Loss of *RB1* induces non-proliferative retinoma: increasing genomic instability correlates with progression to retinoblastoma

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Retinoblastoma clinical observations revealed the role of tumor suppressor genes in human cancer. Knudson’s ‘two-hit’ model of cancer induction. We now demonstrate that loss of both *RB1* tumor suppressor gene alleles initiates quiescent *RB1*⁻/⁻ retinomas with low level genomic instability and high expression of the senescence-associated proteins p16⁰INK⁴a and p130. Although retinomas can remain unchanged throughout life, highly proliferative, clonal and aneuploid retinoblastomas commonly emerge, exhibiting altered gene copy number and expression of oncogenes (*MYCN*, *E2F3*, *DEK*, *KIF14* and *MDM4*) and tumor suppressor genes (*CDH11*, *p75NTR*) and reduced expression of p16⁰INK⁴a and p130. We suggest that *RB1* inactivation in developing retina induces genomic instability, but senescence can block transformation at the stage of retinoma. However, stable retinoma is rarely clinically observed because progressive genomic instability commonly leads to highly proliferative retinoblastoma.

INTRODUCTION

Retinomas are benign retinal tumors that do not progress, found clinically in 2% of persons carrying a mutant *RB1* allele (1). Since retinoma can occur in individuals who carry one mutant *RB1* allele (1) (Fig. 1), and most retinoblastomas contain additional genomic changes after loss of both copies of *RB1* (for review see 2), we suggested that retinoma is a precursor of retinoblastoma with both *RB1* alleles mutated (mutations M1/M2), but no further events (M3-Mn) to drive progression to retinoblastoma (3).

Study of retinoma has been difficult, because the affected eyes generally function and require no therapy, as in the father of a child affected with bilateral (both eyes) retinoblastoma, who was found to have retinoma after he was shown to carry the germline *RB1* mutation detected in his son (Supplementary Material, Fig. S1; Case 24, Supplementary Material, Table S1). Clinically diagnosed retinoma has been

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one cycle of chemotherapy (6,7) caused dramatic dispersion of retinoblastoma into the vitreous, necessitating removal of the eye (Supplementary Material, Table S1: Case 5). Pathological examination showed a solid posterior tumor adjacent to the inner nuclear layer of the retina, with numerous fleurettes characteristic of retinoma (8), from which the retinoblastoma arose (data not shown).

Retrospective review of available archived paraffin-embedded sections of eyes enucleated for retinoblastoma revealed areas with features of retinoma in 15.6% (20/128) (Supplementary Material, Table S1), contiguous with retina and retinoblastoma (Fig. 1A), evidence of clonal progression from less advanced to more advanced neoplasm (9). The retinomas and retinoblastomas were often contiguous and did not arise from separate areas of the retina. The retinomas showed fleurettes (8), monomorphic, round nuclei and absence of necrosis or mitoses. In contrast, the adjacent retinoblastomas contained densely packed cells with little cytoplasm, round nuclei molded to each other, frequent mitoses, evidence of apoptosis and necrosis, and variable proportions of Flexner–Wintersteiner rosettes, characteristic of retinoblastomas (10), and Homer Wright rosettes, common in many neural tumors (11). Thus, retinomas are actually much more available for study than previously thought, since they are found in many eyes enucleated for retinoblastoma. Since we studied random pre-existing sections covering a small area of each eye, we extrapolate that the actual frequency of retinoma is higher than observed.

In addition to the 20 eyes discovered though the review of enucleated eyes, two additional eyes containing retinomas adjacent to retinoblastomas (Cases 9 and 10; Supplementary Material, Table S1) and the previously published retinoma, which was clinically documented to progress to retinoblastoma (5) (Case 1; Supplementary Material, Table S1) were included in subsequent analyses.

**Retinomas are homozygous null for RB1**

We determined the genetic status of RB1 in unaffected retina, retinoma and retinoblastoma. From eyes of two unilaterally affected patients, genomic DNA of adequate quantity and quality was acquired by laser capture microdissection from large, clearly defined areas of retina, adjacent retinoblastoma and retina (Fig. 1B). In Case 2 (Supplementary Material, Table S1), both the retina and retinoblastoma were homozygous for a G to T transversion at position 214 (c.214G>T) resulting in a stop codon (E72X) (Fig. 1C), whereas the adjacent retina and blood (data not shown) were normal. In this case, the M1 event is the c.214G→T mutation, and M2 is loss of heterozygosity resulting in two identical RB1 mutant alleles. In Case 3 (Supplementary Material, Table S1), two different RB1 mutant alleles were detected in the retinoma and adjacent retinoblastoma (c.1333C→T resulting in a stop codon, R445X, and c.298G→T resulting in a stop codon, G100X), while the retina showed the normal DNA sequence which was clinically documented to progress to retinoblastoma (Fig. 1A), evidence of clonal progression from non-affected retina, contiguous with normal retina (RET) and retinoblastoma (RB). Boxes indicate the areas shown in Fig. 2. (B) Microdissection from formalin-fixed, paraffin-embedded tissue retrieved representative samples of retina, retinoma and retinoblastoma for DNA and mRNA isolation. H&E section of the eye of Case 2 is shown before and after laser capture microdissection of retinoma (*). (C) DNA from retina, retinoma and retinoblastoma microdissected from the paraffin-embedded eye was sequenced for the null RB1 point mutation (c.214G>T) that resulted in an early stop codon (E72X), demonstrated in the surgical retinoblastoma specimen. (D) Retinoblastoma surgical tumor of Case 3 was found to be heterozygous for two different RB1 mutant alleles. DNA from retina, retinoma and retinoblastoma microdissected from the paraffin-embedded eye was sequenced for the two alleles, c.1333C→T (R445X) and c.298G→T (G100X).

observed to progress to retinoblastoma (4,5). We examined eyes enucleated for retinoblastoma for presence of underlying retinoma and show the first molecular evidence of clonal progression from senescent retinoma to malignant retinoblastoma. This data indicates that loss of RB1 in a susceptible retinal cell induces genomic instability but may be insufficient for malignant progression because a senescence response to the instability results in non-proliferative retinoma. Clonal change from retinoma to retinoblastoma is accompanied by major changes in proliferation markers and increased genomic instability involving specific oncogene(s) and tumor suppressor gene(s).

**RESULTS**

**Retinomas are common in eyes enucleated for retinoblastoma**

Clinical observation of one key patient revealed that residual tumor with histological features of retinoma remained after one cycle of chemotherapy (6,7) caused dramatic dispersion

**Figure 1.** Retinoma adjacent to normal retina and retinoblastoma in eyes removed as primary treatment for retinoblastoma are homozygous null for RB1. (A) The eye removed from unilateral Case 2 stained with H&E to reveal an area of retinoma (R), contiguous with normal retina (RET) and retinoblastoma (RB). Boxes indicate the areas shown in Fig. 2. (B) Microdissection from formalin-fixed, paraffin-embedded tissue retrieved representative samples of retina, retinoma and retinoblastoma for DNA and mRNA isolation. H&E section of the eye of Case 2 is shown before and after laser capture microdissection of retinoma (*). (C) DNA from retina, retinoma and retinoblastoma microdissected from the paraffin-embedded eye was sequenced for the null RB1 point mutation (c.214G→T) that resulted in a stop codon (E72X), demonstrated in the surgical retinoblastoma specimen. (D) Retinoblastoma surgical tumor of Case 3 was found to be heterozygous for two different RB1 mutant alleles. DNA from retina, retinoma and retinoblastoma microdissected from the paraffin-embedded eye was sequenced for the two alleles, c.1333C→T (R445X) and c.298G→T (G100X).
retinoma. Supporting that the retinomas were \( \textit{RB1}^{-/-} \), no retinoblastoma protein (pRb) was detectable in retinomas and retinoblastomas by immunohistochemistry in nine of nine eyes, which normally expressed pRb in specific cells of the adjacent retinas, as previously documented (12,13).

The \( \textit{RB1} \) mutant alleles were determined in unilateral retinoblastomas (M1 and M2) and blood of bilaterally affected children (M1) (Supplementary Material, Table S1), for most of the cases showing retinomas. All classes of mutations were represented, including nonsense, whole gene deletions, splice leading to premature termination, methylation of the promoter and small exonic deletions and insertions. All of these mutations are predicted to result in no residual pRb. One retinoma patient showed the well-recognized R661W missense mutation (Supplementary Material, Fig. S1; Case 24, Supplementary Material, Table S1) (14,15), associated with reduced penetrance, but otherwise no specific pattern of \( \textit{RB1} \) mutation type was associated with retinoma.

**Retinomas show a consistent staining with Ki67, PCNA and p53**

Immunostaining for Ki67, PCNA and p53 consistently distinguished retinoma from retinoblastoma (Supplementary Material, Table S2). The proliferation markers Ki67 and/or PCNA (data not shown) stained strongly positive in retinoblastomas, but were undetectable in retinomas and retinas (Fig. 2). Although an occasional cell in retinomas stained faintly, strong p53 staining was observed only in a subset of cells in retinoblastomas (Fig. 2).
Retinomas express the senescence proteins p16\textsuperscript{INK4a} and p130

We hypothesized that senescence induced by loss of RB1 alleles might maintain the arrested state of retinomas. Oncogene-induced senescence, characterized by increased expression of p16\textsuperscript{INK4a}, is the form of G1 growth arrest that is associated with human (16) and mouse (17,18) precancerous lesions. Nine of nine retinomas (Supplementary Material, Table S2) showed strong cytoplasmic staining for p16\textsuperscript{INK4a} (Fig. 3), but there was no staining of adjacent retinas or retinoblastomas. Cytoplasmic p16\textsuperscript{INK4a} may act to sequester cyclin dependent kinases that would otherwise enter the nucleus and inactivate pRb and its family members (19). Staining with antibody for the G2 arrest marker cyclin B1 was positive for only a subset of retinoblastoma cells (Supplementary Material, Fig. S2), suggesting that retinoma arrest occurs at G1 or G0, not G2.

Using quantitative real-time RT-PCR, we found that CDKN2A (which encodes p16\textsuperscript{INK4a} and p14\textsuperscript{ARF}) mRNA in Case 4 was highly expressed in both retinoma and retinoblastoma compared with normal retina (Fig. 4), suggesting that a post-transcriptional mechanism accounts for the lack of p16\textsuperscript{INK4a} protein in retinoblastoma. Adequate quality mRNA from microdissected paraffin-embedded tissues could be obtained only from Case 4.

We hypothesized that in the absence of pRb in retinomas, the RB family members p107 and/or p130 may interact with p16\textsuperscript{INK4a} to mediate cellular senescence. Immunostaining for p130 was strong in retina and retinoma but not detected in retinoblastoma, suggesting that loss of p130 may disrupt senescence and release the RB1\textsuperscript{+/−} cells to malignant progression (Fig. 2). Real-time RT-PCR using RNA derived from microdissected tissues from a paraffin-embedded eye showed equal expression of RB1, encoding p107, in retina, retinoma and retinoblastoma (Fig. 4). We did not have sufficient mRNA to similarly study p130 expression.

Retinomas display low level genomic copy number changes that involve more genes and higher levels of amplification in adjacent retinoblastomas

We have reported a pattern of specific genomic copy number changes beyond the loss of RB1 in 84% of surgical retinoblastomas (20), including candidate tumor suppressor genes NGFR (encoding p75\textsuperscript{NGFR}) (5) and CDH11 (21), oncogenes KIF14 (22,23), DEK and/or E2F3 (24), and MYCN. We performed interphase FISH analysis on four eyes containing retinoma and retinoblastoma, using specific probes for these genes and MDM4, another candidate 1q oncogene (25), and their corresponding centromeres. We scored for genomic gain or loss by counting the number of FISH signals in 100 cells per tissue.

Only three of the four eyes analyzed for genomic gain contained unaffected retina in addition to retinoma and retinoblastoma. All three retinas showed the expected normal two copies of all candidate genes tested (MYCN, E2F3, DEK, CDH11, KIF14 and MDM4) and chromosomal centromeres 2, 6, 16 and 1 (Fig. 5 and Supplementary Material, Fig. S3). Genomic copy number variation was distinctly different in adjacent retinas, retinomas and retinoblastomas. All (4/4) retinomas contained a proportion of cells displaying gains in some genes, indicating genomic instability (Fig. 6). In each case, the adjacent retinoblastomas showed increased genomic changes in patterns suggesting progression, in the retinoblastoma compared with the retinoma (Fig. 5, Supplementary Material, Fig. S3 and Fig. 6). The retinomas in Case 3 had median copy number of 3 for KIF14, while retinomas in Case 4 had median copy number of 3 for DEK and E2F3 (chromosome 6p) and for KIF14 and MDM4 (chromosome 1q). Consistent with previous reports of the fraction of retinoblastoma showing genomic gain, KIF14 and DEK showed abnormal copy numbers (median and maximum) in all four retinoblastoma. DEK and E2F3 on chromosome 6p and KIF14 and MDM4 on chromosome 1q tended to be gained together. All (4/4) retinoblastomas showed greater degree gain for candidate genes (up to seven copies in some cases) than retinomas. Gains of KIF14, DEK and E2F3 were seen in Cases 2–4. MYCN was gained only in Case 4: in the retinoma MYCN median copy number was 2 but a few cells showed five copies; in the retinoblastoma MYCN median copy number was 4 with a maximum of six copies. Cases 3 and 4 also showed low level gain of centromeres 1 and 6.

A recent study suggests that MDM4 is an oncogene gained at 1q (25); our results show median copy number > 2 of this gene in only Case 4. Maximum copy number of MDM4 > 2 was observed only in combination with gains of KIF14, while KIF14 showed genomic gains in every case, even when MDM4 remained two-copy (Fig. 5, Fig. 6, Supplementary Material, Fig. S3).

All of our samples exhibited normal copy numbers for CDH11, indicating that this small cohort of tumors either did not involve CDH11 loss, or have lost CDH11 expression by point mutation or small deletions that are not detected by FISH.
Expression of candidate oncogenes and tumor suppressor genes in retinomas is different than in retinoblastomas

We quantified mRNA microdissected from retinoma and retinoblastoma of Case 4 and prenatal retina for potential expression of candidate M3-Mn genes (Fig. 4). p75NTR mRNA was decreased or absent from both retinoma and retinoblastoma, compared with retina. We previously reported abundant p75NTR in 3/3 retinomas but not in retinoblastomas (5), so p75NTR mRNA may have a short half-life in retinoma. The candidate tumor suppressor gene, CDH11, was expressed at equivalent levels in retinoma and retina, and at decreased levels in retinoblastoma. The presence of p75NTR and CDH11 in retinoma suggests that expression of these genes is a feature of the cell of origin, which is lost with the transition to retinoblastoma.

Staining for DEK was observed in retinas, with positive staining stronger in retinoblastomas than retinomas in 2/4 cases (Fig. 2). E2F3 staining was absent from retinas and retinomas, while a fraction of retinoblastoma cells, presumably those in S-phase of the cell cycle, stained positively (Fig. 2). In case 4, we observed a slight increase in KIF14 and E2F3 mRNA between normal retina and retinoma, but 20 to 25-fold increased mRNA in retinoblastoma compared with retinoma, while DEK was equivalently expressed in both retinoma and retinoblastoma (Fig. 4), confirming results of immunostaining.

DISCUSSION

Retinoblastoma has been clinically observed to emerge from stable retinoma (4,5,26,27). The clinical, histological and molecular features and identical RB1 mutant alleles in retinomas and adjacent retinoblastomas are key evidence of the clonal development from retinoma to retinoblastoma, associated with progressive aneuploidy. In most cases, the retinomas were found adjacent to the normal retina, suggesting by nuclear morphology that they grew out of the inner nuclear layer of the retina, consistent with the concept that retinoma arose from normal retina with the acquisition of the M2 RB1 mutation; mild genomic instability would appear to be a consequence of loss of pRb. At some point, the magnitude of genomic instability, perhaps in key genes, resulted in a dramatic change from lack of proliferation to fully proliferative retinoblastoma, displaying marked genomic instability. This is consistent with models of tumor progression (9).

Retinomas have also been called ‘spontaneous regression of retinoblastoma’ (although regression has not been observed), ‘retinoblastoma group 0’ and ‘retinocytoma’ (also used to describe highly differentiated retinoblastoma with characteristic Flexner–Wintersteiner rosettes (10)). Since it is highly unlikely that gross genomic changes could ‘regress’ to normal, the term ‘spontaneous regression of retinoblastoma’ is inappropriate for retinomas. This term may be correctly applied to a very different clinical scenario when rare
Retinoblastomas regress due to infarction of large tumors and show evidence of massive cell death (1). Of all the other terms proposed (retinocytoma, retinoblastoma group 0), only ‘retinoma’ has been uniquely applied to these distinct and important non-proliferative retinal precursors of retinoblastoma.

It is feasible that retinoma and retinoblastoma could be two different endpoints derived from a common \textit{RB1}^{+/−} precursor cell. There are certainly cases of retinoma that stay benign for the lifetime of the individual (1, 26–32). Similarly, it is likely that retinoblastoma can emerge without a detectable retinoma stage. However, our study suggests evolution of each case from retinoma to retinoblastoma, based on the spatial relationships and genomic copy numbers in retina, retinoma and retinoblastoma.

Our findings suggest that p16\textsubscript{INK4a} is upregulated and becomes cytoplasmic after loss of \textit{RB1}^{+/−}, suggesting senescence may inhibit proliferation in retinoma. Arrest of the cell cycle by exogenous p16\textsubscript{INK4a} in other cell types is suggested to depend on function of both pRb and one of the other Rb family members, either p107 or p130, based on expression of p16\textsubscript{INK4a} at near endogenous levels by transfection or infection of various knockout mouse cells or human \textit{RB1}^{+/−} cell lines (33). However, the high levels of p16\textsubscript{INK4a} protein expressed in human retinomas, perhaps in response to genomic instability induced by loss of pRb, may be sufficient to block the cell cycle through p130 or p107, even in the absence of pRb. It is possible that p16\textsubscript{INK4a} is induced in most \textit{RB1}^{−/−} cells. Rare cells may escape the induction of senescence, either by inactivation of p16\textsubscript{INK4a} or no activation of p16\textsubscript{INK4a} in the first place, and go on to clonally progress to retinoblastoma with activation of oncogenes through genomic instability. Clinical evidence however, strongly suggests that the rare retinoma that remains stable and unchanging under observation can sometimes later progress to an active, malignant retinoblastoma, perhaps by failure of senescence.

It is likely that p130, highly expressed in retinoma and shown to be upregulated in experimental situations of genomic instability with or without p16\textsubscript{INK4a} activity (34), is the effector of senescence rather than p107. Retinoblastomas did not stain for p130 (Fig. 3), but did show uniform low p107 mRNA expression, implicating p130 as the key Rb family member differentiating retinoma and retinoblastoma. Although presence of p16\textsubscript{INK4a} suggests G\textsubscript{1} arrest, high expression of p130 and lack of Ki67 expression in retinomas suggests arrest at G\textsubscript{0}. In mouse models of retinoblastoma, loss of both \textit{Rbl1} and \textit{Rbl2} (encoding p130) is a more potent tumorigenic insult than loss of \textit{Rbl1} and \textit{Rbl1} (encoding p107) (35). Studies implicating p130 loss in the pathogenesis of retinoblastoma (36) identified polymorphisms but not
convincing p130 mutations, so the inactivation of p130 may not depend on genomic change.

The patterns of gains and losses of genes in retinoma compared with retinoblastoma is further evidence of their clonal progression, as retinoblastoma commonly displays similar, yet more severe, genomic changes than its adjacent retinoma. KIF14 is the most frequent genomic change in retinoblastoma tumors (20) and is strongly predictive of outcome in breast and lung cancer (23,37). Our data reinforces the key role of KIF14 in transformation. KIF14 is the earliest genomic gain in retinoma and the most frequent and highest copy number alteration in retinoblastoma. The MDM4 gene nearby is only gained (25) in some samples, because the mechanism of genomic gain selected by KIF14 activity sometimes involves large regions of chromosome 1q including MDM4 (Fig. 6, Fig. 5, Supplementary Material, Fig. S3).

We suggest a model for the progression of normal retina to retinoma to retinoblastoma (Supplementary Material, Fig. S4), beginning with inactivation of both alleles of the RB1 tumor suppressor gene (M1 and M2) in a susceptible retinal cell. A limited number of cell cycle divisions occur before cells arrest with a specific aborted differentiation characterized by fleurettes. RB1 inactivation is known to promote genomic instability (38) by E2F deregulation and accumulation of double strand DNA breaks (39). Such disturbance of genomic integrity is consistent with the low-level genomic instability we observe in retinoma (Fig. 6). Subtle genomic changes may lead to gain of oncogenes, such as KIF14, E2F3 and DEK. These genomic changes, however, are not manifested at the level of gene expression, and cells are arrested in the retinoma. Indeed, retinoma can remain quiescent throughout life, as illustrated in Supplementary Material, Fig. S1. However, genomic instability may increase the risk that critical cancer genes will become mutated (M3-Mn) in a retinoma cell and breach the senescence barrier. Some cells may still be halted by other mechanisms, such as initiation of apoptosis by p75NTR. Loss of such protective mechanisms ultimately commonly leads to retinoblastoma, with progressive gain of oncogenes MYCN, DEK/E2F3, and KIF14/MDM4, and loss of potential tumor suppressor genes, including CDH11. The p53 pathway, which is intact in retinoblastoma, may be inadequate to prevent development of retinoblastoma.

Knowledge of the molecular switches that drive a normal cell to dysplasia and eventually to malignancy and metastasis are key to understanding how cancer develops. The multistep model of carcinogenesis with sequential accumulation of genetic mutations is well-defined in the progression of benign to malignant colon tumors (40). An important issue is the timing of genomic instability in cancer development (41). The preneoplastic prostate lesion, prostatic intraepithelial neoplasia (PIN), also shows cell cycle deregulation and genomic instability prior to development of recurrent clonal genomic aberrations and malignant transformation (42,43). Our data suggest that the prototypical tumor suppressor gene, RB1, combined with low level chromosome instability manifest in retinomas, is insufficient for malignant transformation until a critical level of genomic instability perhaps involving key genes, tips the balance to uncontrolled proliferation. A premalignant senescent stage may be common in many or most cancers, but be unrecognized when the tissue supports high frequency progression to malignancy. The senescent precursor lesion may not necessarily require therapeutic intervention, but appropriate surveillance for progression, as has been suggested for small renal masses that resemble renal cell carcinoma but that do not progress (44). Retinomas clinically recognized only in adulthood do not require treatment, but in infancy may be commonly treated as retinoblastoma.

Study of human clinical samples was essential to delineate the molecular pathway from retinoma to retinoblastoma. The mouse models that were designed to understand the role of the RB1 gene in development of retinoblastoma have achieved malignant neural retinal tumors with no recognition of a non-

Figure 6. Summary of copy number of oncogenes and candidate tumor suppressor genes determined by FISH in retinomas and retinoblastomas from the same cases. Colored boxes indicate median copy number; arrows indicate maximum copy number.
proliferative retinoma, and no fleurettes or retinoma-like structures or recognition of a pre-malignant lesion. However, in one model it was recognized that the first step to malignancy was the survival of the \( Rb1^{+/−} \) retinal cell that might progress to retinoblastoma-like tumor (45). That human \( Rb1^{+/−} \) retinal cells survive as retinoma, without transformation despite mild genomic instability, until sequential genomic changes lead to retinoblastoma, was only discovered by study of human specimens. Identification of the oncogenes and tumor suppressor genes important in this progression also depends on study of human disease, from which will emerge relevant therapeutic and prevention targets.

Retinoblastoma, a relatively rare cancer, has dramatically changed the way cancer is studied and understood, through important scientific advances such as the identification of \( Rb1 \) as the first tumor suppressor gene (46) and Knudson’s classic two-hit hypothesis (47) that has become the dogma of cancer genetics. Retinoma now clarifies the idea that the ‘two hits’ are only rate-limiting for the disease, as the loss of \( Rb1 \) does not cause cancer, but only makes it highly likely that retinoblastoma will emerge from the retinoma.

The molecular definition of retinoma clearly demonstrates that the two \( Rb1 \) mutant alleles do not inevitably result in malignancy, but only in genomic instability; that the process can pause at this stage is important and not previously demonstrated. Importantly, Knudson called the two hits (M1/M2) only rate-limiting, consistent with the retinoma/retinoblastoma data we present. Most important for the future of children with retinoblastoma will be detailed understanding the molecular switches that drive retinoma to malignancy, which will be essential for the treatment for retinoblastoma for the presence of retinoma. In each case, a sample of tumor had been removed immediately after enucleation for molecular studies, before the remainder of the tumor was fixed, embedded and sectioned. Retinoma cases are summarized in Supplementary Material, Table S1.

**Materials and Methods**

**Human tissues**

Eyes enucleated for retinoblastoma were obtained from the surgical pathology archives of the Eye Bank of Canada (Toronto, Canada) and the Hospital for Sick Children (Toronto, Canada), and studied with approval from the Research Ethics Boards of the Hospital for Sick Children and University Health Network (Toronto, Canada). Two eyes were obtained from Wills Eye Hospital (Philadelphia, USA). All studies with human tissue were confirmed to be in accordance with the tenets of the Declaration of Helsinki.

**Scoring of retinoma incidence**

Two independent observers (VK, WH) reviewed haematoxylin and eosin (H&E) stained sections of 128 eyes removed as treatment for retinoblastoma for the presence of retinoma. In each case, a sample of tumor had been removed immediately after enucleation for molecular studies, before the remainder of the tumor was fixed, embedded and sectioned. Retinoma cases are summarized in Supplementary Material, Table S1.

**Slide preparation, laser capture microdissection and DNA/RNA isolation**

Tumor sections (5 μm) were deparaffinized in xylene for 5 min, then hydrated in a series of ethanol solutions for 5 min each. Sections were briefly stained (30 s) in haematoxylin, rinsed in sterile water and run through a series of EtOH solutions at increasing concentrations, then in xylene for 3 min. Tissues were heated at 37°C for 10 min and stored in a dessicator until used. Laser capture microdissection to isolate normal retina, retinoma and retinoblastoma tissues was performed by the Ontario Cancer Institute Advanced Optical Microscopy Facility, using an Arcturus Pixcell II. DNA was extracted and purified using a QIAamp DNA Micro Kit (#51306, Qiagen, Mississauga, ON, Canada) and RNA was extracted and purified using the Arcturus Paradise™ Reagent System (#K10300L). DNA and RNA were quantified using an ND-3300 Nanodrop spectrophotometer.

**Rb1 mutation identification**

Fresh surgical retinoblastoma specimens or blood were studied for the \( Rb1 \) mutation (s) in the majority of cases (Supplementary Material, Table S1) as described (15). For Case 2, exon-specific PCR amplification of the \( Rb1 \) gene was performed and sequenced with the Visible Genetics Sequencing Kit using Cy 5.0 or Cy 5.5 labeled sequencing primers. After sequencing, products were separated on the Visible Genetics MicroGene Clipper or Long Read Tower. Sequences were analyzed using GeneObjects software, and compared with the published \( Rb1 \) sequence (Genbank: NM00321). For Case 3, mutation-specific PCR was performed to confirm the mutant \( Rb1 \) alleles in retinoma.

**Gene specific FISH**

FISH verified RP11 and RP1 BAC clones for the areas of interest were selected from the NCBI Clone Registry (http://www.ncbi.nlm.nih.gov/genome/clone/) and from the Invitrogen CTD clone library (Burlington, Canada). The BAC clones selected were RP11-430C7 (MDM4), RP11-12P12 (KIF14), RP11316K24 (CDH11), RP1-298J15 (DEK) and CTD-23247D10 (E2F3).

BAC DNA was extracted from an overnight culture according to standard methods and assessed by gel electrophoresis. DNA was directly labeled with either Spectrum Green or Spectrum Orange (Vysis, Des Plaines, IL) by nick translation using the Vysis Nick Translation Labeling Kit as described by the manufacturer. 200 ng of probe along with 1.5 μg of human Cot-1 DNA was hybridized overnight at 37°C to de-waxed and pepsin-digested paraffin sections. Washed slides were DAPI stained and scored with a Zeiss Axioplan 2 Plus microscope (Zeiss, Toronto, Canada) equipped with appropriate filter sets and image captured with MetaSystems’ ISIS imaging software (MetaSystems, Altlussheim, Germany).

**Quantitative RT–PCR**

Quantitative RT–PCR using TaqMan™ chemistry was performed as previously described for \( p75 \) (5), \( E2F3 \) and...
DEK (24), KIF14 (22), as well as with gene specific primers (Applied Biosystems Incorporated) for CDH11 (Assay ID: Hs0156438_m1), RBL1 (Assay ID: Hs00161234_m1) and CDKN2A (Assay ID: Hs00233365_m1).

Immunostaining

Immunostaining on samples outlined in Supplementary Material, Table S1 was performed as previously described (12), using the antibodies and conditions described in Supplementary Material, Table S3. Immunostaining for p16[INK4a] was visualized on a Zeiss LSM510 confocal microscope (Zeiss, Toronto, Canada).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. The authors declare no competing interests.

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REFERENCES


