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Identification of the Novel Role of pRB in Eye Cancer

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Abstract Cancer of the eye, though relatively rare, can involve multiple areas. Retinoblastoma is the most common primary intraocular cancer in children, with 3–7 cases per million people per year worldwide. Uveal melanoma is the most common primary intraocular cancer in adults, predominately in whites, with annual incidence of six per million people in the United States and Europe. Despite the rarity of retinoblastoma, Knudson's two-hit hypothesis to explain its genesis was substantiated by elegant genetic studies and is viewed as a turning point in cancer research. pRB plays an important role in cell cycle and apoptosis, performing its function through interaction with transcription factors, p53, and MDM2. Unfortunately, advances in eye cancer treatment have not paralleled those in treatment of other sites of cancer. In spite of higher accuracy in early diagnosis, eye-cancer-specific mortalities have remained unchanged for decades, while overall cancer mortality rates have dramatically declined. An extensive literature search revealed that, except for retinoblastoma, few investigations had been done on the pRB pathway in eye cancers even though altered pRB expression has been associated with a number of cancers. Early detection of eye cancer is critical for the prognosis of both vision and survival. Mutation analysis should become an integral part of future management of patients with eye cancer. Characterization of the mutational pattern of RB1 is crucial in identifying predisposition for cancer of many sites including the eye. Furthermore, cost-effective and efficient genetic mutation screen testing methods, which can be used to categorize mutant RB1 carriers, are needed. Illumination of genetic insights can guide clinicians to develop a rational strategy for cancer treatment and help predict prognosis in cancer patients. J. Cell. Biochem. 88: 121–127, 2003.

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The eye has three major parts: the globe (eyeball), the orbit, and the adnexal structures. The eyeball has two important parts: the retina and the uvea. Cancers that affect the eyeball are called intraocular cancers. The orbit consists of the tissues surrounding the eyeball, and cancers of these tissues are called orbital cancers. The adnexal structures include the eyelids and lacrimal (tear) glands. Cancers that develop in these tissues are called adnexal cancers. Cancers of the orbit and adnexa develop from tissues such as muscle, nerve, or skin just as do their counterparts elsewhere in the body.

In children, retinoblastoma (a cancer arising from cells in the retina) is the most common primary intraocular cancer, and medulloepithelioma is the second. Uveal melanoma is the most common primary intraocular cancer in adults, followed by primary intraocular lymphoma. The other eye cancers, which are even rarer, are listed in Table I. For decades, studies of eye cancer have focused on retinoblastoma, uveal melanoma, and metastases within the eye, as well as lymphoma and rhabdomyosarcoma in the orbit and adnexal tissues. Occasionally ocular oncology also includes tumors of dermal, osseous, neural, vascular, hematologic, soft tissue, and mucous membrane, as well as glandular derivations. Intraocular neoplasms usually differ from their counterparts in other tissues in regard to clinical behavior, epidemiological features, and underlying genetics.

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TABLE I. Classification of Primary Malignant Neoplasms in the Eye

Type of eye cancer by anatomic site	Name of the eye cancer
Intraocular cancers	Retinoblastoma[a]
	Uveal melanoma[a]
	Lymphoma (reticulum cell sarcoma)
	Leukemia/lymphoma
	Medulloepithelioma
	Rhabdomyosarcoma
	Leiomyosarcoma
	Neuroepithelial adenocarcinoma
Orbital cancers	Lymphoma
	Rhabdomyosarcoma
	Granulocytic sarcoma
	Multiple myeloma
	Malignant peripheral nerve sheath tumor
	Alveolar soft part sarcoma
	Malignant melanoma
	Fibrosarcoma
	Leiomyosarcoma
	Chondrosarcoma
	Liposarcoma
	Malignant glioma of optic nerve
	Endodermal sinus tumor
Adnexal cancers	Basal cell carcinoma
	Bowen's disease
	Squamous cell carcinoma
	Malignant melanoma
	Sebaceous cell carcinoma
	Trabecular (Merkel) cell carcinoma
	Lymphoma/leukemia
	Kaposi's sarcoma
	Malignant eccrine tumors
	Malignant pilar tumors
	Lacrimal gland carcinoma

[a]Discussed in text.

Retinoblastoma occurs sporadically but is more prevalent in persons with a family history of the disease. The annual incidence of retinoblastoma varies from seven per million in African populations, where it represents 10% to 15% of childhood neoplasms, to 3 to 6 per million in the United States and Europe, where it accounts for 2% to 4% of childhood cancers, to lower than three per million in Asian populations [Parkin et al., 1988]. Despite the rarity of retinoblastoma, elegant genetic studies of the disease led to the identification of the first human tumor suppressor gene, *RB1*; sophisticated statistical analysis of familial versus nonfamilial cases of retinoblastoma resulted in the famous "two-hit" carcinogenetic hypothesis [Knudson, 1996].

Uveal melanoma is another rare type of intraocular cancer with a rate of six new cases per million persons per year in the United States and Europe. Uveal melanoma usually occurs sporadically with an absence of obvious genetic predisposing factors; in a few patients, however, genetic predisposition may be involved. Although cutaneous and uveal melanocytes are both derived from the neural crest and share similar embryological, morphological, and antigenic properties, coexistence of these two diseases may be coincidental. Since 1905, when Parson first described a family with a history of four generations with uveal melanoma—a Victorian detective story of sorts—family history and pedigree analysis have been traditionally used in ophthalmologic genetic studies.

Germline mutations in RB1 are associated with childhood retinoblastomas and a predisposition to osteosarcomas [Hickman et al., 2002]. Somatic mutations in RB1 contribute to the development of several human tumors, including retinoblastomas, osteosarcomas, lung carcinomas, renal cell carcinomas, and bladder carcinomas [Cance et al., 1990; Cordon-Cardo et al., 1997; Dosaka-Akita et al., 1997]. The pRB, protein product of RB1, plays a critical role in cell cycle and apoptosis by governing the passage of cells through the G1 phase-restriction point, promoting terminal differentiation, and preventing cell cycle re-entry.

Most previous reports have focused on either the p53 or the pRB pathway; few have discussed the interplay of these two pathways. The identification of p21WAF1/CIP1 as a p53 target gene indicates that p53 can control the phosphorylation status of pRB and regulate its activity. Both p53 and pRB can interact with the same oncoprotein, MDM2, an indication of an intimate molecular and genetic interaction between the p53 and pRB pathways. This article will focus on the most recent advances in research on pRB and its role in cell cycle, apoptosis, and tumorigenesis with emphasis on its interaction with p53, and we will propose some suggestions for future research in genetic aspects of eye cancer.

Molecular Characteristics of RB1 and pRB

RB1 contains 27 exons within 180 kb of genomic DNA; it is mapped by linkage studies and deletion analysis to the q14 region of human chromosome 13. RB1 produces a 4.8-kb mRNA, within which 2.7 kb encode a protein (pRB) of 928 amino acids. The pRB is a 105-kDa nuclear phosphoprotein belonging to the pocket protein family. RB1 contains three distinctive domains: the N-terminus, the central A and B domains separated by a linker region, and the C-terminal region that degrades with mild proteolysis. The structural integrity of these domains is required for biological functions, including the regula-

tion of growth and differentiation of the cell; biochemical activities, including transcriptional regulation; and interaction with viral and cellular proteins [Zheng and Lee, 2001].

Role of pRB in Cell Cycle

The pRB executes its biological effects by both positively and negatively regulating transcription. Positive gene regulation is associated with cell differentiation. Transcription repression is associated with inhibition of the cell cycle. The E2F family is a group of sequence-specific DNA-binding transcription factors that regulate the timing and level of expression of many genes involved in cell cycle regulation [Zheng and Lee, 2001]. Five of six known E2F family members have a pRB-binding motif embedded in a C-terminal transactivation domain. When bound to this motif, pRB represses transcription by several mechanisms. First, pRB prevents E2F from interacting with factors such as TBP. This simple competitive effect on an activation domain is termed *quenching*. Second, once tethered to a promoter by E2F, pRB can simultaneously bind the activation domain of another activator. In this way, pRB quenches two activators at the same time. Third, the E2F/pRB complex can inhibit other activators even though they are not pRB-binding targets. This type of "direct" or "active" repression is mediated by pRB (not E2F) since a GAL4-RB fusion protein efficiently inhibits promoters bearing GAL4 binding sites. It has been proposed that pRB carries out this function by recruiting one or more of several co-repressors [DiCiommo et al., 2000].

The pRB is phosphorylated by cyclin D-cdk4/t and cyclin E-cdk2. Hypophosphorylated forms of pRB predominate in the early G1 phase and reappear during the M phase. They bind target proteins and arrest cells in G1. Hyperphosphorylated forms of pRB are present from the late G1 phase throughout the S, G2, and M phases. The phosphorylation of pRB is believed to remove the block to G1 phase progression. Phosphorylated pRB no longer interacts with its associated proteins, such as E2F2 and HDACs, and release of pRB from these complexes negates all mechanisms of transcription repression, thus activating the transcription of genes required for S phase entry and DNA synthesis. Meanwhile, E2Fs induce the expression of cyclin E and cyclin A, and, in turn, cyclin E/cdk2 collaborates with cyclin D/cdk4,6 to complete pRB phosphorylation [Kelly et al., 1998; Lundberg and Weinberg, 1998].

Role of pRB in Apoptosis

Apoptosis is one of the consequences of the entry of a cell into S phase unsupported by proliferation signals and factors. Apoptosis is the common result of loss of pRB or deregulation of E2F [Tao and Levine, 1999]. The pRB-deficient mice that die during embryogenesis display extensive apoptosis, an ectopic S phase, and a lack of differentiation in the central and peripheral nervous system, ocular lens, and liver. Because pRB is activated on exit of the cell cycle during differentiation, it is assumed that the function of pRB is to protect differentiating cells from apoptosis [Hickman et al., 2002].

Recent studies using mice chimeric for wild-type and pRB-deficient cells showed that the extensive apoptosis and lack of differentiation that occur in $RB^{-/-}$ cells were suppressed in chimeric mice [Lipinski et al., 2001]. It has been postulated that wild-type cells produce a survival sign that is abrogated in pRB-deficient cells; however, so far no signals in the form of either secreted factors or proteins mediating cell–cell contacts have been characterized. More is known about the cell autonomous mechanisms through which loss of pRB induces apoptosis [Hickman et al., 2002].

Cell death in the $RB^{-/-}$ central nervous system (CNS) and lens is p53-dependent. Studies have observed a strong correlation between loss of pRB and lack of functional p53 in human cancer [Weinberg, 1995]. Isolation of ARF as a transcriptional target of E2F1 sheds light on p53-dependent apoptosis by pRB. It is postulated that E2F1-mediated transcriptional activation of ARF is linked to apoptosis through increased levels of p53 and inactivation of MDM2 [Zhang et al., 1998]. Such a mechanism could account for the induction of cell death in many tissues lacking pRB function. Recently, a gene expression analysis revealed that many potential E2F target genes may be involved in E2F-mediated apoptosis [Stiewe and Putzer, 2000]. It has been suggested that the ability of E2F1, E2F2, and E2F3 to induce apoptosis correlates with their ability to activate expression of APAF1 [Moroni et al., 2001].

Another pRB-associated protein implicated in pRB-regulated apoptosis is Id2, an inhibitor of the basic helix-loop-helix (bHLH) class of transcription factors essential for cell differentiation.

The loss of Id2 has been reported to suppress many defects observed in pRB-deficient embryos [Lasorella et al., 2000]. Some suggest that Id2 and pRB work in a linear pathway, while others think that Id2 and pRB work in two parallel pathways controlling proliferation, differentiation, and apoptosis [Hickman et al., 2002].

Interaction of pRB and p53 via MDM2

The discovery that a single genetic locus, the CDKN2A locus that produces two unrelated proteins, one of which regulates the pRB pathway and other the p53 pathway, provided the first evidence of the interrelationship between these cell growth and cell death pathways [Stewart et al., 2001]. MDM2 was originally discovered as a gene overexpressed in the tumorigenic 3T3DM mouse cell line that stably maintains double minute chromosomes [Fakharzadeh et al., 1991]. MDM2 not only modulates p53 activity but also regulates p53 protein level. The rescue of the embryonic lethality of MDM2 null mice in a p53 null background strongly suggests that MDM2 is an important cellular inhibitor of p53. While MDM2 can bind to p53 at its transactivation domain and inhibit its transactivation activity, p53 can also bind to the promoter region of MDM2 and activate its transcription, thereby forming an autoregulation loop between the expression and function of p53 and MDM2. The MDM2-p53 interaction can also inhibit p53-induced apoptosis. In addition to its interaction with p53, MDM2 can bind to the C-terminus of pRB and inhibit the regulation of E2F1 activity by pRB. The binding of MDM2 to pRB can overcome a pRB-induced G1 arrest in U2-OS cells. Furthermore, MDM2 directly stimulates the transcriptional activity of E2F1. Recent evidence shows that pRB impairs certain functions of MDM2 during the process of forming a trimeric complex with p53; pRB overcomes the ability of MDM2 to inhibit p53-mediated apoptosis [Martin et al., 1995].

pRB Pathway and Tumorigenesis

Knudson's two-hit model specifies the simplest molecular mechanism of hereditary tumorigenesis—the first mutation in an allele of the RB1 exists in the germline and a subsequent somatic mutation in the second normal RB allele causes retinoblastoma formation in the eye. The two-hit model is viewed as a turning point in cancer research. In fibrosarcoma, brain tumors, bladder cancer, breast cancer, and leukemia, pRB is commonly inactivated directly by mutation, a viral oncoprotein, or alteration of proteins in the pathway of pRB regulation. RB1 mutations are predominantly associated with retinoblastoma and small cell carcinoma of the lung, while pRB is inactivated by virus in cervical carcinoma [Harbour et al., 1988]. The developing retina is at nearly 100% risk of forming retinoblastoma when both RB alleles are mutated, but only a few other types of tumors, such as sarcoma and melanoma, are initiated with loss of pRB. Elucidating the mechanisms by which pRB plays a critical role will enable novel therapies and strategies for prevention, not only for retinoblastoma but for other eye cancer and cancer in general.

Epidemiological Studies of pRB and Cancer

Although the two relationships are equally important, there have been far fewer epidemiological studies of pRB and cancer than of p53 and cancer. A search of "pRB and epidemiology and cancer" in the National Library of Medicine's PubMed database returned only 85 hits. In contrast, 1765 hits were found for "p53 and epidemiology and cancer." Among the few pRB studies, aberrant expression of pRB has been associated with and/or are shown to be an independent prognostic marker in esophageal squamous cell carcinoma [Xing et al., 1999], laryngeal carcinoma [Dokiya et al., 1998], large B cell lymphoma [Moller et al., 2000], prostate cancer [Krupski et al., 2000], and choroidal melanoma [Massaro-Giordano et al., 1999], though one study revealed no association of altered expression of pRB alone and death in bladder cancer patients [Jahnson and Karlsson, 1998].

Synergistic effects of concomitant mutations of p53 and RB1 have been reported in non-small cell lung cancer [Dosaka-Akita et al., 1997] and bladder cancer [Cordon-Cardo et al., 1997]. That concomitant alterations in both p53 and pRB increase tumor recurrence and decrease survival in several types of cancer suggests an interaction of pRB and p53 pathways in suppressing tumor development and progression. Other studies have shown an additive growth effect of altered expression of pRB, p53, and MDM2 in non-small cell lung carcinomas [Gorgoulis et al., 2000] and in prostate cancer [Theodorescu et al., 1997], thus supporting the

concept that these molecules constitute a tightly regulated network participating in cell cycle control, apoptosis, and chromosomal stability. One study, however, showed conflicting results regarding p53 and pRB in survival of colorectal cancer patients [Poller et al., 1997].

RB1/pRB and Eye Cancers

Like p53, pRB is expressed ubiquitously and is intimately associated with regulation of the cell cycle and apoptosis. Because pRB acts as a general cancer suppressor and the disruptions of its pathway play a critical role in tumorigenesis, its importance to research in RB1/pRB and eye cancers is no surprise. An extensive literature search of the PubMed database showcased articles on RB1/pRB in retinoblastoma (n = 10,866), uveal melanoma (n = 4), medulloepithelioma (n = 24), rhabdomyosarcoma (n = 61), leiomyosarcoma (n = 13), and orbital sarcoma (n = 1). But no articles were found on RB1/pRB and ocular/intraocular lymphoma, neuroepithelial adenocarcinoma, other orbital cancers, or any type of adnexal cancers. Although tremendous work has been done on pRB and retinoblastoma, further studies are still needed to evaluate the precise role of pRB loss in the development of non-hereditary retinoblastoma. Obviously, little is known about pRB/RB1's role in eye cancers other than retinoblastoma.

Mutations of the RB1 are known to cause both non-hereditary and hereditary forms of the cancer. There is a definite need for cost-effective and efficient methods that can be used to identify mutant RB1 carriers, so that genetic counseling can be available to patients and their relatives. Identification of RB1 mutations contributes to the early diagnosis of retinoblastoma [Najera et al., 2001], and early detection of eye cancer is critical to the prognosis for both vision and survival. The process for such genetic testing should maximize benefits while minimizing risk, and this balance depends on an understanding of the complete role of RB1/pRB and their alterations in eye cancers. Illumination of genetic insights can guide clinicians to develop a rational strategy for cancer treatment.

Gene therapy is an advanced therapeutic technique in which a functioning gene is inserted into a cell to correct a metabolic abnormality or to introduce a new function. Because cancer is the result of mutation or loss of genetic material within cells, an ideal anticancer approach is to introduce genes to correct those aberrations. Restoration of tumor suppressor genes—for example, the *p53* gene—has been initiated. Because of the easy anatomic accessibility of the eye, ocular cancer might be an optimal model for RB1 gene therapy. A full understanding of the role of this gene in eye cancers is key for achieving the goal.

Hereditary retinoblastoma, which is usually diagnosed at a younger age than non-hereditary retinoblastoma, has been found to be associated with germline RB1 mutations [Jakubowska et al., 2001]. Mutation of the *RB1* gene causes retinoblastoma at an early age [Damjanovich et al., 2000]. Early onset of secondary malignancies such as osteosarcoma is associated with mutation of one allele of the RB1 [Chauveinc et al., 2001]. Our previous report indicated that exon location of the mutation, type of mutation, and multiple mutations were associated with early onset of breast cancer [Lai et al., 2002]. Similar studies have not been done for RB1, either alone or in combination with the *p53* gene, in any type of eye cancer other than retinoblastoma. Future studies of the RB1 mutational pattern in relation to age at eye cancer onset are important and warranted. Furthermore, population-based studies are needed to evaluate the prognostic effects of the interaction between RB1 and *p53* genes in eye cancer. Finally, given the very low incidence of any type of eye cancer, multi-center collaborative studies should be encouraged for sufficient numbers of cases to carry out reliable genetic analyses.

CONCLUSION

Partially due to the lag in genetic studies of eye cancer, advances in eye cancer treatment have not paralleled those for other types of cancer. While the overall cancer mortality rate has dramatically declined [Jemal et al., 2002], eye-cancer-specific mortalities have remained unchanged for decades despite higher accuracy in early diagnosis. A long-term, multi-center, randomized clinical trial supported by the National Eye Institute (NEI) and the National Cancer Institute (NCI), called the Collaborative Ocular Melanoma Study (COMS), has concluded that the survival rates for two alternative treatments—radiation therapy and removal of the eye—are about the same [Benson, 2002]. Unfortunately, enucleation, a treatment for eye cancer which dates back to more than a century,

is still in practice as the standard treatment for intraocular cancer. Such a therapeutic approach is incompatible with patients' desire for quality of life in the era of modern medicine. A shift in research toward investigating the genetic basis of eye cancer is therefore urgent.

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