Eye Cancer: Unique Insights into Oncogenesis
The Cogan Lecture

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The major objective of this lecture is to illustrate how eye cancer research has dramatically affected not only patients with these ocular tumors, but also our fundamental understanding of cancer biology and many other aspects of biomedical research. Retinoblastoma and uveal melanoma, the two most common eye cancers in children and adults, respectively, are very different clinically and biologically. Retinoblastoma is a rare childhood eye cancer that usually occurs before 5 years of age, is often hereditary and bilateral, is caused by the mutation of a single rate-limiting gene, and is rarely fatal if treatment of the eye cancer is successful. Uveal melanoma occurs mostly in older adults, is rarely hereditary or bilateral, is caused by an accumulation of multiple genetic lesions with no single disease-causing gene mutation, and is fatal in up to 50% of patients, even when the eye cancer is successfully treated. Despite these profound differences, retinoblastoma and uveal melanoma have provided unique and complementary insights into the cell cycle, differentiation, development, neoplasia, and other fundamental biological processes. Herein, we will consider some of the highlights in ocular oncology research over the past two decades, starting with the identification of the retinoblastoma gene in 1986. We will examine how the molecular genetics of retinoblastoma has fundamentally altered our view of development, cell cycle and cancer biology. We will then explore the role of the retinoblastoma tumor suppressor pathway in uveal melanoma. Finally, we will review recent exciting research in uveal melanoma that may lead to a significant change in our understanding of that cancer, and we will consider the far-reaching implications of this breakthrough in future research, clinical trials, and patient care.

RETINOBLASTOMA

Identification of the Retinoblastoma Gene

Despite its relative rarity, occurring in only approximately 1 patient per 20,000 live births, retinoblastoma has been at the heart of many of the landmark discoveries in cancer research over the past two decades. Most patients with unilateral, unifocal retinoblastoma do not transmit the disease to their children, nor are they predisposed to secondary primary cancers elsewhere in the body. In contrast, patients with bilateral retinoblastoma usually have multiple, bilateral tumors; they transmit the disease in an autosomal dominant pattern; and they have an alarmingly high rate of second primary cancers. Because of the autosomal dominant inheritance pattern, it was widely assumed for many years that retinoblastoma was caused by a dominantly acting oncogene. In 1971, however, Knudson proposed the “two-hit hypothesis,” which heralded a major paradigm shift in retinoblastoma and, indeed, all cancer biology. Knudson’s supposition was that the RB gene inhibits tumor formation in a recessive manner and that inactivation of both copies of the RB gene in a susceptible retinal cell leads to retinoblastoma. In nonhereditary retinoblastoma, the mutation of both RB alleles occurs in the same retinoblast. Since the chance of both events occurring in the same cell is very small, this same-cell mutation explains the low incidence and uniformity. In hereditary retinoblastoma, the first RB mutation is passed in the germline and is present in most or all cells in the body. The second mutation then occurs somatically in a retinal cell that already contains one RB mutation. Because the only somatic mutation is required in any susceptible retinal cell, this mode of mutation explains the multifocality and bilaterality of hereditary retinoblastoma. The presence of the RB mutation throughout the body (including the germline) explains the hereditary transmission and the risk of second primary cancers. In 1986, Knudson’s hypothesis was validated by the identification of the RB gene on chromosome 13, region q14, by a Dryja et al., and this finding was confirmed shortly thereafter by other groups.

The Retinoblastoma Gene and Protein

The RB gene contains 27 exons distributed over approximately 200 kb of DNA. Germline RB mutations tend to occur at CpG dinucleotides and are distributed throughout the gene. RB mutations are also found in many of the second primary cancers found in patients with retinoblastoma, such as osteosarcoma, soft tissue sarcoma, and melanoma. In addition, RB mutations are found in some sporadic cancers of the lung, prostate, breast, and other tissues. The Rb protein, composed of 928 amino acids, is a nuclear phosphoprotein that contains an N-terminal region, a central pocket domain composed of an A box, and a B box, and a C terminus. In virtually all human cancers in which the RB gene is not mutated, the Rb protein is functionally inactivated, suggesting that Rb is a tumor suppressor of fundamental importance in cancer biology. In fact, tumor-causing viruses such as SV40, adenovirus, and human papillomavirus all produce proteins that bind and inhibit Rb, and these oncoproteins are essential for the tumorigenic properties of these viruses. The Rb “pocket” is formed by interaction of the A and B boxes along an extended interface and is essential for the tumor suppressor function of Rb, as evidenced by the disruption of the pocket by virtually all tumor-causing mutations.

Assembly of Multimeric Protein Complexes

The tumor suppressor activity of Rb is due, at least in part, to its ability to inhibit cell division by blocking S-phase entry. This and most other physiologic effects of Rb rely on its ability...
to regulate gene expression by assembling multimeric protein complexes at promoters. Rb does this through its multiple binding sites that allow it to interact simultaneously with several proteins (Fig. 1). The B box within the pocket contains a leucine-x-cysteine-x-glutamate (LxCxE) binding site to which bind the viral oncoproteins and many chromatin remodeling proteins that contain an LxCxE motif. A separate binding site formed by a cleft at the interface of the A and B boxes, through which Rb binds to E2F transcription factors (E2Fs). There may also be other binding sites in the pocket, since chromatin remodeling proteins such as BRG1 do not need an LxCxE motif to bind Rb. The C terminus contains binding sites for the oncoproteins HDM2 and c-Abl. Thus, Rb is able to assemble a large variety of multimeric complexes through its distinct binding sites.

**Transcriptional Repression**

Rb and the other pocket proteins p107 and p130 do not contain a DNA binding domain, but they interact with specific DNA elements through binding to E2Fs to -5, which do contain sequence-specific DNA binding domains. E2F sites are present in many genes involved in the cell cycle, in differentiation, and in apoptosis, and Rb represses these genes by at least two mechanisms (Fig. 2). First, Rb binds and physically blocks the E2F transactivation domain. Second, when Rb is brought to the promoter through interaction with E2Fs, it can actively repress transcription by suppressing the activity of surrounding enhancers on the promoter. Rb does this by recruiting chromatin remodeling proteins to promoters, where they alter local chromatin structure and inhibit access by the transcriptional machinery. Several major classes of chromatin remodeling proteins have been shown to interact with Rb, including histone deacetylases (e.g., HDAC1 to -3), SWI/SNF ATP-dependent nucleosome assembly proteins (e.g., BRG1), polycomb group proteins (e.g., HPC2 and Ring1), DNA methyltransferases (e.g., DNMT1), and histone methyltransferases (e.g., SUV39h). These interactions with chromatin-remodeling proteins are in turn regulated by specific Rb phosphorylation events.

**Stepwise Regulation of Rb by Phosphorylation**

Rb is regulated by phosphorylation and can be phosphorylated at up to 16 serine/threonine-proline recognition sites by cyclin-dependent kinases (CDKs). CDKs are activated by interaction with their cyclin-binding partners and inactivated by CDK inhibitors such as the tumor suppressor p16Ink4a. Early investigators envisaged a binary model of Rb activity in which the protein is active when hypophosphorylated and inactive.
when hyperphosphorylated. However, this model has been found to be overly simplistic. In reality, Rb is phosphorylated in multiple steps throughout the cell cycle. Initial phosphorylation of Rb is catalyzed by cyclin D-CDK4 in early G1, then by cyclin E-CDK2 late in G1 and later by cyclin A-CDK2 in the S phase. These multiple phosphorylation events are necessary for complete hyperphosphorylation and inactivation of Rb. Further, phosphorylation of specific sites appears to regulate distinct Rb functions, suggesting a complex mechanism for regulating of Rb by sequential phosphorylation events.

In 1999, we provided a molecular model for understanding this complex regulation of Rb activity. Rb is phosphorylated in a stepwise series of increasingly energetically unfavorable reactions that are enabled by conformational changes induced by the previous phosphorylation event (Fig. 3). Similar sequential phosphorylation mechanisms have been shown for other proteins such as c-Fos. The initial phosphorylation of Rb at sites in the C terminus by cyclin D-CDK4 triggers an intramolecular conformational change in which the negatively charged C terminus interacts with a positively charged lysine patch in the B box. This interaction displaces LxCxE proteins such as HDACs from their binding site, thereby partially inactivating Rb. This phosphorylation event is probably sufficient to abrogate the ability of Rb to block the G1-to-S transition. This intramolecular interaction also brings CDK2 docking sites in the C terminus into proximity of additional phosphorylation sites in the pocket, thereby providing the phosphorylation of these sites by cyclin E-CDK2. These phosphorylation events cause additional conformational changes that provide access by cyclin-CDK complexes to Ser567, which is otherwise buried within the A–B interface and inaccessible to phosphorylation. Phosphorylation of Ser567 grossly disrupts the tertiary structure of Rb, releasing E2Fs. In support of this model, crystallographic studies of Rb have predicted that phosphorylation of the C terminus would trigger an intramolecular interaction with the pocket, that Ser567 mediates critical contacts between the A and B boxes, and that phosphorylation of Ser567 would destabilize the pocket structure and eliminate the E2F binding site.

To provide a physiologic context for these findings, we later showed that Rb represses cyclin E and other G1-phase cell cycle genes when it is fully active through multimeric complexes containing HDAC and BRG1. After initial phosphorylation by cyclin D-CDK4, Rb dissociates from HDAC but remains bound to BRG1 and E2Fs, in which state it continues to repress cyclin A and other S-phase genes. After cells enter the S-phase, the increasing levels of cyclin E and consequent activation of CDK2 leads to further phosphorylation of Rb, releasing BRG1 and derepressing the cyclin A gene, which then allows cyclin A-kinase complexes to accumulate and maintain Rb in a phosphorylated state during cell cycle progression through the S phase. Thus, the sequential phosphorylation and progressive inactivation of Rb may allow orderly cell cycle progression through the precisely timed activation of cyclins (Fig. 3). However, the role of Ser567 phosphorylation remained unclear for several years.

**The Role of Rb in Apoptosis**

Loss of Rb leads to apoptosis in the retina, lens, and many other tissues through a potent, multifaceted death response, suggesting that there is an inherent pressure for organisms to eliminate aberrant cells that lack Rb. Loss of Rb leads to apoptosis through two major mechanisms. First, since Rb binds the transactivation domain of E2Fs, it directly blocks the ability of proapoptotic E2F1 to transactivate genes involved in apoptosis, such as *ARF* and *p73*. Loss of Rb releases E2F1 from its inhibitory interaction with Rb, allowing it to activate proapoptotic genes. In general, this mechanism is dependent on the p53 pathway. Second, loss of Rb derepresses apoptotic genes that are under basal inhibition by Rb–E2F complexes. This mechanism is largely independent of p53 and does not require E2F transactivation. Using a dominant-negative E2F mutant (dnE2F) that contains the DNA binding domain but not the transactivation domain, we recently identified one of these basally inhibited proapoptotic genes and identified a potentially new Rb-mediated apoptotic pathway.

Reconciling the Antiproliferative and Antiapoptotic Functions of Rb

Because Rb inhibits both cell division and apoptosis, how do cells inactivate Rb to allow normal cell division without triggering apoptosis? We provided a potential explanation for this paradox by showing that the cell cycle and apoptotic functions of Rb are regulated by distinct mechanisms. The CDK sites in the C terminus are phosphorylated every cell cycle and regulate the orderly cell cycle progression, which could explain why Rb-null cells are prone to chromosomal instability.
The Role of Rb in Development and Differentiation

Surprisingly, Rb is not needed for the cell division cycle to function. Cells lacking Rb progress normally through the cell cycle, but they are unable to exit the cycle normally during senescence or differentiation. However, tumors develop in mouse retina lacking Rb/p107 not because of a proliferation defect, but because of an inability of retinal progenitor cells to exit the cell cycle and terminally differentiate. Similarly, we showed that the Rb pathway is essential for melanocyte differentiation. In these studies, the melanocyte differentiation factor MITF was found to induce expression of the p16INK4a tumor suppressor, causing an accumulation of hypophosphorylated (active) Rb and cell cycle arrest. This MITF-INK4A-Rb pathway was required for efficient cell cycle exit and melanocyte differentiation in cultured cells and in vivo (Fig. 5). Thus, the tumor suppressor function of Rb appears to be due, at least in part, to its ability to induce permanent cell cycle exit in cultured cells and in vivo (Fig. 5). Thus, our current view is that Rb is dispensable for regulation of the normal cell cycle, but it is essential for coordinating orderly cell cycle exit associated with differentiation and senescence. Further, Rb may act as a buffer against inappropriate apoptosis during normal cell division but can unleash a potent apoptotic signal under abnormal hyperproliferative or stress conditions. Next, we will explore how uveal melanoma has provided further insights into the role of Rb in melanocyte biology and oncogenesis.

The RB Pathway in Uveal Melanoma

Uveal melanoma is the most common eye cancer and the second most common form of melanoma. Unlike retinoblastoma, uveal melanoma has been extremely recalcitrant to classic molecular genetic analysis. This cancer is rarely hereditary, which has hampered linkage analysis for susceptibility genes. Further, none of the major tumor suppressor genes, including RB, are mutated in uveal melanomas with any significant frequency, as evidenced by the normal expression of the RB protein in most of these tumors. In parallel studies of the p53 pathway, we found no evidence for p53 mutations but frequent disruption of the p53 pathway, such as overexpression of the p53 inhibitor HDM2. Likewise for Rb, we found that the Rb pathway is commonly inhibited in uveal melanomas by inappropriate phosphorylation of Rb. In primary uveal melanomas, we showed that Rb is phosphorylated at the C-terminal CDK sites that regulate the repressor activity of Rb. Since these sites are known to be phosphorylated by...
FIGURE 5. The role of Rb in melanocyte differentiation. (A) The melanocyte differentiation factor MITF transcriptionally activates the p16Ink4a tumor suppressor, which activates Rb by inhibiting CDK4. Active Rb then enforces cell cycle exit at the G1-S boundary, which is required for efficient melanocyte differentiation. (B) Melanocyte differentiation in vivo is hindered when p16Ink4a is not present to activate Rb. Low-power photomicrographs show immunofluorescence analysis of the melanocyte markers S100, DCT, and TRP1 in cutaneous hair follicles of 3-week-old INK4A-wild-type mice and INK4A-null mice. Note that the expression of S100 (green), TRP1 (red), and DCT (red) is greatly diminished in INK4A-null mice. DAPI (blue) indicates cell nuclei. Scale bar, 100 μm.

FIGURE 6. Two molecular classes of uveal melanoma. (A) Unsupervised analysis of primary uveal melanomas using principal component analysis reveals two distinct groups of uveal melanomas based on gene expression patterns. (B) Hierarchical clustering of the top 62 genes that discriminate between class 1 and 2 melanomas shows that the two tumor classes have distinct "molecular signatures." (C) Microarray-based comparative genomic hybridization experiments show that the class 2 signature is strongly associated with monosomy 3 (down arrows) and inversely associated with gain of chromosome 6p (up arrows). Reprinted, with permission, from Onken MD, Worley LA, Ehlers JP, Harbour JW. Gene expression profiling in uveal melanoma reveals two molecular classes and predicts metastatic death. Cancer Res. 2004;64:7205–7209. © 2004 American Association for Cancer Research.
specific cancer genes have been linked to these loci.94 This association, including loss of chromosome 3 and gain of 8q, but no somatic alterations are associated with increased risk of metastasis.88 Further, many uveal melanomas display constitutive activation of the MAPK pathway,89 which activates cyclin D1 expression.90

Of interest, while Rb is phosphorylated in uveal melanomas at the C-terminal sites that regulate cell proliferation, Ser567 is rarely phosphorylated in these tumors (Harbour JW, unpublished data, 2004). This observation is consistent with the very low rate of spontaneous apoptosis observed pathologically in primary uveal melanomas, compared with the high rate of apoptosis in Rb-null retinoblastomas. Hence, uveal melanomas (and many other solid tumors) do not completely abolish Rb activity, but rather, they appear to cause partial inactivation of Rb, to allow cell division without inducing the excessive apoptosis that can accompany complete Rb loss. This phenomenon may explain, at least in part, the extreme resistance to therapy-induced apoptosis in these tumors.91

A NEW MODEL OF UVEAL MELANOMA GENETICS

The Genetics of Uveal Melanoma Metastasis

Up to half of patients with uveal melanoma die of metastatic disease, even when the eye tumor is successfully treated.92 Do the defects in the Rb pathway explain this propensity for metastasis in uveal melanoma? This notion seems unlikely for several reasons. First, Rb pathway abnormalities are found in virtually all uveal melanomas, whether or not they are associated with metastasis (Harbour JW, unpublished data, 2003). Second, for the reasons discussed earlier, disruption of the Rb pathway is probably an early event in melanoma initiation, being required for melanocytes to re-enter the cell cycle before acquiring additional mutations.74,95 A few recurring chromosomal alterations are associated with increased risk of metastasis, including loss of chromosome 3 and gain of 8q, but no specific cancer genes have been linked to these loci.96 This lack of understanding of the metastatic phenotype using traditional methods led us to explore global changes in gene expression.

Two Distinct Molecular Classes of Uveal Melanoma

If Rb pathway alterations occur early in uveal melanoma pathogenesis, what are the later events that determine whether these tumors will metastasize? Several clinical, pathologic, and cytogenetic features have been shown to predict metastatic disease, including larger tumor size, anterior tumor location, older patient age, epithelioid cell type, extracellular matrix deposition in looping patterns, and monosomy 3.95–99 To gain insights into the molecular mechanisms of uveal melanoma metastasis, we used microarray gene expression profiling to analyze primary, uncultured uveal melanomas.100 We found that primary uveal melanomas cluster naturally into two groups that we provisionally refer to as classes 1 and 2, based on gene-expression signature (Fig. 6). Survival analysis showed that patients with class 1 tumors rarely died of metastasis, whereas those with class 2 tumors had a high rate of metastatic death. Correspondingly, class 2 tumors were more likely to exhibit epithelioid cells, looping extracellular matrix patterns, and monosomy 3.100,101 Similarly, the Lohmann laboratory102 in Germany independently found that uveal melanomas form two molecular classes that correspond to chromosome 3 status. The gene signatures from these two studies were remarkably similar, despite differences in study design and patient population, indicating that these molecular signatures may be generalizable to other populations. Further, these were the first large-scale studies of gene expression using primary, uncultured uveal melanomas, which is important because the use of cultured cells for such studies can introduce unpredictable artifacts resulting from the stress and selection that occur in cell culture.103 Several other studies have yielded findings consistent with these two landmark papers.104–108 Thus, there appears to be a robust and profound molecular difference between low-grade, nonmetastasizing (class 1) tumors and high-grade, metastasizing (class 2) tumors. The distinctive gene signatures of these two tumor classes provide a valuable new tool for understanding the biological processes underlying metastasis. The results of preliminary work in our laboratory suggest that previous prognostic markers for metastasis, such as epithelioid cytology and extracellular matrix patterns, may be phenotypic indicators of a more fundamental underlying difference in the differentiation program that is operative in class 2 tumors.109 How these changes in gene expression lead to metastasis is now the subject of intense investigation.

Implications for the Future

If these recent discoveries in uveal melanoma stand the test of time, they may eventually be viewed similarly to the identification of the RB gene and the profound impact that that event had on our understanding of retinoblastoma. The discovery that uveal melanomas form a binary classification of low- and high-grade, metastasizing (class 2) tumors will metastasize? Several clinical, pathologic, and cytogenetic features have been shown to predict metastatic disease, including larger tumor size, anterior tumor location, older patient age, epithelioid cell type, extracellular matrix deposition in looping patterns, and monosomy 3.95–99 To gain insights into the molecular mechanisms of uveal melanoma metastasis, we used microarray gene expression profiling to analyze primary, uncultured uveal melanomas.100 We found that primary uveal melanomas cluster naturally into two groups that we provisionally refer to as classes 1 and 2, based on gene-expression signature (Fig. 6). Survival analysis showed that patients with class 1 tumors rarely died of metastasis, whereas those with class 2 tumors had a high rate of metastatic death. Correspondingly, class 2 tumors were more likely to exhibit epithelioid cells, looping extracellular matrix patterns, and monosomy 3.100,101 Similarly, the Lohmann laboratory102 in Germany independently found that uveal melanomas form two molecular classes that correspond to chromosome 3 status. The gene signatures from these two studies were remarkably similar, despite differences in study design and patient population, indicating that these molecular signatures may be generalizable to other populations. Further, these were the first large-scale studies of gene expression using primary, uncultured uveal melanomas, which is important because the use of cultured cells for such studies can introduce unpredictable artifacts resulting from the stress and selection that occur in cell culture.103 Several other studies have yielded findings consistent with these two landmark papers.104–108 Thus, there appears to be a robust and profound molecular difference between low-grade, nonmetastasizing (class 1) tumors and high-grade, metastasizing (class 2) tumors. The distinctive gene signatures of these two tumor classes provide a valuable new tool for understanding the biological processes underlying metastasis. The results of preliminary work in our laboratory suggest that previous prognostic markers for metastasis, such as epithelioid cytology and extracellular matrix patterns, may be phenotypic indicators of a more fundamental underlying difference in the differentiation program that is operative in class 2 tumors.109 How these changes in gene expression lead to metastasis is now the subject of intense investigation.

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CONCLUSIONS

A cogent argument could be made that the modern era of molecular oncology started with ocular cancer. The discovery of the retinoblastoma gene heralded a revolution in cancer research and ushered in the concept of tumor suppressors. The importance of Rb in developmental, cellular, and cancer biology has stood the test of time and will continue to influence more and more areas of biomedical research, including neuroprotection, stem cell biology, and tissue regeneration. Likewise, the impact of uveal melanoma on cancer biology far outweighs its incidence. This cancer provides an important model for studying the pathobiology of tumor progression and metastasis. Though there are still many gaps in our understanding, we can now begin to construct a provisional sequence of major genetic events in uveal melanoma progression (Fig. 7). It is likely that scientific advances in retinoblastoma and uveal melanoma will continue to benefit patients with these potentially lethal cancers, as well as those with a wide array of other maladies.

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