suitability as a mutation screening method has been confirmed in a large number of single-locus disease. Screening of point mutation in *RB1* gene by DHPLC approach has been described by Houdayer et al. [114].

MLPA is a recently developed technique that allows multiplex detection of copy number changes in genomic DNA sequences. Ordinary multiplex PCR requires one pair of primers for every fragment to be amplified. Given that the efficiency of different primer pairs is not equal, this technique cannot easily be used for relative quantification of target sequences. MLPA reactions are more robust as all fragments are amplified with the use of only one pair of PCR primers. MLPA probes consist of two parts: one synthetic oligonucleotide and one phage M13 derived single stranded DNA fragment (Fig. 9a). Each part contains one of the two sequences recognized by the universal PCR primers. In addition, the M13 derived fragment contains a non-hybridizing stuffer sequence of variable length. The two parts of the MLPA probes are then hybridized to each target sequence, which are enzymatically ligated, and products are amplified using the universal PCR primers (Fig. 9b, 9c and 9d). The stuffer sequence makes amplification products different in length and allows to identify them by size separation. In the analysis, the signal strengths from the probes are represented by corresponding peak areas (Fig. 9e). Peak areas are then compared with those of a control individual. For MLPA analysis of *RB1*, we used a specific probe mix developed by MRC Holland. This mix contains probes for 23 of the 27 exons of the *RB1* gene. No probes are made for exons 5, 10, 15 and 16 due to their close proximity to other exons, while two probes are available for exon 1. In addition to these 24 probes, two probes are available

for genes which are relatively close to *RB1* (*ITMB2*, 48 kb upstream; *CHC1L*, 72 kb downstream) as well as a probe for the *DLEU1* gene located at a distance of 1.6 Mb from *RB1* gene.

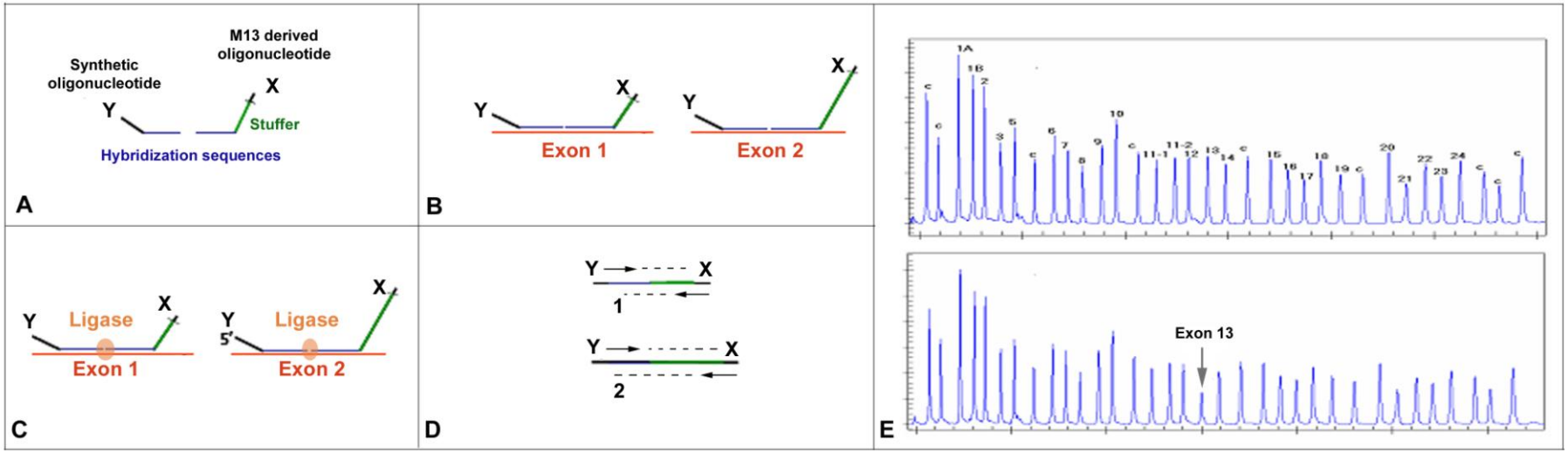

Fig. 9. Principle of MLPA. A) MLPA probes. The synthetic oligonucleotide contains the sequence recognized by the universal primer Y (in dark), while the M13 derived fragment contains the specific sequence for universal primer X (in dark) and the stuffer sequence (in green). Target sequences are indicated in blue. B) Hybridization of MLPA probes. Genomic DNA is denatured and the two parts of each MLPA probe are hybridized to the target sequences. C) Ligation reaction. Only perfectly matched probes are ligated by a thermostable ligase. D) PCR reaction. All the ligated probe products are amplified by PCR using only one primer pair (X and Y). The amplification product of each probe has a unique length and can be identified by size separation. E) Example of fragment analysis by capillary electophoresis. Analysis of a control DNA (upper panel) and a test DNA (lower panel). Compared to a control reaction, the relative peak area of each amplification product reflects the relative copy number of the target sequence of that probe in the analyzed sample. Here, the analysis of test DNA shows a decrease in peak area of exon 13 compared to control, indicating the presence of a deletion.

Methods

Point mutations. Primers and PCR conditions used for single exons and promoter analysis were as described previously [114, 155, 156] Mutation analysis was performed by DHPLC using the Transgenomic WAVE™ (Transgenomic, San Jose, CA, USA). PCR products were denatured at 95°C, reannealed at 65°C for 10 minutes, and cooled to 4°C to generate heteroduplexes. The optimal column temperature for fragment analysis was calculated using the WaveMaker Software (Transgenomic, San Jose, CA, USA). DHPLC analysis was performed at melting temperatures reported in Table 1. Samples with abnormal DHPLC profiles were sequenced on an ABI PRISM 310 genetic analyzer (Applied Biosystems). Promoter, exons 1, 15, 16, 17 and 20 were analysed by SSCP, as described in Sampieri et al.[157].

EXON	PCR: ANNEALING T°	LENGTH (bp)	DHPLC: MELTING T°	SSCP
Promotor	64°	573		20° 4h
Ex 1	64°	311		5° 4h
Ex 2	62°	410	53°	
Ex 3	53°	259	54°	
Ex 4	56°	305	52°	
Ex 5	63°	194	53,8 – 52,2°	
Ex 6	60°	280	51° - 52,2°	
Ex 7	60°	239	54,4°	
Ex 8	56°	316	54° - 56°	
Ex 9	60°	221	53°	
Ex 10	53°	171	54,1° - 54,6°	
Ex 11	56°	245	52° 55°	
Ex 12	60°	183	53° 54°	
Ex 13	60°	237	56°	
Ex 14	60°	211	56,8° - 59,6°	
Ex 15	60°	226		20° 4h
Ex 16	59°	257		5° 4h
Ex 17	53°	339	55,4°	
Ex 18	60°	222	54°- 57°	
Ex 19	62°	294	54° - 56°	
Ex 20	56°	335	54,2°	5° 4h
Ex 21	56°	328	52°	
Ex 22	63°	315		5° 4h
Ex 23	56°	287	55° - 57°	
Ex 24	60°	278	53,5°	
Ex 25	56°	297	56,5° - 59°	
Ex 26	62°	209	55°	
Ex 27	62°	178	57°	

Tab 1. PCR amplification, DHPLC and SSCP analysis conditions.

Deletions/duplications. We also analyzed *RB1* by MLPA using the probe mix P047 (MRC Holland). The analysis was carried out as described by Schouten[158]. Briefly, 100 ng of genomic DNA was diluted with TE buffer to 5 µl, denatured at 98°C for 5 minutes and then hybridized with SALSA Probe-mix at 60°C overnight. Ligase-65 mix was then added and ligation was performed at 54°C for 15 minutes. The ligase was successively inactivated by heat (98°C for 5 minutes). PCR reactions were performed in a 50 µl volume. Primers, dNTP and polymerase were added and amplification was carried out for 35 cycles (30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72 °C). The amplified products were analyzed on an ABI model 310 using Genescan ROX 500 standards and Genescan software. Data from Genescan were copied to Excel files (Windows) where the final results were calculated using Coffalyser software (MRC Holland). Dosage alterations were considered significant if sample values deviated more than 30% from the control.

Results

During this study, 116 Italian patients affected by retinoblastoma tumors were referred to the Medical Genetics Unit of Siena for molecular diagnosis. 81 DNA samples were collected after the first mutational screening published in 2006 [157]. Of these, 9 were familial cases (3 unilateral and 6 bilateral), and 72 were sporadic (36 unilateral and 36 bilateral). All samples were screened by MLPA to detect deletions/duplications. All positive samples were confirmed by qPCR. The negative samples were analyzed by DHLPC in order to detect point mutations. The entire coding region (27 exons), the nearby intronic regions, and the promoter were screened by PCR-mediated techniques, followed by DHPLC or SSCP analysis and then direct sequencing. A total of 44 mutations were detected (13 deletions and 31 point mutations). In particular, 29 patients were found to have bilateral sporadic retinoblastoma, 6 had unilateral retinoblastoma and 9 patients had familial disease. These results are summarized in Table 2 (deletions) and Table 3 (point mutations).

ID	Deletion	Phenotype	
#34	*RB1* gene	F	B
#109	Exon 18	F	B
#57	Exon 14	S	B
#129	*RB1* gene	S	B
#132	Exon 13	S	B
#133	*RB1* gene	S	B
#138	Exon 12	S	B
#317	Exon 12	S	B
#359	Exon 24	S	B
#282	*RB1* gene	S	U
#370	*RB1* gene	S	U
#355	*RB1* gene	S	U
#387	*RB1* gene	S	U

Table 2 Deletions identified by MLPA analysis. *S* Sporadic, *F* Familial, *U* Unilateral, *B* Bilateral.

ID	Location	DNA variation	AA change	Phenotype	
#88	Exon 2	g.5527delG	p.V81fsX110	F	B
#127	Intron 19	g.156692G>A		F	B
#152	Exon 23	g.162237C>T	p.R787X	F	B
#157	Intron 6	g.56841T>G		F	B
#162	Intron 5	g.45789T>A		F	B
#102	Exon 7	g.56884delG	p.V213fsX218	F	U
#115	Intron 22	g.162115G>A		F	U
#59	Exon 23	g.162364C>G	p.S829X	S	B
#118	Intron 20	g.160727T>G		S	B
#122	Intron 6	g.45867G>C		S	B
#134	Exon 19	g.153235 del A	p.G617fsX621	S	B
#139	Exon 15	g.76898 C>T	p.R467X	S	B
#143	Exon 14	g.76430C>T	p.R445X	S	B
#16	Exon 20	g.156788-156789delCA	p.P687fsX690	S	B
#170	Exon 11	g.65386C>T	p.R358X	S	B
#182	Exon 19	g.153252C>T	p.T620M	S	B
#185	Exon 4	g.41974delA	p.V144fsX152	S	B
#190	Exon 15	g.76898C>T	p.R467X	S	B
#193	Intron 12	g.70330G>A		S	B
#198	Exon 20	g.156713C>T	p.R661W	S	B
#202	Exon 7	g.56855G>T	p.E204X	S	B
#205	Exon 17	g.78250C>T	p.R556X	S	B
#221	Exon 14	g.76460G>A	p.R455X	S	B
#225	Exon 23	g.162237C>T	p.R787X	S	B
#229	Exon 17	g.78250C>T	p.R556X	S	B
#245	Exon 23	g.162304delT	p.L809fsX825	S	B
#252	Exon 10	g.64348C>T	p.R320X	S	B
#263	Exon 14	g.76460C>T	p.R455X	S	B
#283	Intron 12	g.70330G>A		S	B
#137	Exon 14	g.76460C>T	p.R455X	S	U
#213	Exon 14	g.76430C>T	p.R445X	S	U

Table 3 Identified point mutations. Nucleotide numbering for genomic DNA is according to GenBank accession number L11910.1. Amino acid numbering refers to the reference sequence for pRB protein, NP_000312. *S* Sporadic, *F* Familial, *U* Unilateral, *B* Bilateral.

Combining the results obtaining from the first screening together with the samples collected later by the Medical Genetics Unit of Siena, a germline mutation in *RB1* gene was detected in 56 (6 sporadic unilateral, 35 sporadic bilateral and 15 familial cases) out of 116 patients. All functional classes of mutations were identified: 32% nonsense, 20% frameshift, 20% splicing, 25% deletion and 3% missense (Fig. 10).

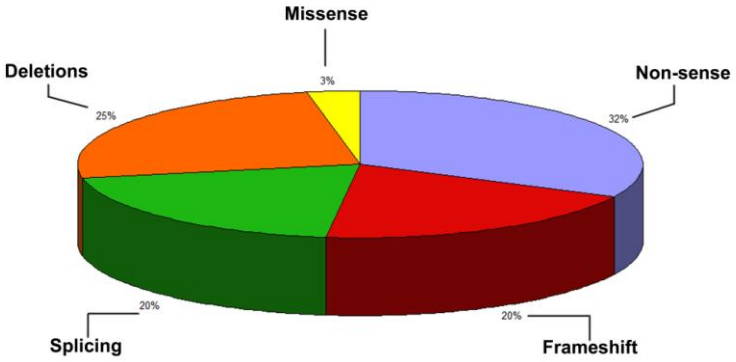

Fig. 10. Distribution of classes of mutations detected in 56 retinoblastoma patients.

The improved methods for *RB1* mutation identification utilized by our lab, specifically the combination of the semi-automated DHPLC method for *RB1* point mutations screening and the MLPA analysis for deletions/duplications detection, led to an increase of the mutation detection rates in all classes of retinoblastoma of our cohort of patients. In particular, the mutation detection rate increased from 0% to 12% among sporadic unilateral cases, from 54% to 71% in sporadic bilateral cases, and from 66% to 83% in familial cases (Fig. 11).

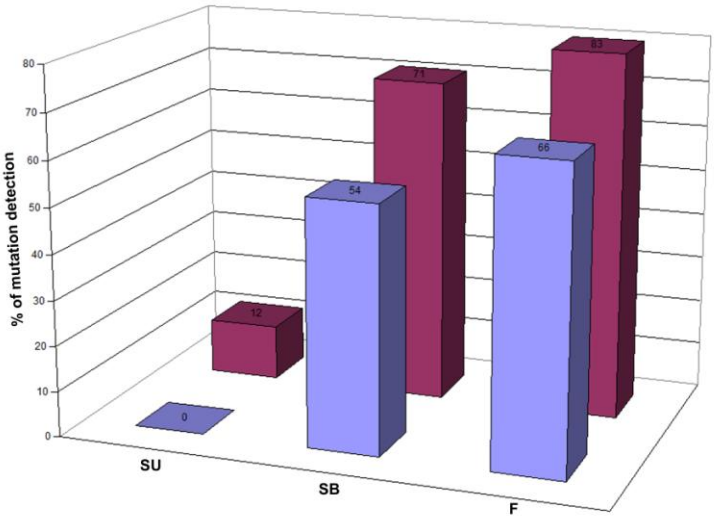

Fig. 11. Mutation detection rate improvement from the first screening (Sampieri et al., 2006) (in light blue) to the optimized combined screening (in purple) for sporadic unilateral (SU), sporadic bilateral (SB) and familial (F) cases.

3.3 Retinoblastoma and mental retardation microdeletion syndrome: clinical characterization and molecular dissection using array CGH.

Caselli R, Speciale C, Pescucci C, Uliana V, Sampieri K, Bruttini M, Longo I, De Francesco S, Pramparo T, Zuffardi O, Frezzotti R, Acquaviva A, Hadjistilianou T, Renieri A, Mari F.

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ORIGINAL ARTICLE

Retinoblastoma and mental retardation microdeletion syndrome: clinical characterization and molecular dissection using array CGH

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Abstract We describe three patients with retinoblastoma, dysmorphic features and developmental delay. Patients 1 and 2 have high and broad forehead, deeply grooved philtrum, thick anteverted lobes and thick helix. Patient 1 also has dolicocephaly, sacral pit/dimple and toe crowding; patient 2 shows intrauterine growth retardation and short fifth toe. Both patients have partial agenesis of corpus callosum. Patient 3 has growth retardation, microcephaly, thick lower lip and micrognathia. Using array-comparative genomic hybridization (CGH), we identified a 13q14 de novo deletion in patients 1 and 2, while patient 3 had a 7q11.21 maternally inherited deletion, probably not related to the disease. Our results confirm that a distinct facial phenotype is related to a 13q14 deletion. Patients with retinoblastoma and malformations without a peculiar facial phenotype may have a different deletion syndrome or a casual association of mental retardation and retinoblastoma. Using array-CGH, we defined a critical region for mental retardation and dysmorphic features. We compared this deletion with a smaller one in a patient with retinoblastoma (case 4) and identified two distinct critical regions, containing 30 genes. Four genes appear to be good functional candidates for the neurological phenotype: *NUFIP1* (nuclear fragile X mental retardation protein 1), *HTR2A* (serotonin receptor 2A), *PCDH8* (prothocaderin 8) and *PCDH17* (prothocaderin 17).

Keywords 13q14 deletion syndrome · Developmental delay · Mental retardation · Retinoblastoma · Array-CGH

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Introduction

Retinoblastoma is the most common intraocular tumor of early childhood, with an incidence of 1/15,000–28,000 live births. Tumor development is caused by inactivation of both alleles of the *RB1* gene located in 13q14.2. In 68% of cases *RB1* is inactivated by point mutations, in 5% *RB1* complete gene deletions have been found, while gross-sized molecular deletions have been found in 10% of cases (Albrecht et al. 2005; Dahiya et al. 2000; Kloss et al. 1991; Lohmann and Gallie 2004; Sampieri et al. 2006). When the deletion involves part of the *RB1* surrounding genome it causes a contiguous gene deletion syndrome characterized by retinoblastoma, developmental abnormalities and peculiar facial dysmorphisms. The first author to suggest a specific facial phenotype associated with 13q14 deletion was Motegi in 1983 (Motegi et al. 1983). He described two patients with retinoblastoma and common facial features including prominent eyebrows, broad nasal bridge, bulbous nasal tip,

large mouth, thin upper lip and long philtrum (Motegi, et al. 1983). A few years later, he described an additional patient, and was able to improve the clinical definition of the syndrome (Motegi et al. 1987). In 1999, Baud et al. (1999) defined the dysmorphic features of 13q14 deletion syndrome. He described a cohort of 22 patients with the following common dysmorphic abnormalities: high and broad forehead, thick and everted ear lobes, short nose, prominent philtrum and thick everted lower lip (Baud et al. 1999). In 2001, Bojinova et al. (2001) extended the facial phenotype associated with the 13q14 deletion syndrome with the description of additional 13 patients characterized by cranial anomalies, frontal bossing, deeply grooved and long philtrum, depressed and broad nasal bridge, bulbous tip of the nose, thin upper lip, broad cheeks, and large ears and lobules. Afterward, a patient with a X:13 translocation and phenotypic features peculiar to the 13q14 deletion syndrome was described (Dries et al. 2003). Finally, in 2004, a patient with retinoblastoma, pinealoma and mild multiple congenital anomalies/mental retardation syndrome (MCA/MR) and a germline 13q14 deletion were reported (Skrypnyk and Bartsch 2004).

All these reported cases were studied by means of cytogenetic analysis. We investigated using array-based comparative genomic hybridization (array-CGH) three patients with retinoblastoma and MCA/MR. Using the same method, we analyzed an additional patient with isolated retinoblastoma and a previously identified RB1 deletion (Sampieri et al. 2006) to attempt to define a minimal critical region for MCA/MR. Patients were selected among the cohort of retinoblastoma cases collected in the biobank of the Medical Genetics Unit of the University of Siena (http://www.biobank.unisi.it). Here we report an accurate clinical and molecular characterization of these patients.

Materials and methods

Array-CGH analysis

Array-CGH analysis was performed using commercially available oligonucleotide microarrays containing approximately 43,000 60-mer probes (Human Genome CGH Microarray 44B Kit. Agilent Technologies, Santa Clara, California), as previously reported (Pescucci et al. 2006). The average resolution is about 75–100 kb.

Real-time quantitative PCR

Real-time quantitative polymerase chain reaction (PCR) was performed to confirm array-CGH data. We used TaqMan Gene Expression Assays by design (Applied Biosystems, http://www.products.appliedbiosystems.com), which provides a pre-designed primers-probe set for real-time PCR experiments. In order to validate the presence of the 13q deletion in cases 1, 2 and 4, we used the TaqMan probe and primers in exon 17 of RB1, as previously described (Sampieri et al. 2006). For validating the presence of the 7q deletion in case 3, we designed the probe in the BC066990 sequence related to the 7q11.21 locus. Forward primer: 5′-GTG CTG TAG TGC AGA ATG TAA CAA A-3′; reverse primer: 5′-CAG AAA GCC AAG AAT AAC-3′; TaqMan probe: 5′-AGG GTG AAC AAA ACC AGT TGA GTT-3′. PCR was carried out using an ABI prism 7000 (Applied Biosystems) in a 96-well optical plate with a final reaction volume of 50 µl. A total of 100 ng (10 µl) was dispensed in each of the four sample wells for quadruplicate reactions. Thermal cycling conditions included a pre-run of 2 min at 50°C and 10 min at 95°C. Cycle conditions were 40 cycles at 95°C for 15 s and 60°C for 1 min, according to the TaqMan Universal PCR Protocol (ABI). The TaqMan Universal PCR Master Mix and Microamp reaction tubes were supplied by Applied Biosystem. The starting copy number of the unknown samples was determined using the comparative Ct method, as previously described (Livak 1997).

RB1 mutation analysis

Genomic DNA was amplified by PCR. Primers and PCR conditions for single exons and promoter analysis have been described previously (Hogg et al. 1992; Houdayer et al. 2004; Scheffer et al. 2000). PCR products were mixed with an equal volume of formamide, denatured by heating at 95°C for 5 min, followed by immediate chilling on ice. Single-strand conformational polymorphism (SSCP) was performed on a Genephor apparatus (Pharmacia Amersham, Little Braunschweig, Germany) using a GeneGel Excel 12.5/24 Kit (Pharmacia Amersham).

Results

Clinical description

Case 1

Patient number 1, a 1-year and 2-month-old female, is the first and only child of healthy unrelated parents (Fig. 1a). At birth, the mother and father were 28 and 33 years old, respectively. Their family history was unremarkable. No teratogen exposure during pregnancy had been reported. The child was born on term by means of caesarean delivery.

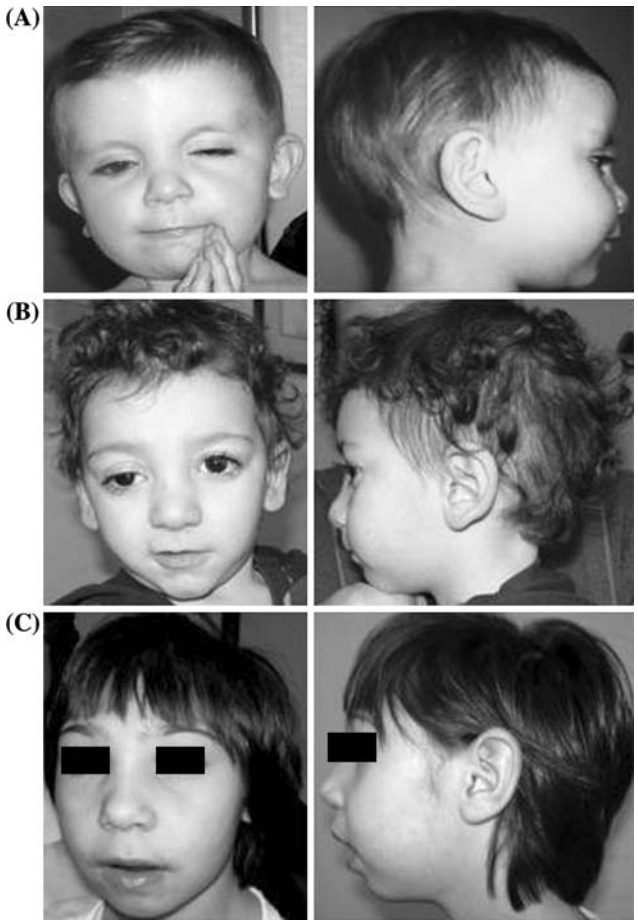

Fig. 1 Face and profile views of the patients. **a** Case 1: patient no. 1 at the age of 1 year 2 months. *Frontal view* showing high and broad forehead, deeply grooved philtrum. *Side view* showing dolicocephaly and thick anteverted lobes and helix. **b** Case 2: patient no. 2 at the age of 2 years 7 months. *Frontal view* showing hypotelorism, long palpebral fissures, epichantic folds, slight unilateral ptosis and thick and everted lower lip. Thick anteverted lobes and helix are showed on the *side view*. **c** Case 3: patient no. 3 at the age of 7 years 6 months. *Frontal view* showing sparse eyebrows in the medial third broad nasal bridge, bulbous tip of the nose, long philtrum, thick and everted lower lip. *Side view* showing large ears and micrognathia

Birth weight was 3,130 g (50th percentile), length was 51 cm (50th percentile) and head circumference was 36 cm (>90th percentile). Bilateral retinoblastoma was diagnosed at 5 months of age. At that time, MRI revealed corpus callosum hypoplasia. ABR and ankle ultrasonography were normal. At our first examination (6 months), psychomotor development was slightly delayed. Her weight was 7.850 g (75th percentile), length 68 cm (90th percentile) and head circumference 46 cm (97th percentile) with dolicocephaly. The patient presented scalp anomalies including widely open fontanelles and an alopecic area on the right temporo-parietal region. High and broad forehead, deeply grooved philtrum, and thick anteverted lobes and thick helix were noted. In addition, she showed sacral dimple and

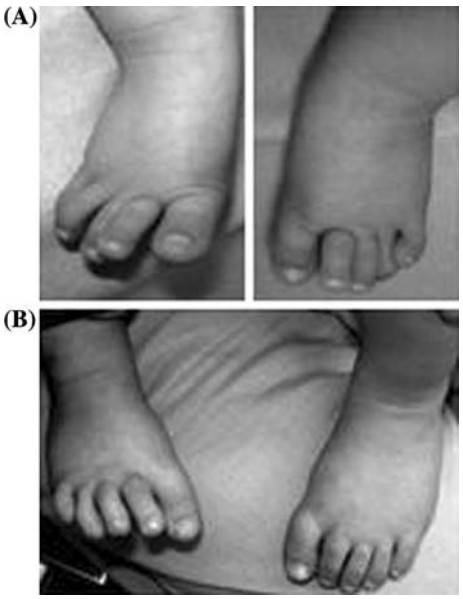

Fig. 2 Toe anomalies in cases 1(a) and 2(b). **a** View of the right feet showing toe crowding. Note clinodactyly of the 5th toe on the left foot. **b** Note short 5th toe with hypoplastic toe nail

clinodactyly of the 5th toe on the left and toe crowding on the right (Fig. 2a). At the age of 11 months, she presented with a relapse in the right eye treated by chemotherapy. Two months later, the right eye was enucleated. In the following months, a relapse in the left eye occurred, which was treated successfully by means of radiotherapy. At our second clinical examination (14 months), her weight was 9 kg (10–25th percentile), length 76–77 cm (50th percentile) and head circumference 49 cm (>97th percentile). Psychomotor delay persisted. Ultrasound cardiac examination was normal (Table 1).

Case 2

Patient number 2, a 2-year and 7-month-old boy, is the third-born of healthy and non-consanguineous parents (Fig. 1b). Intrauterine growth retardation was noted at the 36th week of gestation. He was born on term. At birth, his weight was 2,300 g (<3rd percentile), length was 47 cm (10–25th percentile) and OFC was 32 cm (3rd–10th percentile). He demonstrated a deficit of thermoregulation. On the 11th day, he presented with enterococcus sepsis. During the first months of life, the parents noted iris bilateral heterochromia. Right eye retinoblastoma was diagnosed at 10 months of age. At 1 year of age, a MRI was performed and hypoplasia of the corpus callosum was noted. By 1 year and 5 months of age, he suffered a relapse, which was treated by enucleation. At our first examination (2 years of age) his height was 78 cm (<3rd percentile), weight 8,250 kg (<<3rd percentile) and OFC 44 cm (<<3rd

Table 1 Clinical findings in patients with retinoblastoma, dysmorphic features and developmental delay

Dysmorphic features	Age		
	Case 1 (≠ 133) 1 year 2 month	Case 2 (≠ 129) 2 year 7 month	Case 3 (≠ 76) 7 year 6 month
Thick anteverted lobes	+	+	–
Thick helix	+	+	–
High and broad forehead	+	+	–
Deeply grooved philtrum	+	+/–	–
Short nose	–	+	+
Thick everted lower lip	–	+	+
Cardiac anomaly	–	–	–
Brain anomaly	Partial corpus callosum agenesis	Corpus callosum hypoplasia	–
Skeletal abnormality	Toe crowding	Short V toe	–
Growth retardation	–	+(<3 cnt)	+/–
Other	Dolicocephaly Alopecia Sacral pit	Iris heterochromya	Microcephaly (<<3 cnt) Cutis mormorata epicanthic folds

percentile). He showed hypotonia and particular facial features including high and broad forehead, deeply grooved philtrum, thick and everted lower lip, thick and everted auricular lobes, and thick helix. Moreover, he had short 5th toe with hypoplastic toenail (Fig. 2b). A second clinical examination 7 months later confirmed growth delay: 80 cm in height (<5th percentile), weight 9.0 kg (<<3rd percentile) and OFC 45 cm (<<3rd percentile). The previously noted facial features were still present. There were no abnormalities of other organs and systems. He reached self-governing deambulation at 2 years and 6 months. Presently, he is able to say only few words and he has no sphincter control. An echocardiogram showed minimum aortic reflux, probably due to the infantile infection. X-rays of hands and toes indicated no abnormalities (Table 1).

Case 3

Patient number 3, a 7-year and 11-month-old female, is the first-born of healthy, non-consanguineous parents (Fig. 3c) at birth, the mother was 35 years and the father 47 years old. After bearing this child, the mother later had a spontaneous abortion. However, this patient does have a 19-year-old maternal half-sister suspected to have Gilles De La Tourette syndrome; the mother had a spontaneous abortion after her birth. During gestation, ultrasound study revealed microcephaly. This patient was born on term and her weight was 2,780 g (10–25th percentile); data on length and OFC are not available. Development has been slightly delayed: she reached self-governing deambulation at 20 months of age and was able to say her first words when she was 2 years old. At 2.5 years of age, her mother noted right leucoria. Unilateral retinoblastoma was diagnosed and treated with eye enucleation. At our first examination (5 years and 7 months of age), her weight was 14 kg (<5th percentile), height 109.5 cm (25–50th percentile) and OFC 41 cm (<<3rd percentile). Physical examination showed sparse eyebrows in the medial third, epichantic folds, broad nasal bridge, bulbous nasal tip, long philtrum, thick and everted lower lip, large ears, micrognathia and cutis marmorata. She also showed a moderate mental retardation (Table 1). A second clinical examination at 7 years and 11 months of age confirmed short stature (115 cm, <5th percentile), the same previously described facial features and microcephaly (OFC 42 cm, <<3rd percentile).

Case 4

Patient number 4, a 5-year and 4-month-old female, is the second child of unrelated parents. At birth, the mother was 20 years and the father 26 years old. Paternal history was unremarkable and the first child of the couple is healthy. The mother and a younger brother, however, have been affected by retinoblastoma. The mother presented with retinoblastoma in the left eye at 11 months of age and enucleation was immediately performed. In the younger brother, the diagnosis was made at 40 days of life. In the second child, patient number 4, the gestation of the proband was unremarkable and no teratogen exposure was reported. Multifocal retinoblastoma in the left eye was diagnosed at 2 years and 7 months. After four cycles of chemotherapy, the tumors showed good regression but three relapses occurred and enucleation was performed.

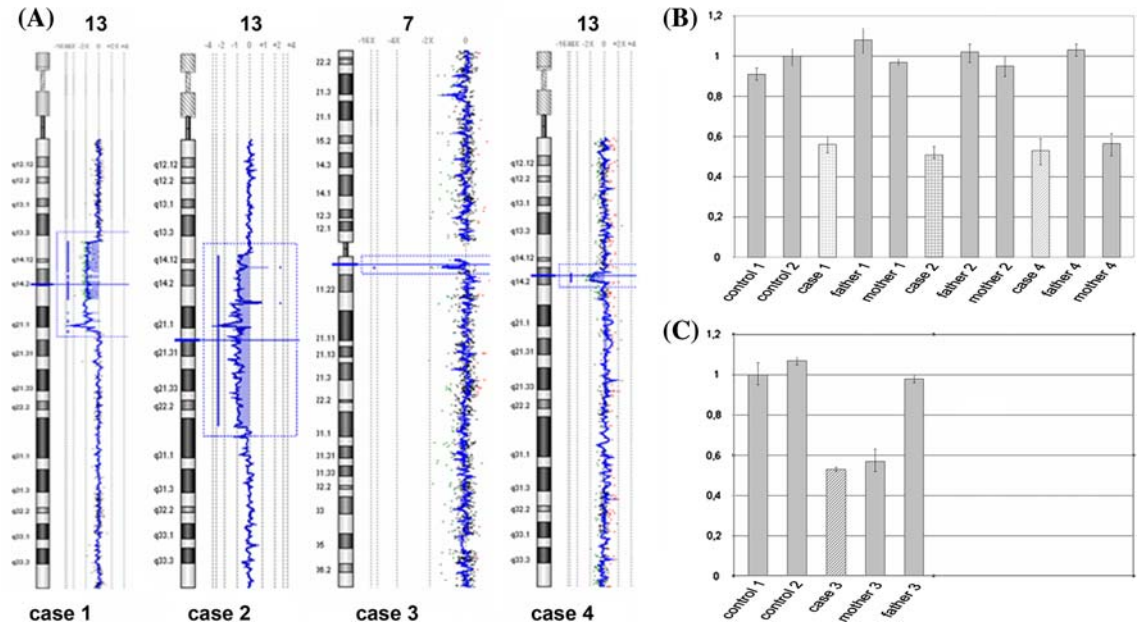

Fig. 3 Patients' molecular data. **a** Array CGH ratio profiles. On the left, chromosome ideogram for each case. On the right, the log2 ratio of the chromosome probes plotted as a function of chromosomal position. Oligos with a value of zero represent equal fluorescence intensity ratio between sample and reference. Each *dot* represents a single probe (oligo) spotted on the array. Copy number loss shifts the ratio to the left (value of about –2X). **b** and **c** Real-time quantitative PCR validation experiment. **b** RB1 ddCT ratios and standard deviations of two different controls and of patients 1, 2 and 4 and their parents. Patients 1, 2 and 4 and the mother of 4 show a ddCT ratio of about 0.5, indicating the presence of a single copy of RB1 (deletion). The parents of patients 1 and 2 and the father of patient 4, as the controls, show ddCT ratios of about 1.0, which indicates a double copy of the gene. **c** BC066990 sequence ratios and standard deviations of two different controls and of patient 3 and her parents. The patient and the mother show ddCT ratio of about 0.5, indicating the presence of a single copy of BC066990 sequence (deletion); while the father, like the controls, shows ddCT ratios of about 1.0 indicating a double copy of the sequence

During ophthalmological follow-up, no foci were noted in the right eye. On clinical examination, neither mental retardation nor dysmorphisms have been noted. Isolated unilateral retinoblastoma was the unique clinical sign.

Molecular characterization

Point mutation analysis of promoter and coding sequences of the *RB1* gene in the four cases did not reveal any alterations.

Oligonucleotide array-CGH analysis with an averaged spatial resolution of approximately 75 kb was performed on DNA from the four patients. The analysis of ratio profiles in cases 1, 2 and 4 revealed a different sized interstitial deletion in the long arm of chromosome 13. Based on the array findings, the deleted region observed in case 1 was found to extend approximately 19 Mb [46,XXdel(13)(q13.3q21.2)]. The proximal breakpoint is mapped in 13q13.3 (last oligonucleotide present located in 40.34 Mb, first deleted in 40.40 Mb position), while the distal breakpoint is located between 59.29 and 59.36 Mb in 13q21.2 (last oligonucleotide deleted and first present, respectively) (Fig. 3a). The deleted region seen in case 2 is approximately 36 Mb in size [46,Xydel(13)(q14.11q31.1)]. In this case, the proximal breakpoint is mapped in 13q14.11 (last oligonucleotide present located in 43 Mb, first deleted in 43.24 Mb position), while the distal breakpoint is located between 79.27 and 79.80 Mb in 13q31.1 (last oligonucleotide deleted and first present, respectively) (Fig. 3a). In contrast, the array-CGH analysis of case 3 revealed a 200-kb proximal deletion on chromosome 7q [46,XX del(7)(q11.21)](Fig. 3a).

The array-CGH analysis of case 4 allowed us to identify an interstitial deletion of approximately 1.7 Mb [46,XX del(13)(q14.2)]. In this case, both breakpoints are located in 13q14.2 (last oligonucleotide present located in 47.35 Mb, first deleted in 47.44 Mb position while last oligonucleotide deleted in 49.10 Mb position and first oligonucleotide present in 49.17Mb position). In order to confirm array findings, real-time Quantitative PCR experiments were performed in the patients and their parents. In all cases, the deletion was confirmed. Identified deletions of the long arm of chromosome 13 were de novo in patients 1 and 2 and inherited in 4. The 7q microdeletion in patient 3 was inherited from the unaffected mother (Fig. 3b, c).

Discussion

Two distinct syndromes are associated with deletions involving different regions of the long arm of chromosome 13. One of these syndromes is caused by a more distal deletion that involves band q32 and is phenotypically characterized by the presence of severe mental retardation, major malformations and digital anomalies (Brown et al. 1993, 1995). The other one is due to a proximal deletion that involves band q14 and is associated with retinoblastoma and mental retardation. Baud et al. defined the peculiar facial traits of this syndrome that is characterized by anteverted ear lobes, high and broad forehead, and a prominent philtrum (Baud et al. 1999; Motegi et al. 1987, 1983).

The facial features of patients 1 and 2 suggest the 13q14 deletion syndrome described by Baud et al. 1999 (Table 1). In particular, both patients have high and broad foreheads, deeply grooved philtrum, thick and anteverted lobes and thick helices. These characteristics are absent in patient 3. As expected on the basis of clinical examination, array CGH analysis confirmed a 13q14 deletion syndrome in patients 1 and 2 but not in 3. Our findings confirm that 13q14 deletion syndrome is characterized by specific facial features and that this diagnosis may be strongly suspected on a clinical ground even before the genetic test.

A more complex situation is present in case 3. The presence of the small 7q deletion in the unaffected mother suggests that this deletion is not responsible for the clinical phenotype in this patient. In addition, a known copy-number polymorphism (CNP) is located at a distance of 80 kb from our deletion (http://www.projects.tcag.ca/bioxrt/), and the deletion is included in a region of highly homologous duplicated sequences (Sharp et al. 2005). However, three different imprinted regions located on the long arm of chromosome 7 have already been described (http://www.geneimprint.com/site/genes-by-species.Homo+sapiens.imprinted-All). Consequently, additional analyses are necessary to investigate whether the deleted region is imprinted and to definitively rule out the involvement of this small deletion in the clinical phenotype of the patient.

Deletions identified in cases 1 and 2 partially overlap and allowed us to define a minimal critical region for mental retardation and dysmorphic features of about 16 Mb that includes 39 known genes.

Comparison of this critical region with the 13q14.2 deletion present in case 4 with isolated retinoblastoma allowed us to exclude a central region containing 9 known genes. Consequently, we identified two distinct critical regions, a centromeric sub-region of about 4 Mb and a telomeric one of about 10 Mb. Gene content analysis of the centromeric sub-region showed the presence of 14 known genes (Fig. 4, Table 2). Among them, *NUFIP1* is of particular interest due to its putative role in central nervous system (CNS) development and to its preferential brain expression. The *NUFIP1* gene encodes for a nucleo-cytoplasmatic RNA binding protein: FMRP interacting protein 1. NUFIP1 interacts with FMRP, the protein disrupted in Fragile X Mental Retardation (Bardoni et al. 2003). In particular, *NUFIP1* could be involved in the regulation of local protein synthesis near active synapses in association with FMRP (Bardoni et al. 2003). Due to its role in synaptic plasticity, *NUFIP1* could be a good candidate gene for mental retardation in our patients. The *HTR2A* gene is located within the centromeric deleted sub-region. This gene codifies for a receptor of serotonin. Disturbances in

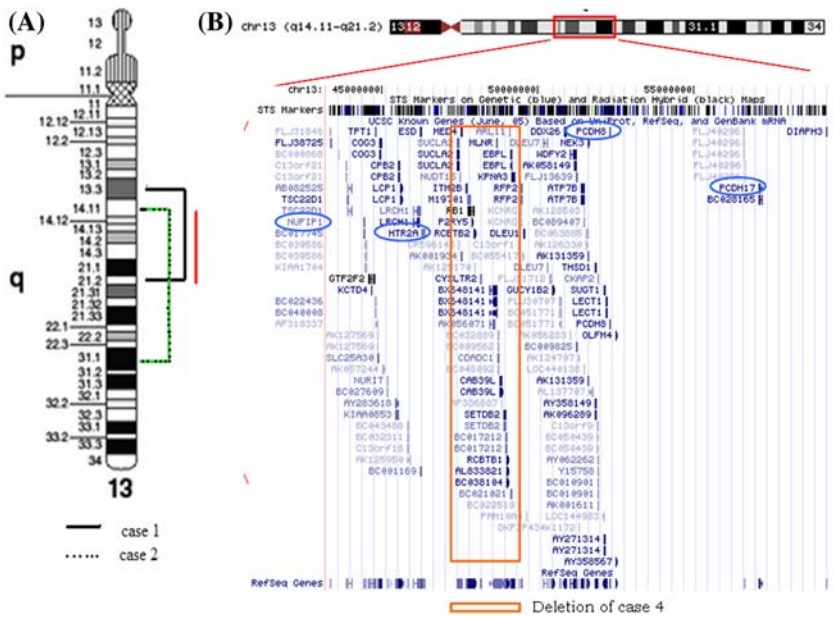

Fig. 4 Gene content of the deleted region (UCSC Genome Browser; http://www.genome.ucsc.edu). **a** Comparison between deletions observed in cases 1 and 2 and minimal critical region. **b** Gene content of the two critical sub-regions. *Orange rectangles* delimit deleted region identified in case 4. *Circled genes* are discussed in the text

Table 2 Centromeric critical region

Gene symbol	Gene name	Note
TSC22D1	TSC22 domain family 1	
NUFIP1	Nuclear fragile X mental retardation protein 1	
GTF2F2	General transcription factor IIF, polypeptide 2	
KCTD4	Potassium channel tetramerisation domain containing protein 4	
TPT1	Histamine-releasing factor	
COG3	Component of Golgi transport complex 3	
NURIT	Testis-specific leucine zipper protein nurit	
SLC25A30	Solute carrier family 25, member 30	
ZC3H13	Zinc finger CCCH-type containing 13	
CPB2	Plasma carboxypeptidase B2 isoform a	
LCP1	Lymphocyte cytosolic protein 1	
LRCH1	Leucine-rich repeats and calponin homology	
ESD	Esterase D	
HTR2A	Serotonin receptor 2A	Polymorphisms have been studied in association with ADHD, Alzheimer disease and behavioral disorders

Table 3 Telomeric critical region

Gene symbol	Gene name	Note
RFP2	Ret finger protein 2 isoform 2	Involved in B-cell chronic lymphocytic leukemia in 13q14 deletion
KCNRG	Potassium channel regulator	Candidate for B-cell chronic lymphocytic leukemia and prostate cancer tumor suppressor
DLEU1	Deleted in lymphocytic leukemia, 1	Leukemia associated protein 1 may act as tumor suppressor
DLEU7	Deleted in lymphocytic leukemia, 7	
DDX26	DEAD/H box polypeptide 26	
WDFY2	WD repeat and FYVE domain-containing protein 2	
GUCY1B2	Guanylate cyclase 1, soluble, beta 2	
ATP7B	ATPase beta polypeptide	Homozygous mutations have been associated with Wilson disease
NEK3	Serine/threonine-protein kinase Nek 3	
THSD1	Thrombospondin type I domain-containing 1	
TMAP	Tumor-associated microtubule-associated protein	
SUGT1	Suppressor of G2 allele of SKP1	
LECT1	Leukocyte cell-derived chemotaxin 1	
PCDH8	Protocadherin 8	
PCDH17	Protocadherin 17	
DIAPH3	Diaphonous protein homolog 3	

the serotonergic neurotransmission system may be responsible for behavioral disorders (Bruce et al. 2005). In particular, a polymorphism in the HTR2A gene was associated with the remission of attention deficit/hyperactivity disorder (ADHD) (Li et al. 2006). Disruption of serotonin receptor activity may contribute to CNS disorders that have been associated with impaired development.

Gene content analysis of the deleted telomeric sub-region showed the presence of 16 known genes (Fig. 4, Table 3). Among them, *PCDH8* and *PCDH17* may be good candidates for the generation of the neurological phenotype in our patients. These genes belong to the protocadherin gene family and codify for integral membrane proteins, which are thought to function in signaling pathways and in cell

adhesion in a CNS-specific manner. The role of Pcdh8 in the nervous system was investigated in rat hippocampus (Yamagata et al. 1999). Antibodies against Pcdh8 attenuate basal synaptic transmission and completely inhibit long-term potentiation in hippocampal slices (Yamagata et al. 1999). The expression and function of the *PCDH17* gene is not well known.

To date, all 48 cases with a 13q14 microdeletion reported in the literature have been characterized at the cytogenetic level. This is the first report of characterization at the molecular level, using array-CGH, of patients with retinoblastoma and mental retardation, and a critical region for mental retardation is defined. Further experiments are necessary to narrow this critical region and to dissect the syndrome, thus identifying the gene(s) responsible for the neurological phenotype in these patients.

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3.4 Genomic differences between retinoma and retinoblastoma.

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ORIGINAL ARTICLE

Genomic differences between retinoma and retinoblastoma

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Abstract

Introduction. Genomic copy number changes are involved in the multi-step process transforming normal retina in retinoblastoma after *RB1* mutational events. Previous studies on retinoblastoma samples led to a multi-step model in which after two successive *RB1* mutations, further genomic changes accompany malignancy: 1q32.1 gain is followed by 6p22 gain, that in turn is followed by 16q22 loss and 2p24.1 gain. Retinoma is a benign variant of retinoblastoma that was initially considered a tumor regression, but recent evidences suggest that it rather represents a pre-malignant lesion. Genetic studies on retinoma tissue have rarely been performed. *Materials and methods.* We investigated by Real-Time qPCR, copy number changes of candidate genes located within the 4 hot-spot regions (*MDM4* at 1q32.1, *MYCN* at 2p24.1, *E2F3* at 6p22 and *CDH11* at 16q22) in retina, retinoma and retinoblastoma tissues from two different patients. *Results.* Our results demonstrated that some copy number changes thought to belong to early (*MDM4* gain) or late stage (*MYCN* and *E2F3* gain) of retinoblastoma are already present in retinoma at the same (for *MDM4*) or at lower (for *MYCN* and *E2F3*) copy number variation respect to retinoblastoma. *CDH11* copy number is not altered in the two retinoma samples, but gain is present in one of the two retinoblastomas. *Discussion.* Our results suggest that *MDM4* gain may be involved in the early transition from normal retina to retinoma, while *MYCN* and *E2F3* progressive gain may represent driving factors of tumor progression. These results also confirm the pre-malignant nature of retinoma.

Retinoblastoma (RB) is the most common pediatric intraocular neoplasm initiated by inactivation of both alleles of the *RB1* tumor suppressor gene after two successive mutations (M1 and M2) [1]. Although the loss of *RB1* is a prerequisite for retinoblastoma initiation, further genomic changes (M3-Mn) may drive to malignancy by activating oncogenes and inactivating tumor suppressor genes [2].

Previous studies performed on RB tumor samples by conventional/microarray comparative genomic hybridization (CGH) or quantitative multiplex polymerase chain reaction (QM-PCR) reported recurrent genomic gains/amplifications at 1q32, 2p24, 6p22 and losses at 16q22 [3–9]. The characterization of these genomic imbalances have led to identify candidate oncogenes and tumor suppressors: *MDM4* and *KIF14* at 1q32, *MYCN* and *DDX1* at 2p24, *E2F3* and *DEK* at 6p22, *CDH11* and *RBL2* at 16q [10]. On the basis of the frequencies of these genomic changes, a multi-step model for RB progression has been proposed [9]. In this model, loss of both *RB1* alleles (M1-M2) is not sufficient to initiate tumor formation. The two most common frequent genomic changes are gains at 1q32 and 6p22 and they are therefore considered M3 and M4 molecular events necessary to drive malignant transformation. 16q22 loss and 2p24 gain/amplification are less frequent and they are reported as alternate M5 events accompanying tumor progression [9].

Retinoma is considered a benign variant of retinoblastoma based on clinical and histopathological evidence [11,12]. The distinctive clinical characteristics include a translucent, greyish retinal mass protruding into the vitreous, "cottage-cheese

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calcification" (75%) and retinal pigment epithelial migration and proliferation (60%) [11]. Histological features include foci of photoreceptor differentiation (fleurettes), abundant fibrillar eosinophilic stroma, absence of mitotic activity, foci of calcification [12]. Individuals carrying a constitutive *RB1* mutation can manifest either retinoma or retinoblastoma or, more rarely, a combination of both in different eyes or in the same eye as two separate foci [13]. The reported proportion of stable retinomas among carriers of a germline *RB1* mutation, ranges from 1.8 to 10% [11–15]. These lesions have been initially considered spontaneous tumor regression because they resemble retinoblastoma that have involuted after radiotherapy and chemotherapy [16–19]. However, Gallie and colleagues pointed out that these lesions did not show convincing clinical evidences of tumor regression and proposed that they rather represent a stage in the pathway to retinoblastoma development [15–20]. There are few reported cases of clinically diagnosed retinomas that have undergone malignant transformation [14–21].

Studies aimed at clarifying retinoma/retinoblastoma relationship at molecular level are rarely performed. This is principally due to the fact that retinoma tissue is very difficult to obtain since patients are not treated and retinoma/retinoblastoma mixed tissues are rarely reported in enucleated eyes. In three such cases, the expression of the pro-apoptotic neurotrophin receptor p75 ($p75^{NTR}$) has been evaluated [21]. The authors found that $p75^{NTR}$ protein was expressed in both normal retina and retinoma tissue, while it was absent in retinoblastoma, leading to the suggestion that $p75^{NTR}$ loss might accompany malignancy. $p75^{NTR}$ is a member of tumor necrosis family receptors which binds to neurotrophins, and plays a key role in maintaining the balance between survival and death in the developing retina [22–26]. Although $p75^{NTR}$ expression is lost, no gene copy number changes have been detected in retinoblastoma suggesting that it could be due to point mutations or epigenetic events [9].

In the present work, we used Real-Time qPCR to profile genomic imbalances at the four retinoblastoma hot-spot regions (1q32.1, 2p24.1, 6p22 and 16q22) in two human eye samples with areas of retinoma adjacent to retinoblastoma.

Materials and methods

Pathology and Immunohistochemistry

Eyes enucleated for retinoblastoma were obtained from the archives of the Department of Human Pathology and Oncology of the University of Siena (Siena, Italy). After surgery, enucleated eyes were immersion-fixed in buffered formalin for 48h. After fixation, sampling, paraffin embedding and cut were performed according to the usual pathological methods. In addition to clinically diagnosed retinoma (*Case 1*), 30 different eyes enucleated for retinoblastoma (at least two different paraffin blocks for each case, at least four different sections for each block) were analyzed by hematoxylin-eosin (H&E) staining and by $p75^{NTR}$ immunostaining to identify areas of retinoma. Five-micron-thick sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked with 3% H_2O_2 in a Tris-buffered saline solution for 15 min, and non-specific binding was blocked with normal goat serum for 5 min. Slides were immersed in Target Retrieval Solution (DAKO Cytomation, Glostrup, D) for antigen unmasking and heated 3 times for 5 minutes in a microwave oven at 750 W. Slides were incubated with the primary monoclonal antibody for $p75^{NTR}$ (Novocastra Laboratories, Newcastle-upon-Tyne, UK) diluted 1:50 for 1 hour and with the primary monoclonal antibody for Ki67 diluted 1:100 (Lab Vision, Suffolk, UK) for 1 hour. Secondary antibody (Lab Vision, Ultra Vision Large Volume Detection System, anti-polyvalent, HRP, Fremont, CA, USA) incubation was performed for 20 min. The binding reaction was detected using 3,3′-diaminobenzidine (DAB) (DAKO Corporation, Carpinteria, CA, USA). Slides were then lightly counterstained with Harry's hematoxylin. Both internal negative (sclera cells) and omitted antibody controls were used. Retina ganglion cells were used as internal positive control.

Laser capture microdissection and DNA extraction from tissue samples

Retinoblastoma, retinoma and retina tissues were identified on H&E-stained sections. Five-micron-thick sections were deparaffinized, rehydrated and stained with Mayer hematoxylin and yellow eosin, dehydrated with xylene. Slides were observed through an inverse microscope. Cells of different tissues were isolated by laser capture microdissection (Arcturus PixCell II, MWG-Biotech, Florence, Italy). Selected cells adhere to the film on the bottom of the cap (Arcturus, MWG-Biotech) and are immediately transferred into a standard microcentrifuge tube containing digestion buffer and proteinase K (20 μg/ml) (Qiagen, Hilden, Germany). DNA was extracted by the use of QIAmp® DNA Micro Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The Hoechest dye binding assay was used on a DyNA Quant™ 200 Fluorometer (GE Healthcare) to determine the appropriate DNA concentration.

Whole genome amplification

Whole genome amplification was performed using the GenomePlex® Complete Whole Genome Amplification (WGA) kit (Sigma-Aldrich, UK) according to the manufacturer's protocol. Briefly, after DNA extraction from microdissected tissue cells, 100 ng of template DNA were incubated at 95°C for 4 min in 1x fragmentation buffer, and the sample was cooled on ice. The sample was further incubated with the Library Preparation Buffer and Library Stabilization solution at 95°C for 2 min and the sample was cooled on ice. One microliter of Library Preparation Enzyme was added and the mix incubated at 16°C for 20 min, 24°C for 20 min, 37°C for 20 min, and 75°C for 5 min. The resulting sample was amplified using WGA polymerase, after initial denaturation at 95°C for 3 min and for 14 cycles at 94°C for 15 s and at 65°C for 5 min. Amplification products were purified using GenElute™ PCR Clean-up kit (Sigma-Aldrich) according to the instructions of the suppliers. The appropriate DNA concentration was determined by a DyNA Quant™ 200 Fluorometer (GE Healthcare).

Real-Time quantitative PCR

Real-Time quantitative polymerase chain reaction (PCR) was performed to detect genomic copy number imbalances. We used a pre-designed set of primers and probes specific for real time PCR experiments provided by the Assay-by-Design service (Applied Biosystems, Foster City, CA, http://www.products.appliedbiosystems.com). Primers and probes were designed for *MDM4*, *MYCN*, *E2F3* and *CDH11* (Table I). PCR was carried out using an ABI prism 7000 (Applied Biosystems) in a 96-well optical plate with a final reaction volume of 50 µl. PCR reactions were prepared from a single Mix consisting of: 2X TaqMan Universal PCR Master Mix, 20X gene Assay Mix, 20X RNAaseP Mix (internal reference) and HPLC pure water. A total of 100 ng of DNA was dispensed in each sample well for triplicate reactions. Thermal cycling conditions included a pre-run of 2 min at 50°C and 10 min at 95°C. Cycle conditions were 40 cycles at 95°C for 15 s and 60°C for 1 min according to the TaqMan Universal PCR Protocol (Applied Biosystems). Normal retina has been used for the purpose of calibration. The starting copy number of the unknown samples was determined using the comparative Ct method, as previously described [27].

Results

Clinical description

Case 1 is a 6 years and 3 months old male, third child of healthy and non-consanguineous parents. At the age of 1 year and 9 months strabismus was noted, but the parents referred that the first ophthalmoscopic evaluation was normal. At 2 years and 6 months, during periodical control, a retinoma was diagnosed in the right eye with the typical appearance of a whitish translucent mass without dilated vessels (Figure 1). The retinal lesion remained stable for 11 months; the child underwent monthly examination under general anesthesia for the risk of malignant transformation of retinoma in early childhood. At 3 years and 5 months, during an ophthalmoscopic follow-up, a whitish creamy mass was noted on the retinoma surface with dilated feeder vessels indicating the malignant transformation. The retinoblastoma was firstly treated with conservative therapy, i.e. 4 cycles of chemoreduction (Carboplatin and Etoposide) combined with focal therapy (thermotherapy and argon laser therapy). A relapse was noticed 3 months later and other 2 cycles of chemotherapy were performed followed by stereotactic radiotherapy. The tumor remained in complete remission until the age of 5 years, when a new relapse appeared at the same site, so right eye enucleation was performed. During ophthalmoscopic follow-up no neoplastic foci were identified in the left eye.

Case 2 is a 3 years and 7 months old female, second child of healthy and non-consanguineous

Table I. Primers and probes sequences for Real-Time qPCR.

Gene symbol	Band	Primers (5′-3′)	TaqMan probe (5′-3′)	Primers & Probe location
MDM4	1q32.1	CAGCAGGAGCAGCATATGGTATA CTACTGGGACGTCAGAGCTTC	GGAGATCTTTTGGGAGAA	Exon 3
MYCN	2p24.1	CGTCCGCTCAAGAGTGTCATC TTTCTGCGACGCTCACTGT	CTTGAGCCCCCGAAACT	Exon 2
E2F3	6p22	GTACCATAAGGGAAGACTTCTTTTAACTGT GAAGCAATGTCCATTAAGGGAAGTTAAAATTAA	AACTCGGAATACGAACTTT	Intron 2
CDH11	16q22	AGAGAGAGCCCAGTACACGTT ACCAATCGGCCACTGGAG	ATGGCTCAGGCGGTGG	Exon 2

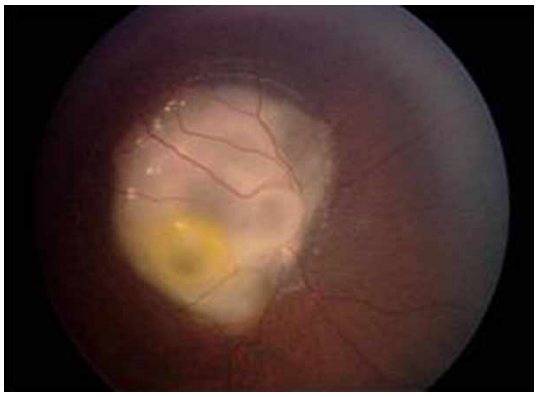

Figure 1. Ophthalmoscopic examination of Case 1. Ophthalmoscopic examination at 2 years and 6 months showing cystic retinoma on the right fundus oculi overwhelming the optic nerve head.

parents. At the age of 2 years and 5 months, her mother noted leukocoria in the right eye. At the ophthalmoscopic evaluation, a large tumor was present in the nasal area of the retina. Echografic examination showed the presence of a large mass with calcifications. Right eye enucleation was performed due to clinical diagnosis of eso- and endophytic retinoblastoma. The ophthalmoscopic follow-up did not identify any neoplastic focus in the remaining eye.

Histology

In *Case 1* a relatively small, eso- and endophytic retinoblastoma was present in the posterior area of the eye infiltrating focally both the choroid and the prelaminar optic nerve (pT2c) (Figure 2a). On the other side of the optic nerve, in part merged with RB cells, a cystic area was identified. Cells surrounding cystic spaces showed retinoma typical features, with plentiful cytoplasm, regular, smaller and light nuclei and signs of photoreceptor differentiation (fleurettes) (Figure 2c and g). In contrast, RB tissue contained cells densely packed with little cytoplasm, partly arranged in Homer Wright rosettes (Figure 2c and h).

In *Case 2* large, eso- and endophytic retinoblastoma was present in the posterior area of the eye (Figure 3a). The tumor growth did not infiltrate the choroid nor the optic nerve (pT2a). In the central part of the neoplasm, in part merged with RB cells, a partly cystic area was identified. Cells surrounding cystic spaces showed abundant cytoplasm, regular, smaller and light nuclei, typical features of retinoma (Figure 3c and g). Retinoma tissue also showed the presence of fleurettes, while RB cells were arranged in Homer Wright rosettes (Figure 3g and h).

$p75^{NTR}$ and Ki67 immunohistochemistry

In both cases, normal retina showed strong and consistent immunostaining positivity for $p75^{NTR}$ (Figure 2b and f; Figure 3b and f). The immunoreaction was located on the cellular surface of neuron bodies and their elongations. Differently, retinoblastoma cells showed absence of $p75^{NTR}$ staining (Figure 2b and d; Figure 3b and d). Immunopositivity was present in the vascular walls of veins, arteries and capillaries. In the areas occupied by RB cells, immunostaining was scanty and in the form of spikes of positivity, representing residues of neuron bodies and their elongations. Retinoma tissues adjacent to retinoblastoma were positive for $p75^{NTR}$ immunostaining (Figure 2b and d; Figure 3b and d). Furthermore, immunostaining for the proliferation marker Ki67, showed positivity in retinoblastomas and undetectable signal in retinomas of both cases (Figure 2e and 3e). Such immunohistochemical features supported the morphological diagnosis of retinoma.

Gain and loss of chromosome regions

To identify genomic differences between retinoma and retinoblastoma, we profiled genomic copy number changes at four gain-loss "hot-spot" regions by Real Time qPCR in retina, retinoma and retinoblastoma tissues from two different patients. To avoid tissue contamination, we isolated retina, retinoma and retinoblastoma cells by laser capture microdissection. Due to the low amount of DNA obtained from microdissected paraffin-embedded tissues, we used whole genome amplification to pre-amplify DNA samples. Probes and primers for qPCR were designed on the following candidate oncogenes/oncosuppressors because literature data on their function or connection to p53 pathway inactivation were intriguing regard to a possible involvement in retinoma/RB transition: *MDM4* (1q32.1), *MYCN* (2p24.1), *E2F3* (6p22) and *CDH11* (16q22) [10–28]. In both cases, retinoblastoma and retinoma showed the same level of *MDM4* gain respect to retina (for *Case 1*, ddCT ratio: 2.35 ± 0.14 in RB and 2.41 ± 0.13 in RN, indicating 5 copies; for *Case 2*, ddCT ratio: 1.62 ± 0.09 in RB and 1.83 ± 0.01 in RN, indicating 3 copies) (Figure 4a). In the two cases, progressive *MYCN* copy number gain was detected in retinoma (3 copies) and in retinoblastoma (5 copies in *Case 1* and 6 copies in *Case 2*) (Figure 4b). ddCT ratio values were 2.57 ± 0.05 (RB) and 1.55 ± 0.24 (RN) in *Case 1* and 3.05 ± 0.13 (RB) and 1.69 ± 0.14 (RN) in *Case 2*. For *E2F3*, in *Case 1* no copy number changes were detected (ddCT ratio: 1.32 ± 0.13 in RB and 1.34 ± 0.06 in RN), while

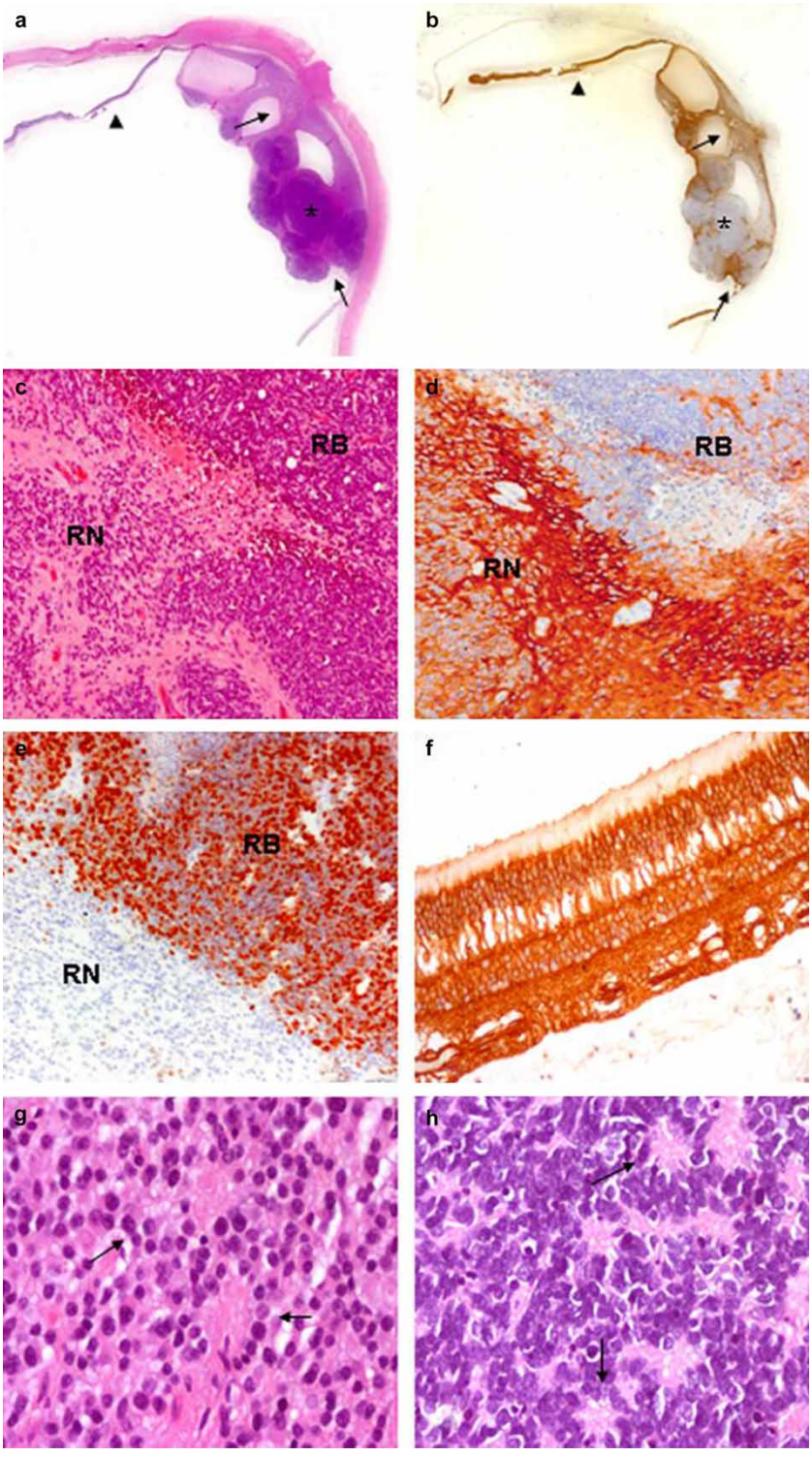

Figure 2. Histology and immunohistochemistry of Case 1. a. H&E staining and b. $p75^{NTR}$ immunohistochemistry of the whole eye. Normal retina (arrow-head), retinoblastoma (asterisk) and retinoma (arrows) are shown. c. H&E staining (original magnification 100x) of retinoma (RN) and retinoblastoma (RB). d. Positive immunostaining for $p75^{NTR}$ (original magnification 100x) in retinoma (RN), while retinoblastoma cells (RB) are not stained as well as inflammatory cells inside the tumor. Spikes of positivity inside the tumor represent residues of neuron bodies and their elongation and vessel walls. e. Widespread Ki-67 nuclear cell immunostaining in RB cells indicates a high proliferation index, while retinoma cells are negative (original magnification 100X). f. Higher magnification of normal retina stained with $p75^{NTR}$ (original magnification 200X). $p75^{NTR}$ positivity in the retina is diffuse and includes neuron bodies and elongate processes of all the retina layers; outer segments of cones and rods are not stained. g. Higher magnification of retinoma (H&E staining, original magnification 200X) showing fleurettes (see arrows). h. Higher magnification of retinoblastoma (H&E stain, original magnification 200X) displaying Homer Wright rosettes (see arrows).

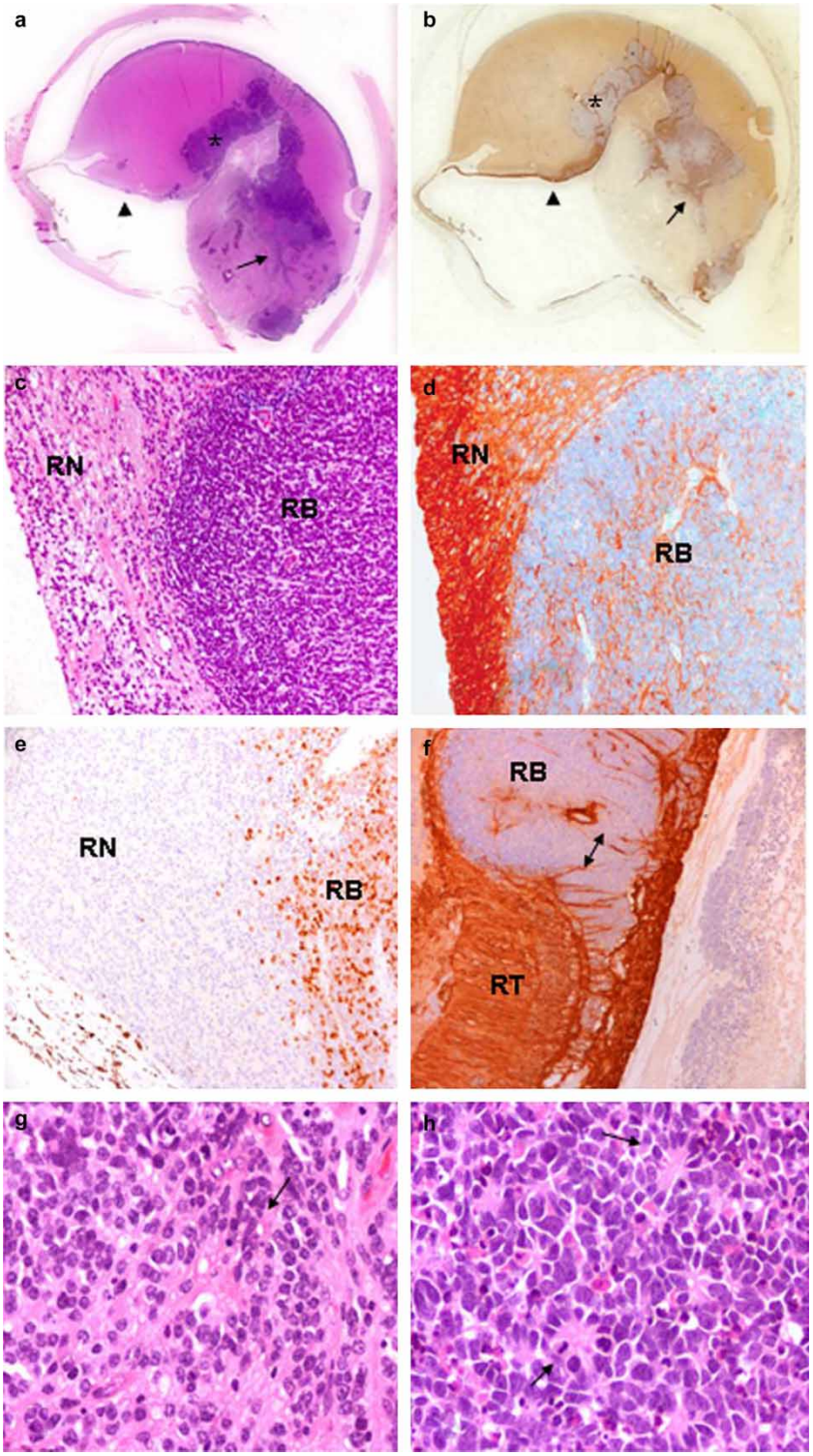

Figure 3. Histology and immunohistochemistry of Case 2. a. H&E staining and b. $p75^{NTR}$ immunohistochemistry of the whole eye. Normal retina (arrow-head), retinoblastoma (asterisk) and retinoma (arrow) are shown. c. H&E staining (original magnification 100x) of retinoma (RN) and retinoblastoma (RB). d. Positive immunostaining for $p75^{NTR}$ (original magnification 100x) in retinoma (RN), while retinoblastoma (RB) cells are not stained. Spikes of positivity inside the tumor represent residues of neuron bodies and their elongation and vessel walls. e. RB cells show a widespread Ki-67 nuclear cell immunostaining indicating a high proliferation index, while retinoma cells are not stained (original magnification 100X). f. Normal retina with an intraretinal RB focus stained with $p75^{NTR}$ (original magnification 200X). In the normal retina $p75^{NTR}$ positivity is diffuse, while in the neoplastic area positivity is restricted to vessel walls. g. Higher magnification of retinoma (H&E stain, original magnification 200X) showing fleurettes (see arrows). h. Higher magnification of retinoblastoma (H&E stain, original magnification 200X) with Homer Wright rosettes (see arrows).

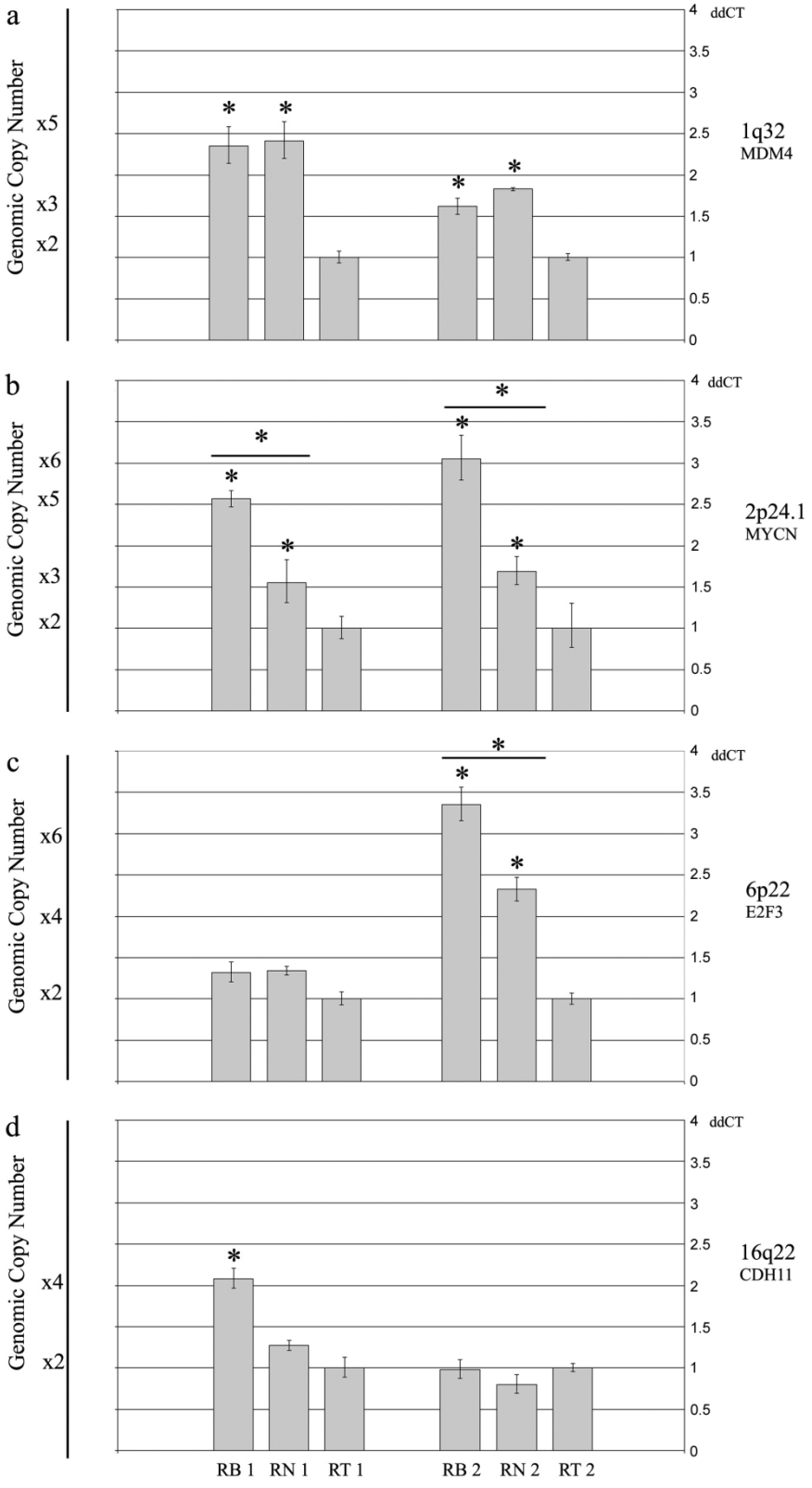

Figure 4. Genomic changes detected by Real-Time quantitative PCR. ddCT ratios and standard deviations obtained for retinoma (RN), retinoblastoma (RB) and retina (RT) of Case 1 (left) and Case 2 (right). For each graph, genomic copy number is indicated on the left (gain: 3-7 copies; amplification $\geq$7 copies). The asterisk shows when the difference is statistically significant (t-test, $p \geq 0.05$). a. Both cases show MDM4 gain in retinoma and retinoblastoma at the same number of copies. b. Progressive gain of MYCN from retinoma to retinoblastoma respect to normal retina in both cases. c. Case 1 does not show copy number changes, while Case 2 displays gain of E2F3 in retinoma and amplification in retinoblastoma compared to retina. d. No genomic changes were detected in the two retinoma tissues. One of the two retinoblastoma samples (Case 1) presents gain of CDH11.

in *Case 2*, we identified copy number gain in retinoma (5 copies) and amplification in retinoblastoma (7 copies) (ddCT ratio: 3.36 ± 0.09 in RB and 2.32 ± 0.09 in RN) (Figure 4c). For *CDH11*, copy number gain (4 copies) was detected only in retinoblastoma of *Case 1* (Figure 4d). ddCT ratio values were: 2.08 ± 0.08 (RB) and 1.27 ± 0.07 (RN) in *Case 1* and 0.98 ± 0.16 (RB) and 0.80 ± 0.20 (RN) in *Case 2*.

Discussion

Retinoma is a retinal lesion highly associated with retinoblastoma but lacking malignant characteristics [11]. These lesions have been initially called "spontaneous regression" of retinoblastoma. However clinical evidences do not support this hypothesis and suggest that retinoma rather represents a step towards retinoblastoma development [11–21]. Very recently, during the revision process of the present manuscript, Dimaras and colleagues published interesting data clarifying this issue [29]. They demonstrated that retinomas display inactivation of both *RB1* alleles, absence of proliferative markers (Ki67 staining), low level of genomic instability and high expression of the senescence-associated proteins (p16INK4a and p130). Diversely, adjacent retinoblastomas show reduced expression of p16INK4a and p130 and increased genomic changes, indicating progression from retinoma.

Here, we investigated copy number changes at four "hot spot" regions for tumor development in two human eye samples with areas of retinoma adjacent to retinoblastoma. In the first case, there was clinical evidence of the presence of a cystic retinoma, already decribed by L. Zografos [30], that later progressed to retinoblastoma. The second case of retinoma was identified by retrospective histopathological review of 30 eye samples enucleated for retinoblastoma (1/30, 3%). This percentage of retinomas in enucleated eyes is lower respect to the percentage reported by Dimaras (20/128, 15.6%), but this discrepancy could be due to the different number of reviewed eye samples [29]. In both our cases, immunostaining for the pro-apoptotic neurotrophin receptor $p75^{NTR}$ and the proliferation marker Ki67 clearly distinguished retinoma areas from retinoblastoma, in accordance with previous data [21–29].

Genomic copy number changes represent fundamental steps in the development of retinoblastoma [9]. To detect such changes at the four "hot spot" loci, we employed Real Time quantitative PCR. This technique, differently from FISH, does not allow an analysis at single cell level but determines gene copy number on a whole tissue basis. Quantitative PCR allowed us to clearly detect the relative proportion of gene copy number changes between the three tissues: retina, retinoma and retinoblastoma. Our results showed progressive gain of *MYCN* (2p24) and *E2F3* (6p22) copy number from retinoma to retinoblastoma, confirming that retinoma represents a transition step to tumor development (Figure 4b and c).

Since both *Case 1* and *2* display progressive *MYCN* gain from retinoma to retinoblastoma, these results emphasize the role of *MYCN* in malignant progression. In a previous study, Bowles et al. showed amplification of *MYCN* in 3% of 87 primary retinoblastoma and low-level gains in further 13% [9]. They also found that *MYCN* gains/amplifications are more common in cell lines that in primary retinoblastoma suggesting that this copy number changes confer a selective advantage for cell growth [9]. This characteristic could represent a driving force in retinoma/retinoblastoma transition.

Furthermore, in one of the two cases, we found *E2F3* gain in retinoma and amplification in retinoblastoma. Gain of *E2F3* gene has been found in 70% of retinoblastoma primary tumors [9]. E2F3, together with E2F1 and E2F2, belongs to a subclass of E2F factors that act as transcriptional activators through the RB1-dependent G1/S phase transition [31]. Moreover E2F3 represses p14/ARF, an important tumor repressor in the p53 pathway [32,33]. Consequently *E2F3* amplification may contribute to retinoblastoma growth from retinoma by eliminating p53 apoptotic process.

MDM4 showed gain in both retinoma and retinoblastoma of both cases, at the same number of copies. A recent study revealed an increased *MDM4* copy number in 65% of human retinoblastoma [28]. Our results not only confirm the importance of *MDM4* gain in retinoblastoma development, but interestingly suggest that it may be involved in the early transition to retinoma (Figure 4a). *MDM4* is a negative regulator of p53 transcription and stabilizes the E3 ubiquitin ligase *MDM2* which tags p53 for degradation [34]. Increased p53 degradation could be one mechanism that contributes to inactivate the apoptotic process in retinoblastoma. Functional studies are necessary to confirm the involvement of *MDM4* in the early phases of malignant progression and whether it represents a "driving" gene or a "passenger" gene whose amplification is a consequence of the amplification of another strong candidate located at 1q, *KIF14*. In fact, Dimaras et al. recently reported that *KIF14* shows gains even more frequently and at higher levels respect to *MDM4* [29].

Since loss of *CDH11* has been reported in 45% of primary retinoblastoma [35], this gene has been

proposed as candidate oncosuppressor. However, we did not find *CDH11* loss. On the contrary, we identified copy number gain in one case of retinoblastoma (Figure 4d). *CDH11* overexpression has been reported in other tumor types like prostate cancer, rhabdomyosarcoma and invasive breast cancer [36–38], suggesting that also the overexpression may contribute to cancerogenesis.

The results reported above significantly change the multi-step model of retinoblastoma progression proposed by Bowles on the basis of the frequency of genomic changes found in retinoblastoma tissues [9]. According to this model, after two successive *RB1* mutations (M1 and M2), the following further genomic changes accompany malignancy: 1q32.1 gain (M3), 6p22 gain (M4), 16q22 loss (M5a) and 2p24.1 gain (M5b) (Figure 5a). In accordance with very rencent data, we found that 1q32.1 gains, thought to belong to early stages of retinoblastoma, are already present in retinoma [29]. More interestingly, even the 6p22 and 2p24 gains thought to belong to later retinoblastoma stages are already present in retinoma, although at lower number of copies respect to retinoblastoma (Figure 5b). In accordance with the previous model, no genomic changes were detected at 16q22 region in the two retinomas (Figure 5). In one case, we found 16q22 gain in retinoblastoma tissue, confirming that copy number variations in this region are involved in later stages of tumor progression (Figure 4d) [9].

It is important to note that, while *Case 2* was enucleated at diagnosis, *Case 1* was treated by chemotherapy and radiotherapy before enucleation and this may have at least in part influenced the pattern of genetic alterations. Further studies are therefore necessary to confirm these data. Unfortunately they are limited by sample availability. Only a collaborative effort by different centers will allow to perform these studies on a larger number of samples and to definitively characterize molecular events underlying retinoma and retinoblastoma. The accomplishment of this goal is important not only because it will clarify the pathogenic mechanisms of tumor development, but also because it will lead to the discovery of targets that will represent the basis of therapies aimed to control the malignant progression of retinoma.

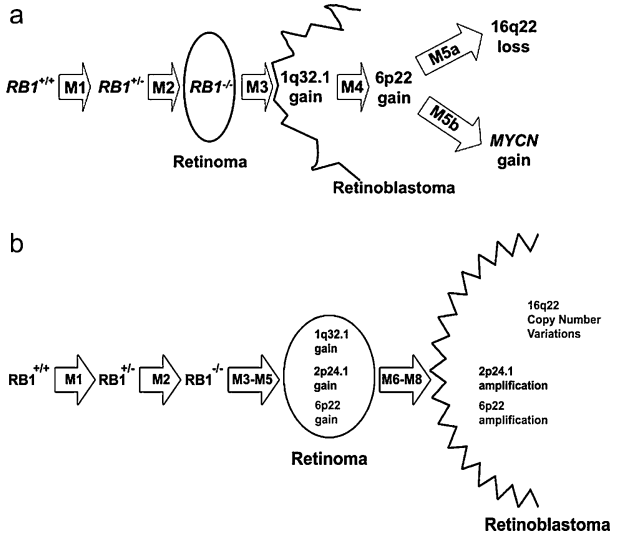

Figure 5 a. Tumor multi-step model from Bowles et al., 2007. b. Retinoblastoma tumor progression model based on our results. In light grey, copy number changes found in only one of the two samples.

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3.5 Array Comparative Genomic Hybridization in retinoma and retinoblastoma tissues.

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Array Comparative Genomic Hybridization in retinoma and retinoblastoma tissues.

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ABSTRACT

In retinoblastoma, two *RB1* mutations are necessary for tumor development. Recurrent genomic rearrangements may represent subsequent events required for retinoblastoma progression. Array-CGH was performed in 18 eye samples, 10 from bilateral and 8 from unilateral retinoblastoma patients. Two unilateral cases also showed areas of retinoma. The most frequent imbalance in retinoblastomas was 6p gain (40%), followed by gains at 1q12-q25.3, 2p24.3-p24.2, 9q22.2, 9q33.1 and losses at 11q24.3, 13q13.2-q22.3, 16q12.1-q21. Bilateral cases showed a lower number of imbalances respect to unilateral cases (p=0.002). Unilateral cases could be divided into a low (≤4) and a high level (≥7) chromosomal instability group. The first group presents younger age at diagnosis (mean, 511 days) respect to the second one (mean, 1606 days). In one retinoma case ophthalmoscopically diagnosed as a benign lesion no rearrangements were detected, while adjacent retinoblastoma displays seven aberrations. The other retinoma case identified by retrospective histopathological examination shares three rearrangements with adjacent retinoblastoma. Two other gene-free rearrangements are retinoma specific. One rearrangement, dup5p, is retinoblastoma specific and includes the *SKP2* gene. Genomic profile therefore indicates that the first retinoma is a pretumoral lesion, while the other represents a subclone of cells bearing "benign" rearrangements overwhelmed by another subclone presenting aberrations with higher "oncogenic" potential. In summary, this study shows that bilateral and unilateral retinoblastoma have different chromosomal instability that correlates with the age of tumour onset in unilateral cases. This is the first report of genomic profiling in retinoma tissue shading light on the different nature of lesions named "retinoma".

INTRODUCTION

Retinoblastoma (RB, OMIM#180200) is the most common primary intraocular malignancy in children, initiated by the inactivation of both alleles of the *RB1* tumor suppressor gene.[1,2] Approximately 40% of RB patients carry a predisposing germ-line mutation transmitted as an autosomal dominant trait. In these patients, inactivation of the second *RB1* allele occurs in the retinal cells and generally results in multiple and often bilateral tumors. In the remaining 60% of children, both mutational events occur in the same retinal cell leading to unilateral sporadic RB.[3]

Retinoma (RN), a benign retinal lesion, is considered the precursor of retinoblastoma.[4,5] Differently from retinoblastoma that is typically opaque white, retinoma appears as a translucent grey retinal mass, frequently associated with calcification and retinal pigment epithelial hyperplasia.[6] Histopathology of retinoma includes foci of photoreceptor differentiation (fleurettes), momomorphic round nuclei, abundant fibrillar eosinophilic stroma and absence of mitotic activity.[7] Recently, it has been demonstrated that the two mutational events inactivating *RB1* gene are already present in retinomas.[4] Using quantitative PCR and FISH on specific candidate genes, it has been also shown that retinomas display low level copy number changes involving higher levels of amplification in adjacent retinoblastomas.[4,5] The study by Dimaras et al. in retinoma importantly clarified that the two hits in *RB1* (M1-M2) do not inevitably cause a malignant phenotype but only genomic instability.[4] At some point this instability can lead to further genomic rearrangements (M3-Mn) that result in tumor progression.[4,8]

Cytogenetic and conventional/microarray comparative genomic hybridization (CGH) studies have detected recurrent genomic alterations in RB: gain of 1q, 2p, 6p and 13q and loss of 16q.[9] These data strongly suggest that these changes may represent M3-Mn events driving tumor progression in RB. In this scenario, retinoma represents a very interesting tissue to study the timing of genomic instability in RB development. However, molecular studies in this lesion are limited by sample availability since patients with only retinoma are not treated and the coexistence of retinoma and retinoblastoma in enucleated eyes is not frequently observed.[4,5]

Array-based CGH technology, designed for detecting segmental genomic alterations at high resolution level, have enabled to profile human cancer genomes, defining regions/genes involved in cancer development and progression.[10-12] To date, genomic rearrangements in RB tissues have been principally investigated by cytogenetic and conventional CGH and only one array-CGH study has been published.[13-18] To our knowledge, genome wide studies in retinoma tissues have never been performed.

Here, we used high resolution array-CGH technique to analyse genomic rearrangements in 18 retinoblastoma eye samples, 10 from bilateral and 8 from unilateral patients. In two unilateral cases, we could also investigate genomic imbalances in two areas of retinoma adjacent to retinoblastoma.[5] In one case (#16), clinically diagnosed retinoma has been observed to progress to retinoblastoma, while in the other case (#15) retinoma was identified by retrospective histopathological examination.[5]

MATERIALS AND METHODS

Tissue samples collection

We collected 18 formalin-fixed paraffin-embedded (FFPE) eye samples from enucleated retinoblastoma patients archived in the Department of Human Pathology and Oncology of the University of Siena. After surgery, enucleated eyes were immersion-fixed in buffered formalin for 48h. After fixation, sampling, paraffin embedding and cut were performed according to the usual pathological methods. The group of samples included 10 bilateral (1 familial and 9 sporadic) and 8 sporadic unilateral cases. For each patient, we have the corresponding DNA sample extracted from blood stored in the Italian Retinoblastoma Biobank (http://www.biobank.unisi.it). Samples #1-18 of the present study correspond to RB biobank samples: 15, 58, 143, 185, 190, 225, 134, 133, 234, 263, 79, 268, 242, 296, 297, 206, 253 and 279. A germ-line mutation in *RB1* have been identified in 8/10 patients with bilateral tumors. No mutations have been detected in the 8 unilateral cases. Mutational screening has been performed by a combination of both DHPLC and MLPA analysis. Two unilateral cases presented areas of retinoma adjacent to retinoblastoma.[5]

Laser capture microdissection and DNA extraction from tissue samples

Normal retina, retinoma and retinoblastoma tissues were identified on H&E-stained sections. Five-micron-thick sections were deparaffinized, rehydrated and stained with Mayer haematoxylin and yellow eosin, dehydrated with xylene. Slides were observed through an inverse microscope. Cells of the three different tissues were isolated by laser capture microdissection (Arcturus PixCell II, MWG-Biotech, Florence, Italy). Selected cells adhere to the film on the bottom of the cap (Arcturus, MWG-Biotech) and are immediately transferred into a standard microcentrifuge tube containing digestion buffer and proteinase K (20µg/ml) (Qiagen, Hilden, Germany). DNA was extracted by the use of QIAmp® DNA Micro Kit according to the

manufacturer's protocol (Qiagen, Hilden, Germany). The Hoechest dye binding assay was used on a DyNA Quant™ 200 Fluorometer (GE Healthcare) to determine the appropriate DNA concentration.

Whole genome amplification

Whole genome amplification was performed using the GenomePlex® Complete Whole Genome Amplification (WGA) kit (Sigma-Aldrich, UK) according to the manufacturer's protocol. Briefly, after DNA extraction from microdissected tissue cells, 100 ng of template DNA were incubated at 95°C for 4 min in 1x fragmentation buffer, and the sample was cooled on ice. The sample was further incubated with the Library Preparation Buffer and Library Stabilization solution at 95°C for 2 min and then cooled on ice. One microliter of Library Preparation Enzyme was added and the mix incubated at 16°C for 20 min, 24°C for 20 min, 37°C for 20 min, and 75°C for 5 min. The resulting sample was amplified using WGA polymerase, after initial denaturation at 95°C for 3 min and for 14 cycles at 94°C for 15 sec and at 65°C for 5 min. Amplification products were purified using GenElute™ PCR Clean-up kit (Sigma-Aldrich) according to the instructions of the suppliers. The appropriate DNA concentration was determined by a DyNA Quant™ 200 Fluorometer (GE Healthcare). Amplified DNA fragments from normal retina, retinoma and retinoblastoma samples varied in length from 200 bp to 500 bp.

Array-comparative genomic hybridisation

Array CGH analysis was performed using commercially available oligonucleotide microarrays containing about 99,000 60-mer probes with an estimated average resolution of approximately 25 kb (Human Genome CGH Microarray 105A Kit, Agilent Technologies). DNA labelling was performed using the Agilent Genomic DNA Labeling Kit Plus (Agilent Technologies according to the Agilent protocol (Oligonucleotide Array-Based CGH for Genomic DNA Analysis 2.0v). Genomic DNA (3.5 µg) was mixed with 5 µl of 2.5X Random primers solution (Agilent Technologies) and Nuclease-free water to a total volume of 31 µl. The mix was denaturated at 95° C for 3 min and then incubated in ice/water for 5 min. Each sample was added with 10 µl 5X Buffer, 5 µl of 10X dNTP nucleotide mix, 3 µl of Cy5-dNTP (retinoblastoma/retinoma sample) or 3 µl of Cy3-dNTP (normal retina sample) and with 1 µl of Klenow Fragment (Agilent Technologies). The samples were incubated at 37° C for 3 hrs. Labeled samples were subsequently purified using CyScribe GFX Purification kit (Amersham Biosciences) according to the manufacturer's protocol. Test and control DNAs were pooled and mixed with 25 µg of Human Cot I DNA (Invitrogen), 26 µl of Blocking buffer (Agilent Technologies) and 130 µl of Hybridization buffer (Agilent Technologies). Before hybridization to the array, the mix was denatured at 95° C for 5 min then

pre-associated at 37°C for 1 hrs. Probes was applied to the slide using an Agilent microarray hybridization station. Hybridization was carried out for 40 hrs at 65°C in a rotating oven (20 rpm). The array was disassembled and washed according to the manufacturer protocol with wash buffers supplied with the Agilent 105A kit. The slides was dried and scanned using an Agilent G2565BA DNA microarray scanner.

Image and data analysis

Image analysis was performed using the CGH Analytics software v. 3.4.40 with default settings. The software automatically determines the fluorescence intensities of the spots for both fluorochromes performing background subtraction and data normalization, and compiles the data into a spreadsheet that links the fluorescent signal of every oligo on the array to the oligo name, its position on the array and its position in the genome. The linear order of the oligos is reconstituted in the ratio plots consistent with an ideogram. The ratio plot is arbitrarily assigned such that gains and losses in DNA copy number at a particular locus are observed as a deviation of the ratio plot from a modal value of 1.0. DNA sequence information refers to the public UCSC database (Human Genome Browser, http://genome.ucsc.edu, May 2004 Assembly).

Statistical analysis

We used the MannWhitney U test to compare means of continuous variables between the two groups (p values ≤ 0,05 were considered significant).

RESULTS

Array CGH analysis in retinoblastoma samples

Using array-CGH, we investigated genomic rearrangements in 18 eye tissues, 10 from bilateral and 8 from unilateral retinoblastoma patients. Array-CGH analysis identified genomic rearrangements in 12/18 tumor samples (~67%).

In total, we found 64 genomic aberrations, mostly gains (47 gains vs 17 losses) (Tab. 1). The number of rearrangements was significantly different (Mann-Whitney U test, p=0.002) between the two groups of patients: bilateral cases showed a lower number of imbalances (mean, 1; range, 0-4) respect to unilateral cases (mean, 7; range, 2-24) (Tab. 1).

Recurrent imbalances involved chromosomes 1, 2, 6, 9, 11, 13 and 16 (Fig. 1) (Tab. 2). In 7/18 (40%) samples the entire p arm of chromosome 6 was duplicated (Tab. 2). The other cases bearing overlapping rearrangements allowed to define minimal common regions of gain (MRG) or loss (MRL): dup(1)(q12;q25.3) (4/18 samples; 22%), dup(2)(p24.3;p24.2) (4/18 samples; 22%), dup(9)(q22.2) (3/18 samples; 17%), dup(9)(q33.1) (2/18 samples; 11%), del(11)(q24.3) (2/18 samples; 11%), del(13)(q13.2-q22.3) (2/18 samples; 11%), and del(16)(q12.1-q21) (2/18 samples; 11%) (Fig. 1) (Tab. 2).

These regions have been studied for gene content to identify candidates involved in RB progression. We firstly searched for known oncogenes/tumor suppressors (Tab. 2). Based on annotated gene function, we selected additional candidates (Tab. 2). Priority was given to genes participating in pRB pathway and to genes playing a role in the mechanisms of cell proliferation, differentiation, apoptosis or senescence (Tab. 2).

Correlating molecular and clinical data, we found that in unilateral cases the number of rearrangements is associated with age at diagnosis (Tab. 3). The group with low level chromosomal instability (≤4 chromosomal aberrations) presents a younger age at diagnosis (mean, 511 days; range, 90-958 days), while the group with high level chromosomal instability (≥7 chromosomal aberrations) an older age at diagnosis (mean, 1606 days; range, 1326-1828 days) (Tab. 3).

Array CGH analysis in retinoma samples

Two cases affected by unilateral retinoblastoma (#15 and #16), showed areas of retinoma adjacent to the tumor. Retinoma of case #16 was clinically diagnosed as a benign lesion that after eleven months underwent malignant transformation, while retinoma of case #15 was identified by retrospective histopathological examination. Detailed clinical and histopatological data of the two lesions have been already described in Sampieri et al.[5]

Array-CGH analysis did not detect any genomic rearrangement in retinoma of patient #16. Differently, five genomic rearrangements were identified in retinoma of case #15 (Tab. 4). Among them, three are in common with adjacent retinoblastoma (dup5q13.2, dup6p, dup8p23.1), while the remaining two (dup1q32.2 and dup13q31.2) were exclusively detected in retinoma (Tab. 4). As concern the common rearrangement on 6p, array-CGH log ratio values indicated that the level of gain was progressively increased from retinoma (log ratio ~0,5) to retinoblastoma (log ratio ~1,0) (Tab.4). One rearrangement, dup5p, is present only in retinoblastoma (Tab. 4).

These regions have been studied for gene content in order to identify candidates involved in retinoma/retinoblastoma transition. We searched for known oncogenes/tumor suppressors, for genes related to pRB pathway and for genes involved in proliferation, differentiation, apoptosis or senescence (Tab. 4).

Discussion

The loss of *RB1* function, by mean of two mutational events (M1/M2), is considered the first rate-limiting step in retinoblastoma development.[1,4] Several studies have suggested that genomic imbalances (M3/Mn) involving specific oncogenes/tumor suppressors are required for malignant transformation of RB.[9] In order to characterize such genomic changes, we investigated by high resolution array-CGH a series of 18 tumor (10 bilateral and 8 unilateral) and 2 retinoma samples from enucleated RB patients.[5] To our knowledge this is the first genome wide study in retinoma tissue.

In RB samples, we detected a total of 64 rearrangements, 47 gains and 17 losses (Tab. 1). Bilateral cases showed a lower number of imbalances (mean, 1) compared to unilateral cases (mean, 7), with statistic significance (p=0,002) (Tab. 1). These results suggest that, beyond the inactivation of both *RB1* alleles, different molecular mechanisms may be involved in tumor progression of hereditary RB. Alternatively to chromosomal instability (CIN), other genetic alterations can drive cancer progression, including subtle DNA sequence change such as microsatellite instability (MIN), chromosomal translocations, single gene amplification or deletions. All these changes have been described in retinoblastoma but systematic correlations with clinical data have never been performed.[5,19-22]

In accordance with previous data, we found recurrent imbalances on chromosomes 1, 2, 6, 13 and 16 (Tab. 2).[9] Three previously undescribed recurrent rearrangements were identified, two on chromosome 9 and one on chromosome 11 (Tab. 2).

Gains of 6p showed the highest frequency (40%), confirming that it represents the most common changes observed in retinoblastoma.[9] The rearrangement contains 461 genes including 3 known oncogenes: *IRF4*, *DEK*, and *PIM1* (Tab. 2). We further selected two members of the pRB pathway that have an essential role for cell cycle G1/S transition: the pRB-regulated transcription factor E2F3 and cyclin CCND3, involved in pRB phosphorylation (Tab. 2).[23,24] Previous studies reporting more focused gains at 6p22 lead to deeply investigate genes within this region.[14] By QM-PCR and microarray expression analysis on RB tissues, it has been demonstrated that *DEK* and *E2F3* are the most common gained genes and that they show overexpression.[25,26] Furthermore, Dek and E2f3 are overexpressed in Tag-RB murine tumors. [9] These results indicate that both *DEK* and *E2F3* represent strong candidates and that a combination of genes on 6p, instead of a single one, probably contribute to RB progression.

The MRG on chromosome 1 (dup1q12-q25.3) contains 497 genes (Tab. 2). We selected *MUC1* since its overexpression, as found in human carcinomas and certain hematologic malignancies, induces transformation and resistance to apoptosis (Tab. 2).[27,28] Other interesting

candidates are *MCL1*, encoding a potent multidomain antiapoptotic protein of the BCL2 family, and *SHC1,* a key intracellular signalling molecule that participates in the transforming activity of oncogenic tyrosine kinases (Tab. 2).[29,30] By array-CGH, Zielinski et al. also found recurrent 1q imbalances narrowing one MRG at 1q22 and indicated *SHC1* as candidate.[18] Two previously identified strong candidates on 1q, *KIF14* and *MDM4*, are not included within the identified MRG. By gene specific quantitative PCR approach, both genes have been found gained in RB.[5] In addition, their overexpression in well documented in RB tissues.[31,32] *KIF14* and *MDM4* may therefore play an important role in RB progression, regardless of 1q status.

The MRG defined on chromosome 2 contains only four genes, including the known oncogene *MYCN* and *DDX1,* a gene that encodes a DEAD box protein probably involved in pre-mRNA 3'-end processing and that has been shown to possess oncogenic properties (Tab. 2).[33] Importantly, *MYCN* and *DDX1* have been found coamplified and overexpressed in retinoblastoma and neuroblastoma cell lines and tumours.[34]

The two small MRGs detected on chromosome 9, dup(9)(q22.2) and dup(9)(q33.1), contain two genes and one gene, respectively (Tab. 2). None are reported as oncogenes/tumor suppressors and no obvious candidates have emerged.

Only two genes lie in the MRL defined on chromosome 11 (Tab. 2). The transcription factor ETS1 is involved in control of cellular proliferation, cell senescence and death, and tumorigenesis.[35] Its expression is correlated with more malignant carcinomas and is a negative prognostic indicator.[36] The MRL on chromosome 13 contains 123 genes (Tab. 2). Beside *RB1*, this region bears a newly characterized tumour suppressor gene, *ARLTS1*.[37] It encodes for a pro-apoptotic protein of the Ras superfamily involved in the pathogenesis of various types of tumours: two SNPs have been found to influence familial cancer risk for B-CLL and BRCA1/BRCA2 negative breast cancers, whereas DNA hypermethylation and genomic deletions have been identified as a mechanism of *ARLTS1* down-regulation in CLL, lung cancers and ovarian tumours.[38-41]

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The MRL on chromosome 16 contains the *RBL2* gene, encoding RB family member p130 (Tab. 2). *RBL2* expression is reduced in RB tissues and is one of the genes that can be ablated along with Rb1 to cause retinal tumour formation in mice.[42-44] This region also includes the familial cylindromatosis tumour suppressor gene *CYLD*, whose loss inhibits the apoptotic pathway by activating NF-kappaB (Tab. 2).[45]

Correlating array-CGH results with age at diagnosis we observed that, among unilateral cases, a higher number of chromosomal aberrations is associated with an older age (Tab. 3). These results are in accordance with a previous CGH study reporting that unilateral retinoblastomas from

children with an older age showed significantly more genetic abnormalities respect to retinoblastomas from children with a young age.[15] This could be due to the fact that a too high level of genomic instability may lead to suppression of tumor growth, resulting in a delayed disease onset.[46] Reversely, it is also possible that an intrinsic slow growing rate of the tumor allows the accumulation of a higher number of chromosomal aberrations.

Sample #18, with the oldest age at diagnosis, displays the highest number of genomic aberrations (24), all of small size (0,11-1,98 Mb) (Tab. 1) (Tab. 3). Interestingly, dup18q21.1 contains *SMAD2* (Sma- and Mad-related protein 2), encoding a protein with sequence similarity to Mad2 gene product in Drosophila, a key component of the spindle checkpoint.[47,48] It has been demonstrated that hyperactivation of Mad2 by E2F1 leads to chromosomal instability and aneuploidy in cells in which the Rb pathway is disrupted.[49]

Array-CGH analysis in retinoma tissues revealed striking different results in the two cases. Retinoma from sample #16 did not present any rearrangements, while 7 alterations were detected in the adjacent retinoblastoma (Tab. 1). Interestingly, one of the rearrangements (del16q12.1-q21) contains the *RBL2* gene. In a recent paper, Dimaras et al. found p130 highly expressed in retinoma and not in retinoblastoma, suggesting that it represents a key factor differentiating the two lesions.[4] The authors also hypothesized that retinoblastoma can emerge from stable retinoma by failure of senescence and that p130 may represent the effector of such mechanism.[4,50]

Diversely, in case #15, retinoma showed five genomic rearrangements respect to normal retina, three in common with retinoblastoma (Tab. 4). Concerning the common imbalance on 6p, the level of gain was higher in retinoblastoma respect to retinoma, reinforcing the importance of candidate genes such as *DEK* and *E2F3* in malignant progression. The imbalance found in both tissues on 5q includes *BIRC1*, an interesting candidate gene for early retina/retinoma transition since it encodes for a protein known to act as inhibitor of apoptosis directly suppressing caspases (Tab. 4).[51] Two rearrangements were found exclusively in retinoma and they do not contain any known gene (Tab. 4). Chromosomal gain on 5p, present only in retinoblastoma, includes *SKP2* (p45), an oncogenic protein found overexpressed in cancer (Tab. 4).[52] It displays S-phase-promoting function and is implicated in the ubiquitin-mediated proteolysis of the Cdk inhibitor p27.[53-55] It has been demonstrated that cell cycle arrest through the inhibition of cdk2 activity by p27 is critical for pRB-induced senescence.[56] By-pass of senescence could be therefore involved in malignant transformation of retinoblastoma through the pRB–SKP2-p27 pathway.

The different genomic profiles obtained in the two lesions named “retinomas”, sharing the same hystopathological appearance, indicate that they indeed represent different entities. Case #16, that was clinically diagnosed as retinoma and that after eleven months has been observed to

progress to retinoblastoma, is a pretumoral lesion that has not yet acquired chromosomal aberrations. In a previous work, by Real Time qPCR we showed that retinoma displays gene specific low level gains, with higher levels in adjacent retinoblastoma.[5] These results suggest that increased genomic instability, including chromosomal aberrations and progressive gene amplification, accompanies retinoma/retinoblastoma transition.

Differently, the other case (#15), without a clinically detectable stage of retinoma and identified by retrospective histopathological examination, represents a further step in retinoblastoma progression. It appears as a subclone of cells bearing "benign" rearrangements that has been overwhelmed by another subclone presenting aberrations with selective growth advantage, leading to outgrowth of the tumour. These data underline that only specific sets of chromosomal rearrangements can lead a tumor cell precursor to overcome the selection barrier and generate a fully malignant phenotype.

In conclusion, array-CGH analysis performed in 18 RB revealed a different chromosomal instability level between bilateral and unilateral cases. Among the unilateral group, a bimodal distribution of chromosomal changes was observed and it correlates with age of diagnosis. Already characterized recurrent genomic aberrations have been confirmed and three new have been detected, indicating candidate genes for RB progression. Finally, this study represents the first report of genomic profiling in retinoma tissues and provides the basis for investigation of the role of chromosomal instability in retinoma/retinoblastoma transition.

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Legends to figures

Fig 1. Overview of rearranged chromosomal regions in 18 retinoblastomas as detected by array-CGH. Green lines on the left of each chromosome represent losses and red lines on the right represent gains.

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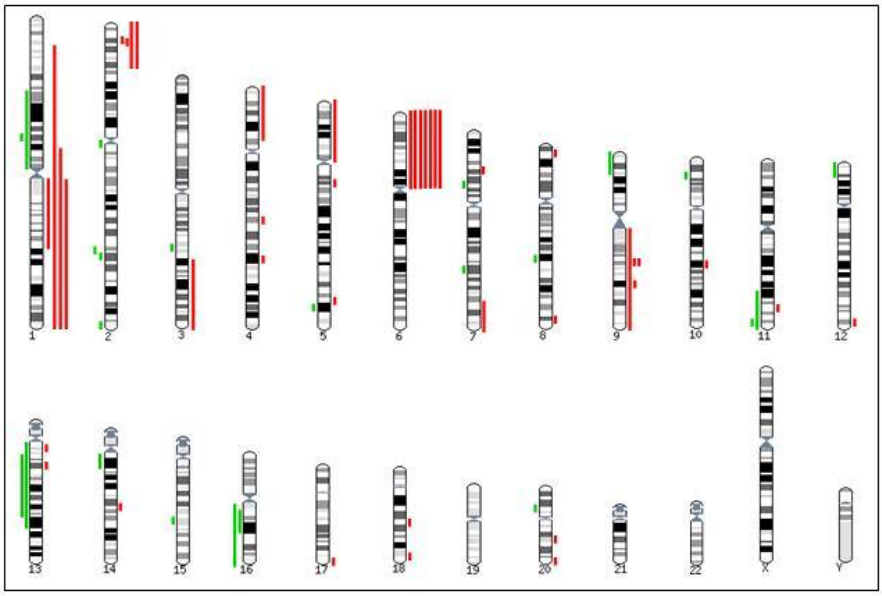

254x190mm (72 x 72 DPI)

Case	Phenotype	N° of aberrations	aCGH gains	aCGH losses
#1	B	0	/	/
#2	B	0	/	/
#3	B	0	/	/
#4	B	0	/	/
#5	B	0	/	/
#6	B	0	/	/
#7	B	1	2 p24.3-p24.2{2,15Mb}	
#8	B	2	6p25.3-p11.1{58,7Mb}	5q34{13,83Mb}
#9	B	2	2p24.3{3,75Mb} 6p25.3-p11.1{58,7Mb}	
#10	B	4		2q32.1{0,43Mb} 4q28.3{0,40Mb} 7q31.1{0,70Mb} 8q21.3{0,35Mb}
#11	U	2	6p25.3-p11.1{58,7Mb}	13q12.11-13q31.2{69,77Mb}
#12	U	2	2p25.3-p22.3){34,85Mb} 6p25.3-p11.1{58,7Mb}	
#13	U	4	1q12-q25.3{38,17Mb} 3q26.1-q29{32,80Mb} 9q12-q34.3{50,30Mb}	13q13.2-q22.3{44,90Mb}
#14	U	4	1p35.3-q44{217,70Mb} 6p25.3-p11.1{58,7Mb} 7q34-q36.3{21,30Mb}	9p24.3-p23{12,59Mb}
#15	U	4	5p15.33-p12{46,14Mb} 5q13.2{0,70Mb} 6p25.3-p11.1{58,7Mb} 8p23.1{0,60Mb}	
#16	U	7	1q21.1-q44{104,50Mb} 2p25.3-p22.3{35,55Mb}	1p32.1-p12{58.4Mb} 4p16.3-p14{37.6Mb} 11q22.3-q25{28.0Mb} 12p13.33-p13.1{14.2Mb} 16q12.1-q21{7.1Mb}
#17	U	8	1q12-q44{104,50Mb} 6p25.3-p11.1{58,7Mb} 9q22.2{0,23Mb} 20q13.33{0,24Mb}	7p13{0,66Mb} 14q11.2-q21.1{23,25Mb} 15q23{0,30Mb} 16q11.2-q24.3{43,66Mb}
#18	U	24	5q33.1{0,35Mb} 7p15.2{0,23Mb} 8q24.23{0,28Mb} 9q22.2{0,58Mb} 9q33.1{0,31Mb} 10q23.1{0,15Mb} 11q23.3{0,63Mb} 12q24.32{0,16Mb} 13q12.12{0,22Mb} 13q13.3{0,31Mb} 14q22.3{0,49Mb} 17q25.3{0,28Mb} 18q21.1{1,98Mb} 18q22.3-q23{1,59Mb} 20q13.12{0,20Mb}	1p21.3{0,30Mb} 2q11.2{0,28Mb} 2q31.33{0,13Mb} 2q37.3{0,13Mb} 3q25.1{0,31Mb} 4q26{0,39Mb} 10p12.33{0,11Mb} 11q24.3{0,58Mb} 20p11.21{0,43Mb}

Tab. 1. Chromosomal aberrations detected by array-CGH in 18 retinoblastomas. B, bilateral cases. U, unilateral cases. Size of rearrangements is given in curly brackets.

Chromosomal imbalances	Breakpoints	Frequence	N° genes	Oncogenes/tumor suppressors	Other candidate genes
Dup(6)(p25.3;p11.1){58,7Mb}	108,083 58,827,841	40% (7/18)	*461*	*IRF4, DEK, PIM1*	*E2F3, CCND3*
Dup(1)(q12;q25.3){38,17Mb} *	141,465,960 179,620,513	22% (4/18)	*497*	*/*	*MCL1, SHC1, MUC1*
Dup(2)(p24.3;p24.2){2,15Mb}*	15,120,360 17,242,742	22% (4/18)	*4*	*MYCN*	*DDX1*
Dup(9)(q22.2){0,23Mb}*	90,484,233 90,687,380	17% (3/18)	*2*	*/*	*/*
Dup(9)(q33.1){0,31Mb}	116,974,701 117,251,019	11% (2/18)	*1*	*/*	*/*
Del(11)(q24.3){0,58 Mb}*	127,676,090 128,202,918	11% (2/18)	*2*	*/*	*ETS1*
Del(13)(q13.2;q22.3){44,90 Mb}*	33,623,259 78,516,556	11%(2/18)	*123*	*RB1, ARLTS1*	*/*
Del(16)(q12.1;q21){7,02 Mb}*	50,674,625 57,636,204	11% (2/18)	*67*	*/*	*CYLD, RBL2*

Tab.2 Recurrent genomic imbalances identified by array-CGH analysis. * minimal overlapping regions.

Case	N° rearrangements	Chr instability group	Age at diagnosis (days)
#11	2	≤ 4	90
#12	2	≤ 4	743
#13	4	≤ 4	285
#14	4	≤ 4	480
#15	4	≤ 4	958
#16	7	≥ 7	1326
#17	8	≥ 7	1663
#18	24	≥ 7	1828

Tab. 3 Correlation between the number of genomic rearrangements and age at diagnosis in unilateral cases.

Chromosomal imbalances	Breakpoints	RN	RB	N° genes	Oncogenes/tumor suppressors	Other candidate genes
Dup(1)(q32.2){0,28Mb}	205,507,621 205,754,022	+	-	0	/	/
Dup(5)(p15.33;p12){46.14Mb}	110,640 46,008,694	-	+	121	/	*SKP2*
Dup(5)(q13.2){0.70Mb}	69,741,318 70,422,297	+	+	7	/	*BIRC1*
Dup(6)(p25.3;11.1){58.7Mb}	126,650 58,721,961	+*	+*	461	*IRF4, DEK, PIM1*	*E2F3, CCND3*
Dup(8)(p23.1){0,60Mb}	7,261,418 7,789,937	+	+	7	/	/
Dup(13)(q31.2){0,38Mb}	87,587,852 87,622,748	+	-	0	/	/

Tab. 5. Genomic rearrangements identified in retinoma and retinoblastoma of case #15.

Position of oligonucletides and genes refers to UCSC Genome Browser, http://genome.ucsc.edu, on Human May 2004 Assembly.

* log ratio values: ~0,5 in retinoma; ~1,0 in retinoblastoma.

4.Discussion and future perspective

The aim of this project was to investigate the role of germline and somatic factors in the variable phenotypic expression of retinoblastoma. We therefore screened our cohort of patients for germline *RB1* mutations and then we investigated the somatic events in both tumor tissue and the benign lesion retinoma.

Constitutive mutations of the *RB1* gene are associated with a predisposition to retinoblastoma. The complexity of the mutations and the fact that the vast majority of mutations are unique and spread over the entire coding sequence has hindered clinical implementation of molecular diagnosis. Precise identification of the *RB1* mutations that account for retinoblastoma in each family is predicted to enhance the quality of clinical management of the affected patient and relatives at risk [159]. If the proband's *RB1* gene status is determined by molecular testing, only those relatives with the mutation require clinical surveillance, whereas those proven to be noncarriers require no further examinations. If a family's mutation is known, prenatal molecular testing allows careful planning of perinatal management for infants with *RB1* mutations, including premature delivery to facilitate early treatment and optimize visual outcome[124]. Large deletions involving the *RB1* gene were the first mutations known to cause hereditary predisposition to retinoblastoma [14, 160]. However, until recently, few gross deletions have been reported in patients with retinoblastoma. This changed only after quantitative multiplex PCR facilitated the identification of gross alterations [111].

We decided to use a combined strategy based on the recently described multiplex ligation-dependent probe assay (MLPA) together with denaturing high-performance liquid chromatography (DHPLC) method for *RB1* screening in order to achieve efficient and high throughput coverage of the *RB1* mutational spectrum. Using this approach, we identified the molecular cause in 56/116 (48%) retinoblastoma patients. Our observed detection rates of 83% in the familial cases, 71% in the sporadic bilateral cases, and 12% in the sporadic unilateral cases, are similar to previous reports [114, 161, 162]. The reasons why mutations are not detected in all cases remains controversial. Aside from technical limitations of mutation detection, epigenetic changes to the *RB1* locus could contribute to this phenomenon to an underestimated degree [163]. Other described causes such as mosaicism or non-coding sequence variants may also play a role in lowering mutation detection rate[99]. To detect the subset of missed mutations, RNA and protein studies would be very helpful. First, these studies could detect subtle changes, as in missense, silent or intronic point mutations. Second, it remains possible that alternative mechanisms of *RB1* gene inactivation are present, such as aberrant

phosphorylation of pRB, degradation of pRB by proapoptotic molecules or the presence of viral proteins that sequester the pRB protein from its partner molecules [164].

Our pattern of mutations confirms inactivating mutations are predominant (32%), followed by deletions (25%), frameshift and splicing mutations (both 20%). In contrast, missense mutations have a very low frequency (3%) (Fig. 10). The distribution of *RB1* gene mutations reported in previous screening studies suggested two different spectra of mutations by country of origin. In certain South American countries (Argentina, Brazil, Colombia, Cuba and Ecuador), as well as Russia, the United Kingdom and Germany, the incidence of nonsense nucleotide substitutions is higher than in the United States, France and Spain [100, 165]. Our previous study, together with the most recent data, places the Italian retinoblastoma population in the first category. The differences in incidence of mutation type suggest the presence of predisposing genetic backgrounds. The regional incidence of mutations could be explained by distinct DNA repair mechanisms underlying nonsense and frameshift mutations [166, 167].

Interestingly, we found an even higher percentage of large deletions (25%) than previously reported (average of 13%) [111-113]. Because these deletions are oncogenic, our results illustrate the importance of a sensitive protocol for molecular screening of hereditary susceptibility to retinoblastoma. In fact, during these years, the techniques adopted for molecular investigation has strikingly improved the mutation detection rate from 0% to 12% in unilateral sporadic cases, 54% to 71% in bilateral sporadic cases and from 66% to 83% in familial retinoblastoma patients (Fig. 11).

Accurate clinical characterization of a large cohort of patients is mandatory in order to detect subtle phenotypic differences. For our patients, we plan to perform a detailed clinical characterization including age of onset, number of foci, laterality, tumor dimension and degree of differentiation, and response to therapy. In this cohort of patients, we will analyze *RB1* constitutive mutations and specific functional polymorphisms in *RB1* itself or in cell cycle checkpoint and apoptosis genes. Recently, it has been demonstrated that a single nucleotide polymorphism (SNP) in exon 4 of *TP53* gene, resulting in the presence of either arginine or proline at codon 72, leads to a change in apoptotic potential and alters prognosis in squamous carcinomas [168-171]. Furthermore, Kochethu et al. investigated the role of this SNP in chronic lymphocytic leukaemia (CLL) and they found that it is associated with increased susceptibility to CLL [172]. In the *MDM2* gene promoter has been found an SNP (SNP309), associated with accelerated tumor formation in both hereditary and sporadic cancers [173].

MDM2 encodes an ubiquitin protein ligase that regulates the stability of p53 by targeting it for proteosomal degradation. SNP309 increases the affinity of the *MDM2* promoter for the transcriptional activator Sp1, resulting in higher levels of *MDM2* mRNA and Mdm2 protein and a subsequent attenuation of the p53 pathway. Since *MDM2* is involved in regulating the expression levels of p53 which are associated with the apoptotic potential, it is a good potential modifier of retinoblastoma. In addition, it has been recently demonstrated that Mdm2 promotes also the ubiquitin-dependent degradation of pRb [174, 175]. We plan to investigate the effect of the two functional polymorphisms, in *TP53* and in *MDM2*, on the age of tumour diagnosis. The molecular results will be correlated with clinical data to clearly establish the genotype-phenotype correlation between *RB1* constitutive mutations type and phenotype.

Large-scale molecular deletions have been found in about 10% of retinoblastoma cases [94, 115]. When the deletion involves part of the genome surrounding *RB1*, it causes a contiguous gene deletion syndrome characterized by retinoblastoma, developmental abnormalities and peculiar facial dysmorphisms [118]. We decided to characterize four patients with retinoblastoma, dysmorphic feature and developmental delay using oligo arrays of 44,000 probes and compare them with another one with isolated retinoblastoma in order to identify the critical region of the 13q14 deletion syndrome and candidate genes possibly responsible for mental retardation. At present, the critical region that we have identified is still very large and includes numerous genes (30). From *in silico* analysis, four genes seem to be good functional candidates for the neurologic phenotype: *NUF1P1* (nuclear fragile X mental retardation protein 1), *HTR2A* (serotonin receptor 2A), *PCDH8* and *PCDH17* (protocadherin 8 and 17, respectively). Further experiments are necessary to narrow this critical region and thus identifying the gene(s) responsible for the neurological phenotype in these patients.

Retinoma is a benign lesion associated with retinoblastoma but lacking malignant characteristics [145]. It has initially been called "spontaneous regression" of retinoblastoma, however clinical evidence does not support this hypothesis and suggests that retinoma represents a precursor of retinoblastoma. To test this model, we obtained both retinoblastoma and the adjacent retinoma tissue from two patients for which both were available. Next, we employed quantitative real-time PCR (qPCR) to identify rearrangements of smaller size at four genes known to be involved in retinoblastoma pathogenesis (*MDM4* at 1q32.1, *MYCN* at 2p24.1, *E2F3* at 6p22 and *CDH11* at 16q22). In both retinomas, qPCR detected *MDM4* gains similar to those seen in the matched retinoblastoma tissue (3 copies in *Case#1* and 5 copies in *Case#2*). *MDM4* is a negative regulator of p53 transcription and stabilizes the E3 ubiquitin

ligase *MDM2* which targets p53 for degradation [170]. *MDM4* is implicated in retinoblastoma pathogenesis because copy number gains of this gene are found in 65% of human retinoblastoma[93]. Our results indicate that *MDM4* gains may be involved in the initial transition to retinoma. We also identified *MYCN* copy number gains, at a lower number in the retinomas (3 copies in both *Case#1 and #2*) compared to the retinoblastomas (5 copies in *Case#1* and 6 in *Case#2*). Amplification of *MYCN* has already been shown in 3% of 87 primary retinoblastomas and low-level gains in a further 13% [140]. Authors suggested that *MYCN* gains/amplifications confer a selective advantage for cell growth. In *Case#2,* we also identified progressive *E2F3* copy number gains in retinoma (5 copies) and retinoblastoma (7 copies) compared to normal retina. Gains of *E2F3* gene have been found in 70% of retinoblastoma primary tumors[140]. E2F3, together with E2F1 and E2F2, comprise to a subclass of E2F factors that act as transcriptional activators through the RB1-dependent G1/S phase transition[176] . Moreover E2F3 represses $p14^{ARF}$, an important tumor suppressor in the p53 pathway[177, 178]. Consequently, high-level *E2F3* gains may promote the transition from retinoblastoma to retinoma by eliminating p53-dependent apoptosis.

Array CGH is a powerful method for the analysis of genomic imbalances in tumor tissues. Cytogenetic and previous studies recently detected recurrent genomic alterations in retinoblastoma. After *RB1* loss (M1-M2), these imbalances could represent the subsequent events (M3-Mn) critical for tumor progression. To define these events, retinoma tissue is a very valuable tissue for the study of retinoblastoma initiation. We performed array CGH analysis in the two retinoma tissues previously investigated by qPCR. Our data revealed striking differences between the two cases. *Case#1* retinoma did not show any genomic rearrangement respect to normal retina. On the contrary, retinoblastoma tissue showed 7 genomic rearrangements. One rearrangement at 16q12.1-16q12 includes the *RBL2* gene (p130), an efficient inducer of cellular senescence when the major arrest pathway determined by pRb/$p16^{INK4a}$ is abolished. Recently, is was shown that p130 is highly expressed in retinoma and not in retinoblastoma, and the authors suggest that retinoblastoma can emerge from stable retinoma after overcoming the senescence response. p130 could indeed represent the effector of such mechanism[142]. The analysis performed in the *Case#2* retinoma showed 5 genomic rearrangements compared to normal retina. Among them, three are shared with retinoblastoma (dup5q13.2 , dup6p, dup8p23.1), while the remaining two (dup1q32.2 and dup13q31.2) are unique to retinoma. Gene content study of retinoma-specific rearrangements revealed that dup1q32.2 is already reported as copy number polymorphism (http://projects.tcag.ca/bioxrt/) and 13q31.2 region does not contain any known gene

(http://genome.ucsc.edu). We also identified retinoblastoma-specific rearrangements, including dup5p. The duplicated region contains a large number of candidate genes, including *SKP2* (p45), that may determine retinoma progression to malignancy. *SKP2* has S-phase-promoting functions and is implicated in the ubiquitin-mediated proteolysis of the Cdk inhibitor p27 [179-181]. The cell cycle arrest through the inhibition of cdk2 activity by p27 is critical for pRB-induced senescence [91]. Perturbation of the pRB-SKP2-p27 pathway could promote tumor development through enabling senescence bypass.

Our results reveal that the two lesions called retinoma share the same histopatological appearance, but they are indeed different entities. *Case#1* was diagnosed as retinoma and it transformed to retinoblastoma after eleven months. Genomic investigation by array CGH revealed that the retinoma lesion did not yet acquire chromosomal aberrations, but it did have gene-specific copy number gains identified by qPCR analysis that accompany the malignant transition from retinoma to retinoblastoma. *Case#2* retinoma was found after a retrospective histopathological examination. The presence of several rearrangements not shared with the adjacent retinoblastoma suggests that it could represent a clone of cells bearing benign chromosomal alterations and that only a specific sets of rearrangements is able to lead to tumor progression.

To date, five cytogenetic and conventional CGH studies [129, 130, 182-184] and one array-CGH[138] have been reported and all of them have suggested that genomic imbalances (M3-Mn) involving oncogenes/tumor suppressors are required for malignant transformation of retinoblastoma. We used high resolution array CGH of a series of 18 tumors (10 bilateral and 8 unilateral) to identify genomic imbalances and perform correlation studies. We detected a total of 64 rearrangements, 47 gains and 17 losses. In line with previous data, we found recurrent imbalances on chromosomes 1, 2, 6, 13 and 16 [139]. We also identified three novel rearrangements, two on chromosome 9 and one on chromosome 11. We correlated array CGH results with the phenotype and the age at diagnosis of our patients. We observed that bilateral cases showed a lower number of imbalances (mean, 1) compared to unilateral cases (mean, 7), with statistic significance (p=0,002). These data suggest that, beyond the inactivation of both *RB1* alleles, different molecular mechanisms may be involved in tumor progression of hereditary retinoblastoma. Alternatively to chromosomal instability (CIN), other genetic alterations can drive cancer progression, including subtle DNA sequence change such as microsatellite instability (MIN), chromosomal translocations, single gene amplification or deletions [140, 185, 186].

As concern age at diagnosis, in accordance with a previous CGH study [129], our data show a correlation between higher number of chromosomal aberrations and older age among the unilateral cases. An explanation to this correlation could be that a too high level of genomic instability may lead to suppression of tumor growth, resulting in a delayed disease onset [187]. On the contrary, it is also possible that an intrinsic slow growing rate of the tumor allows the accumulation of a higher number of chromosomal aberrations.

The imbalance found at the highest frequency (40%) is gain of 6p, confirming that it represents the most detected change in retinoblastoma [128, 140]. The region contains 3 known oncogenes: *IRF4*, *DEK*, and *PIM1*. We also selected as interesting genes, the transcription factor E2F3 and cyclin CCND3 for their role in G1/S transition during the cell cycle. Previous studies reported gains at 6p22 and they demonstrated that *DEK* and *E2F3* are the most common gained and upregulated genes [188, 189]. Our data together with the other findings suggest that both *DEK* and *E2F3* represent strong candidates and that a combination of genes on 6p, instead of a single one, probably contribute to RB progression.

The minimal region of gain (MRG) identified on chromosome 1 contains 497 genes. Two previously identified strong candidates on 1q, *KIF14* and *MDM4*, are not included within the identified MRG [93, 190]. However, using the qPCR analysis we detected gene amplification of *MDM4* gene in two of our samples, suggesting that *KIF14* and *MDM4* may therefore play an important role in retinoblastoma progression, regardless of 1q status. Furthermore, *ABL2* is the only known oncogene contained in the region. We also selected *MUC1* since its overexpression, as found in human carcinomas and certain hematologic malignancies, induces transformation and resistance to apoptosis [191]. Other interesting candidates are *MCL1*, encoding a potent multidomain antiapoptotic protein of the BCL2 family, and *SHC1*, a key intracellular signalling molecule that participates in the transforming activity of oncogenic tyrosine kinases (Ravichandran, 2001).

The MRG defined on chromosome 2 contains only four genes, including the known oncogene *MYCN* and *DDX1*, a gene that encodes a DEAD box protein probably involved in pre-mRNA 3'-end processing and that has been shown to possess oncogenic properties[192]. The two rearrangements detected on chromosome 9 contain a total of three genes, none of them reported as oncogenes/tumor suppressor genes.

The transcription factor ETS1 lies in the region lost in chromosome 11. This protein is involved in control of cellular proliferation, cell senescence and death, and tumorigenesis [193] and its expression is described as a negative prognostic indicator in malignant carcinomas [194].

We identified in two samples a deletion on chromosome 13, that comprises *RB1* gene. The minimal region of loss (MRL) defined contains 123 genes. One interesting gene is *ARLTS1*, a recently characterized tumor suppressor gene that encodes for a pro-apoptotic protein of the Ras superfamily involved in the pathogenesis of various tumours [195].

We detected a deletion on chromosome 16 containing the *RBL2*, that encodes the retinoblastoma family member p130. Its expression is reduced in human retinoblastoma tissues and in mouse models, loss of both *Rb1* and *Rbl2* is a more potent tumorigenic insult than loss of *Rb1* and *Rbl1* (encoding p107)[141]. Beside *RBL2*, this region includes the familial cylindromatosis tumour suppressor gene *CYLD*. Its loss is associated with the NF-kappaB-dependent inhibition of apoptosis[196] .

The identification of chromosomal regions and individual genes involved in retinoblastoma pathogenesis is fundamental to uncovering novel "druggable" targets and to revealing the genomic natural history of this type of cancer. Further work on the genomic and genetic changes in a larger number of retinomas is of great relevance. Unfortunately, this work is limited by the availability of tissues affected by this benign tumor, since it is not progressive and does not require surgery. Use of mouse models will certainly be helpful in defining the critical genes in the minimal regions of gain or loss. Since the mouse/human genome comparisons have previously unveiled highly concordant genomic alterations in other tumor types and have allowed the identification and validation of critical cancer-contributing genes, it is tempting to predict that the M3-Mn events responsible for retinoblastoma progression will become successful therapeutic targets.

5. References

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