Molecular Basis of Low-Penetrance Retinoblastoma

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Retinoblastoma is a malignant tumor of the retina that occurs primarily in young children as a result of mutations in the retinoblastoma gene (RB), the first tumor suppressor gene to be identified. In about 35% to 40% of patients with retinoblastoma, an RB gene mutation is present in the germline, resulting in hereditary transmission of the disease. Most families with hereditary retinoblastoma demonstrate autosomal dominant inheritance with almost complete penetrance and high expressivity. However, some families display an inheritance pattern characterized by reduced penetrance and expressivity. Recent advances in our understanding of the structure and function of the retinoblastoma protein (pRB) now provide new insights into the molecular basis of this low-penetrance form of retinoblastoma. Low-penetrance retinoblastoma mutations either cause a reduction in the amount of normal pRB that is produced (class 1 mutations) or result in a partially functional mutant pRB (class 2 mutations).


Retinoblastoma has played a critical role in our understanding of hereditary predisposition to cancer. Retinoblastoma occurs as a result of germline, hereditary mutations in 35% to 40% of patients, whereas the other cases are caused by somatic, non-hereditary mutations. Clinical observations of age at diagnosis, tumor number, tumor laterality, and family history led Knudsen in 1971 to postulate the 2-hit hypothesis, a mathematical model that predicted the existence of recessive cancer genes or tumor suppressor genes. Central to this hypothesis was the concept that both cellular alleles of the putative retinoblastoma (RB) gene must be inactivated in a developing retinoblast for malignant transformation. Subsequent work confirmed the Knudsen hypothesis by identifying the RB gene on chromosome 13q14 and showing that this gene is mutated in retinoblastomas, as well as other types of cancer. The RB gene was thereby established as the prototypical tumor suppressor gene. Most retinoblastoma families demonstrate autosomal dominant inheritance with almost complete penetrance and high expressivity. However, some families display a different inheritance pattern characterized by reduced penetrance and expressivity. Recent advances in our understanding of the structure and function of the retinoblastoma protein (pRB) now provide new insights into the molecular basis of this low-penetrance form of retinoblastoma. These insights have improved the accuracy of diagnostic testing and family counseling for retinoblastoma and may eventually aid in establishing new treatment strategies.

In most families with retinoblastoma, 80% to 90% of gene carriers develop eye tumors (high penetrance), and most affected individuals develop multiple bilateral tumors (high expressivity). However, in some families a significant proportion of carriers remain unaffected (reduced penetrance), and many affected individuals have only unilateral retinoblastoma or benign retinocytomas (reduced expressivity). The diseased-eye ratio, the ratio of the number of eyes containing tumors to the number of mutation carriers in a family, was devised to quantitatively identify low-penetrance retinoblastoma families by taking into ac-
mutations may not cause tumors with low-penetrance mutations. Approximately 60% of second hits that are tumorigenic with full-penetrance help to explain the phenomenon of low-penetrance retinoblastoma, since mutation are sufficient to suppress tumorigenesis. This mechanism could predictions are based on the assumption that 2 copies of a low-penetrance hit involves a full-penetrance vs a low-penetrance mutation. These predicted result of the 2 hits (tumor vs no tumor) is indicated when the first RB gene locus at chromosome 13q14, with unaffected car-

In recent work, the molecular nature of these weak RB gene alleles has become more clear. Nine different RB gene mutations have now been described in low-penetrance retinoblastoma families (Table 1 and Table 2), 8,12,13-20 These mutations fall into 2 functional classes: mutations that reduce the level of expression of normal pRB (class 1) and mutations that result in a mutant pRB that is partially inactivated (class 2). 21 Virtually all low-penetrance mutations reported to date can be classified according to this scheme.

MOLECULAR MECHANISMS OF LOW-PENETRANCE RETINOBLASTOMA

In this model, mutations that result in only 1 copy of the weak allele, such as nondisjunction without reduplication or a small intragenic mutation, would lead to retinoblastoma (Figure 1). Importantly, these mechanisms account for only about 40% of somatic mutations in retinoblastoma. 14 In contrast, mutations that lead to retention of both alleles, such as nondisjunction with reduplication or mitotic recombination, would be tu-
mogenic in full-penetrance retinoblastoma but not in the low-penetrance form. Since this latter group of mutations accounts for up to 60% of somatic mutations in retinoblastoma, 14 the weak allele model could at least partly account for the reduced penetrance and expressivity in low-penetrance retinoblastoma families.

In one of the first reports of low-penetrance retinoblastoma, Strong et al 13 described a family that transmitted a constitutional chromosomal deletion involving the RB gene locus at chromosome 13q14, with unaffected car-

Class 2 low-penetrance mutations do not necessarily re-
duce the level of protein expression, but rather they af-

MOLECULAR MECHANISMS OF LOW-PENETRANCE RETINOBLASTOMA

Class 1 mutations, which appear to be less common than class 2 mutations, do not alter the structure or function of pRB, but they cause a reduction in the expression level of the normal protein (Table 1). Two subtypes of class 1 mutations have been described: promoter mutations and splice site mutations. Point mutations within the RB gene promoter can cause low-penetrance retinoblastoma interfering by blocking the binding sites for transcription factors, such as SP1, ATF, and other proteins. 15,16 The ATF and SP1 binding sites are known to be required for normal pRB expression 22; hence, these mutations presumably interfere with assembly of the transcriptional machinery at the promoter and thereby reduce efficient gene expression. Another interesting class 1 mutation affects a splice site at exon 21. 37 A G-to-A transition at the last base in exon 21 results in no change in the encoded amino acid (glutamine), but it reduces the match of the exon boundary acceptor site to the splicing consensus. Consequently, most of the transcribed messenger RNA from the mutant allele encodes a nonfunctional protein lacking exon 21, but about 10% of the messenger RNA encodes the normal protein. Presumably, this class of mutations causes low-penetrance retinoblastoma because 1 copy of the mutant RB allele produces a low level of pRB that is insufficient to prevent tumorigenesis. 21

Figure 1. Chromosomal events leading to tumorigenesis in retinoblastoma (RB) according to the Knudsen 2-hit hypothesis. 3 The first hit or mutation of the RB gene occurs in either the germline or a somatic cell (eg, retinoblast). The second hit, which is always a somatic event that disrupts the remaining RB allele, usually involves one of the indicated mechanisms (their approximate frequencies in retinoblastoma tumors are indicated). 3 The predicted result of the 2 hits (tumor vs no tumor) is indicated when the first hit involves a full-penetrance vs a low-penetrance mutation. These predictions are based on the assumption that 2 copies of a low-penetrance mutation are sufficient to suppress tumorigenesis. This mechanism could help to explain the phenomenon of low-penetrance retinoblastoma, since approximately 60% of second hits that are tumorigenic with full-penetrance mutations may not cause tumors with low-penetrance mutations. RB indicates RB gene mutation; wt, wild type.

GENETIC MECHANISMS OF LOW-PENETRANCE RETINOBLASTOMA

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cell cycle progression at the G1-to-S phase transition. This interaction of pRB is due to, at least in part, its ability to inhibit binding sites and varied functions. The tumor suppressor function of pRB, which is a complex molecule with multiple binding proteins, is now confirmed the importance of these regions in tumor suppression.\(^*\) Furthermore, pRB was noted to inhibit cell cycle progression through binding to proliferative factors such as E2F. However, binding of pRB to histone deacetylases (HDAC), chromatin remodeling adenosine triphosphatases (eg, BRG1), and other proteins is also critical for its ability to control the cell cycle. In addition, pRB can also suppress tumorigenesis through mechanisms other than cell cycle control, such as by inducing differentiation. The ability of pRB to induce differentiation may be linked to its ability to bind proteins such as Id2 and myoD. Some low-penetrance mutations appear to result in the production of a mutant pRB that cannot control the cell cycle but can still induce differentiation.

**STRUCTURE AND FUNCTION OF pRB**

The RB gene encodes a 105-kd nuclear phosphoprotein, pRB, which is a complex molecule with multiple binding sites and varied functions. The tumor suppressor function of pRB is due to, at least in part, its ability to inhibit cell cycle progression at the G1-to-S phase transition. This cell cycle regulatory function of pRB is largely due to its ability to bind and inhibit the E2F family of transcription factors that activate genes involved in cell division (Figure 2).\(^*\) The E2Fs bind to pRB in the pocket domain and also in the carboxy-terminal region of the protein. Thus, mutations at either of these locations could reduce binding to E2F.

Many other proteins bind to the pocket domain of pRB and may play a role in pRB-mediated cell cycle control. For example, pRB actively represses transcription of cell cycle genes by recruiting histone deacetylases (HDAC) and adenosine triphosphatases (eg, BRG1) to the promoters, where these enzymes remodel local chromatin structure into an inactive state.\(^*\) The expanding role of the retinoblastoma protein (pRB) in tumor suppression. Initially, pRB was noted to inhibit cell cycle progression through binding to proliferative factors such as E2F. However, binding of pRB to histone deacetylases (HDAC), chromatin remodeling adenosine triphosphatases (eg, BRG1), and other proteins is also critical for its ability to control the cell cycle. In addition, pRB can also suppress tumorigenesis through mechanisms other than cell cycle control, such as by inducing differentiation. The ability of pRB to induce differentiation may be linked to its ability to bind proteins such as Id2 and myoD. Some low-penetrance mutations appear to result in the production of a mutant pRB that cannot control the cell cycle but can still induce differentiation.

**Table 1. Class 1 Low-Penetrance Retinoblastoma Mutations That Reduce Expression of Normal Retinoblastoma Protein**

<table>
<thead>
<tr>
<th>Mutation No.</th>
<th>Diseased-Eye Ratio</th>
<th>DNA Alteration</th>
<th>Location</th>
<th>Functional Significance</th>
<th>Reference</th>
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<tbody>
<tr>
<td>1</td>
<td>0.88</td>
<td>G→A at −198</td>
<td>Promoter</td>
<td>SP1 binding site</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>G→T at −189</td>
<td>Promoter</td>
<td>ATF binding site</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>0.34</td>
<td>G→C at −149</td>
<td>Promoter</td>
<td>Transcription factor binding site</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>0.64</td>
<td>G→A at 2215</td>
<td>Exon 21</td>
<td>Exon splice site</td>
<td>17</td>
</tr>
</tbody>
</table>

*G indicates guanine; A, adenine; T, thymine; C, cytosine; SP1, promoter specific factor 1; and ATF, activating transcription factor.

**Table 2. Class 2 Low-Penetrance Retinoblastoma Mutations That Partially Inactivate the Retinoblastoma Protein**

<table>
<thead>
<tr>
<th>Mutation No.</th>
<th>Diseased-Eye Ratio</th>
<th>AA Consequence</th>
<th>Domain Affected</th>
<th>Nuclear Localization</th>
<th>LXCXE Binding</th>
<th>E2F Binding</th>
<th>Colony Suppression</th>
<th>Differentiation</th>
<th>Temperature Sensitive</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.65</td>
<td>Delete AA127-166 (exon 4)</td>
<td>Amino-terminal (G2/M kinase binding site)</td>
<td>Yes</td>
<td>Minimal</td>
<td>NR</td>
<td>Reduced</td>
<td>Yes</td>
<td>Yes</td>
<td>NR</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>Delete Asn480 (exon 16) Arg661Trp (exon 20)</td>
<td>A box</td>
<td>Yes</td>
<td>Yes</td>
<td>Reduced</td>
<td>Minimal</td>
<td>Yes</td>
<td>Yes</td>
<td>NR</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>Cys712Arg (exon 21)</td>
<td>B box</td>
<td>Yes</td>
<td>Yes</td>
<td>Reduced</td>
<td>Minimal</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>Cys712Arg (exon 21)</td>
<td>B box (adjacent to LXCXE binding site)</td>
<td>Carboxy terminal (NLS, E2F and MDM2 binding sites)</td>
<td>Reduced</td>
<td>NR</td>
<td>Reduced</td>
<td>NR</td>
<td>Minimal</td>
<td>NR</td>
</tr>
<tr>
<td>5</td>
<td>0.78</td>
<td>Delete AA830-887 (exon 24-25)</td>
<td>Reduced</td>
<td>NR</td>
<td>Reduced</td>
<td>NR</td>
<td>Minimal</td>
<td>NR</td>
<td>NR</td>
<td>12</td>
</tr>
</tbody>
</table>

*AA indicates amino acid; LXCXE, amino acid motif; NLS, nuclear localizing signal; and NR, not reported.

**Figure 2.** The expanding role of the retinoblastoma protein (pRB) in tumor suppression. Initially, pRB was noted to inhibit cell cycle progression through binding to proliferative factors such as E2F. However, binding of pRB to histone deacetylases (HDAC), chromatin remodeling adenosine triphosphatases (eg, BRG1), and other proteins is also critical for its ability to control the cell cycle. In addition, pRB can also suppress tumorigenesis through mechanisms other than cell cycle control, such as by inducing differentiation. The ability of pRB to induce differentiation may be linked to its ability to bind proteins such as Id2 and myoD. Some low-penetrance mutations appear to result in the production of a mutant pRB that cannot control the cell cycle but can still induce differentiation.
As an example of a low-penetrance mutation within the pocket, Cys712Arg (conversion of cysteine at residue number 712 to arginine) is a missense mutation that occurs within the B box. The fact that the mutant protein can undergo phosphorylation (a characteristic requiring a relatively normal protein conformation to be recognized by specific kinases) suggests that this mutation subtly destabilizes pRB without grossly disrupting its structure. One potential insight into this mutation is its proximity to Lys-713, which is a key residue in the LXCXE binding site. Mutation of Lys-713 leads to a partially defective pRB protein that cannot bind LXCXE-containing proteins such as HDAC and has reduced tumor suppressor capacity (J.W.H., unpublished data, January 2001). The Cys712Arg mutant also has reduced binding to E2F, and it may directly or indirectly destabilize the E2F binding site. Thus, this low-penetrance mutation appears to partially inactivate pRB by reducing its binding affinity to LXCXE proteins and E2F.

THE CARBOXY-TERMINAL REGION

The importance of the carboxy-terminal region of pRB (amino acids 793-928) in tumor suppression was initially overlooked because most RB gene mutations truncate the protein upstream of this region, thereby masking its tumor suppression function. However, it is now clear that the carboxy-terminal region is important for tumor suppression and contains several functional sites, including a nuclear localizing signal, a second binding site for E2F, bindings site(s) for HDM2 and c-abl, 7 consensus cyclin-dependent kinase (CDK) phosphorylation sites, and CDK docking sites (Figure 3). A low-penetrance mutation in the carboxy-terminal region has provided important insights into the tumor suppressor function of this portion of the protein. This mutation results in an in-frame deletion of exons 24 and 25, excising amino acids 830 through 887 and thereby removing the nuclear localizing signal, the HDM2/c-abl binding site(s), and part of the E2F binding site. The mutant protein, as expected, has no binding to HDM2 and reduced binding to E2F. Interestingly, it still has some residual nuclear localization, since sequences in the pocket can also mediate nuclear entry. Thus, this mutant may retain enough E2F binding and nuclear localization to suppress tumors when expressed in a biallelic state. It is also possible that HDM2 binding constitutes a semisensational function in pRB-mediated tumor suppression, and loss of this function may become manifest as a tumor only in the monoallelic state.

THE AMINO-TERMINAL REGION

The amino-terminal region of pRB (amino acids 1-378) is the least understood portion of the protein. This region contains 6 consensus CDK phosphorylation sites, which may play a role in regulating pRB in the cell cycle. In addition, this region contains binding sites for MCM7 (a replication licensing factor), a novel kinase that phosphorylates both pRB and histone H1 in the G1/M phase of the cell cycle, and other proteins (Figure 3). Mice that have
been genetically engineered to lack pRB die before embryonic day 16 with severe developmental and apoptotic defects. Interestingly, expression of pRB lacking the aminoterminal region delayed the embryonic lethality, but it did not prevent it. Similarly, this aminoterminal–deficient mutant delayed but did not prevent the pituitary tumors seen in mice heterozygous for RB gene deletion. These results suggest that the aminoterminal region is important but not completely essential for pRB function and tumor suppression. Consistent with this notion, a mutation in the aminoterminal region has been shown to result in low-penetrance retinoblastoma, but mutations specific for this region are rare in full-penetrance retinoblastoma. This mutation causes an in-frame deletion of exon 4 (amino acids 127-166), removing the G2/M kinase binding site. The mutant protein also has reduced E2F binding affinity, but it can suppress colony formation in cultured tumor cells and has other tumor suppressor properties of normal pRB. Thus, this low-penetrance mutation appears to disrupt 1 or more semiessential functions in the aminoterminal region of pRB.

TERTIARY STRUCTURE OF pRB

We might overlook important molecular mechanisms of low-penetrance retinoblastoma if we examine only the linear proximity of mutations to binding sites and functional domains. The tertiary structure of pRB is enormously complex, and mutations at key structural residues can have profound effects on the overall protein structure. The recent crystallographic solution of pRB showed that the pocket domain, which consists of the conserved A and B boxes, forms a highly complex multifunctional unit that is held together by the A-B interface. This interface is critical to the pocket structure and is formed by highly conserved amino acid residues from both the A and B boxes that form a hydrophobic core. Mutations that disrupt the structural core of the A-B interface globally destabilize pRB and lead to a nonfunctional protein. As an example, Ser-567, a tumor-derived mutation in patients with bilateral retinoblastoma, forms part of a critical hydrogen bond network at the A-B interface. Alteration of this residue by amino acid substitution leads to a nonfunctional protein that cannot bind E2F or suppress tumor colony formation. Interestingly, we recently showed that phosphorylation of this site also disrupts the A-B pocket and may act as a potentially reversible mechanism for inactivating pRB during the cell cycle. In contrast to this inactivating mutation, low-penetrance mutations subtly affect tertiary structure in a manner that allows the protein to retain some residual function. For example, Arg661Trp is among the most frequently reported low-penetrance mutations. Arg-661 is located in the B box and participates in hydrogen bonding with amino acid residues in the A box. Thus, Arg-661 may help to stabilize the A-B interface, but it does not appear to be a part of the core structure of the interface. Thus, mutation of this residue may partially destabilize the A-B interface without grossly disrupting the pocket structure. This possibility is supported by the finding that the Arg661Trp mutant protein can be phosphorylated, and it approximates normal tumor suppressor activity at reduced temperature, suggesting that the protein may be relatively unstable but not grossly disrupted. It is also intriguing that this mutant has minimal E2F binding activity but can still suppress tumor colony formation. This appears to be a result of its ability to induce differentiation, rather than its inhibition of the cell cycle. It will be of interest to determine whether the Arg661Trp mutant induces differentiation by retention of binding to proteins involved in the differentiation program, such as Id2 and myoD.

Another low-penetrance mutation in the A box results in an in-frame deletion of Asn-480. This residue is not part of a known binding or structural motif, but recent work has provided several clues to understanding the functional consequences of this mutation. The mutant protein can undergo phosphorylation, and it achieves normal tumor suppressor activity at reduced temperature, suggesting that deletion of Asn-480 reduces protein stability but does not globally disrupt protein structure. However, the protein has reduced binding to LXCXE proteins and minimal binding to E2F. The partial activity of this mutant pRB may derive, at least in part, from its residual pocket activity, which could allow it to interact with pocket-binding differentiation factors such as Id2 and myoD. The ability of this mutant to promote differentiation has not been investigated. It may also be noteworthy that Asn-480 is adjacent to Phe-482, a highly conserved and hydrophobic residue that is normally buried within the protein, suggesting that it may play an important structural role. Deletion of Asn-480 could potentially cause Phe-482 to become exposed or otherwise compromised as a structural element.

CONCLUSIONS

Studies of low-penetrance retinoblastoma have yielded important new insights into the cellular function of pRB. These studies have already led to improved diagnostic testing and family counseling for retinoblastoma, and they may eventually inspire novel therapeutic strategies. Although the basis for low-penetrance retinoblastoma was initially attributed to immunologic factors or other mechanisms, it is now clear that most cases of low-penetrance retinoblastoma are due to a special class of mutations involving the RB gene. A classification scheme originally proposed by Otterson et al can be further subdivided to account for virtually all low-penetrance mutations reported to date. The common theme in all of these mutations is a reduction in the quantity or quality of cellular pRB activity. Insufficient quantity of normal pRB may result from mutations in the promoter or splice site sequences. In contrast, pRB may be partially disabled by subtle mutations that globally reduce the stability and binding affinity of the protein or that locally perturb semiessential functions. The tumor suppressor activity of pRB derives both from its ability to arrest the cell cycle and to induce differentiation. Some low-penetrance mutations appear to compromise preferentially one or the other of these functions, suggesting that regulation of the cell cycle and differentiation may play cooperative roles in tumor suppression by pRB. Further studies are needed to more clearly understand the mechanism of low-penetrance mutations and to apply this knowledge to improved care of patients with retinoblastoma.
19. Onadim Z, Hogg A, Baird PN, Cowell JK. Oncogenic point mutations in exon 20 of the RB1 gene in families showing incomplete penetrance and mild expression of the retinoblastoma phenotype. 

20. Cowell JK, Bia B. A novel missense mutation in patients from a retinoblastoma family showing only mild expression of the tumor phenotype. 


