ARTICLE

Retinoblastoma: Revisiting the Model Prototype of Inherited Cancer

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Hereditary retinoblastoma is an autosomal dominant disorder caused by mutations in the *RB1* gene. Analysis of this rare condition has helped to elucidate the mechanisms underlying hereditary cancer predisposition in general. As identification of *RB1* gene mutations has become a part of clinical management of patients with retinoblastoma, there is now a wealth of data. In this article, we summarize the current knowledge on the relations between the genotype and phenotypic expression. Moreover, detailed analysis of genotype–phenotype relations shows that hereditary retinoblastoma has features of a complex trait.

KEY WORDS: retinoblastoma; genotype–phenotype associations; mutation; hereditary cancer predisposition

INTRODUCTION

Retinoblastoma Phenotype

Retinoblastoma (OMIM# 180200) is a malignant tumor of the eye that originates from developing cells of the retina. The estimated incidence is between 1 in 15,000 and 1 in 20,000 live birth children [Suckling et al., 1982]. Diagnosis is based on clinical signs and symptoms and is usually made under the age of five years. Most often, the first presenting sign is a white pupillary reflex (leukocoria). Strabismus is the second most common sign, and may accompany or precede leukocoria. In adults, retinoblastoma is extremely rare and may originate from retinomas, which are benign precursor lesions. About 60% of patients have retinoblastoma in only one eye (unilateral retinoblastoma). In some of these patients, multiple tumor foci can be discerned (unilateral multifocal retinoblastoma). Most patients with unilateral retinoblastoma have sporadic disease, i.e., no other case of retinoblastoma has been noted in their family. About 40% of patients have retinoblastoma in both eyes (bilateral retinoblastoma). Often, these patients show more than one focus per eye (bilateral multifocal retinoblastoma). Generally, diagnosis of children with bilateral retinoblastoma is made earlier than diagnosis of children with unilateral retinoblastoma (median age at diagnosis 11 and 22 months, respectively). Only 10% of all patients have a positive family history of retinoblastoma (familial retinoblastoma). It is important to examine the retina of all first-degree relatives of retinoblastoma patients, as this may identify retinomas or retinal scars, which

It is important to examine the retina of all first-degree relatives of retinoblastoma patients, as this may identify retinomas or retinal scars, which may indicate predisposition to retinoblastoma, even though full malignant retinoblastoma did not develop.

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Dr. Brenda L. Gallie is an ophthalmologist who has focused on the rare cancer in children, retinoblastoma. These studies have contributed significantly to the basic molecular understanding of cancer in general, to applications for molecular diagnosis in healthcare, and to clinical trials with novel Internet use. From 15 years of translational research toward developing efficient and effective technologies to identify mutations for retinoblastoma families, Dr. Gallie has founded a not-for-profit reference lab, Retinoblastoma Solutions, to facilitate mutation testing on a worldwide basis to optimize outcome for families. She is presently Director of the Retinoblastoma Program at The Hospital for Sick Children and Head of the Division of Cancer Informatics at the Ontario Cancer Institute, Princess Margaret Hospital, University of Toronto, Toronto, Canada. She continues to care for children with retinoblastoma, leads a basic science group working to identify precisely why children get retinoblastoma, and is initiating world clinical trials with novel informatics tools and tests for *RB1* mutations through Retinoblastoma Solutions.

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may indicate predisposition to retinoblastoma, even though full malignant retinoblastoma did not develop. Patients with familial or bilateral retinoblastoma have an increased risk of specific neoplasms outside of the eye (second tumors) including osteogenic sarcoma, soft tissue sarcoma, and malignant melanoma [Eng et al., 1993]. The risk for a second tumor to develop is further increased in patients who have received external beam radiation for treatment of bilateral retinoblastoma [Eng et al., 1993; Abramson and Frank, 1998].

Compared to other solid neoplasms, retinoblastomas are small at the time of initial diagnosis. Treatment of retinoblastoma depends on tumor stage, the number of tumor foci (unifocal, unilateral multifocal, or bilateral disease), localization and size of the tumor(s) within the eye, presence of vitreous seeding, and the age of the child. Treatment options include removal of the eye (enucleation), external-beam radiation, chemotherapy, cryotherapy, photocoagulation, and brachytherapy with episcleral plaques. Following successful treatment of intraocular tumors, children still require frequent follow-up examinations for early detection of new intraocular tumors and early treatment of recurrent tumors. If tumor cells have not yet invaded extraocular tissues, treatment is successful in most patients. Metastasizing retinoblastoma has been fatal until recently, when aggressive therapy appears to have cured some children.

Genetics of Retinoblastoma

Environmental factors play no discernable role in the etiology of retinoblastoma [Buckley, 1992], although certain parental occupations appear to increase the risk of retinoblastoma in the children [Bunin et al., 1990]. The importance of hereditary factors has been recognized since the 19th century. Familial aggregation of retinoblastoma was noted as early as 1821 [Kaelin, 1955]. After treatment improved, more patients reached adult age and had children with retinoblastoma, showing an autosomal dominant mode of inheritance. Initially, all cases were regarded as hereditary, but later it was recognized that in a significant proportion of patients with sporadic retinoblastoma, the etiology is nonhereditary [Vogel, 1954]. Knudson [1971] proposed a model to explain the genetic mechanisms underlying hereditary and nonhereditary retinoblastoma. According to his hypothesis, both the hereditary and nonhereditary forms of retinoblastoma are caused by two mutations (two-hit hypothesis):

1. Heritable retinoblastoma arises when the first mutation is inherited via germinal cells. Tumor foci are initiated by the second mutation in somatic retinal cells. Many children with heritable retinoblastoma have new germinal mutations, and both parents are normal. Tumors may be unilateral or bilateral.
2. In the nonhereditary form of retinoblastoma, the two mutations occur in somatic retinal cells. Only one eye is affected.

Experimental evidence established that the two mutations that are required for retinoblastoma to occur target one gene locus on chromosome 13q14 [Cavenee et al., 1983; Godbout et al., 1983]. Second mutations that result in tumor formation often result in loss of the normal allele and, concomitantly, loss of heterozygosity (LOH) at polymorphic loci. In 1986, the cDNA of the *RB1* gene was identified, starting from a cloned fragment of genomic DNA that was found to be homozygously deleted in a retinoblastoma [Dryja et al., 1986; Friend et al., 1986]. The *RB1* gene (GeneBank accession number L11910) consists of 27 exons that are scattered over 183 kb of genomic sequence on chromosome 13q14. At its 5′-end, the *RB1* gene has a CpG-island, which is normally unmethylated. The promoter region contains binding motifs for transcription factors Sp1 and ATF, but no TATA or CAAT elements. In tissues investigated so far, the gene is transcribed into a 4.7-kb mRNA with no convincing evidence of alternative splicing. The open reading frame, which starts in exon 1 and is terminated in exon 27, spans over 2.7 kb and is followed by a 2-kb untranslated region. Homologs of the human *RB1* gene with a high level of sequence similarity in translated regions have been identified in a variety of organisms, including fish. The part of the gene that encodes the domains for the A/B pocket (see below) also has a homolog in higher plants (mat3) [Umen and Goodenough, 2001].

The *RB1* gene encodes pRb, a 928–amino acid nuclear phosphoprotein that migrates at 110 kD in SDS-PAGE when hypophosphorylated. pRb is part of a small family of nuclear proteins that includes p107 and p130. These proteins share significant sequence similarity in two discontinuous areas that constitute the A/B pocket (pocket proteins). Conditional on the phosphorylation status at multiple serine and threonine residues in other regions of the protein, the A/B pocket can bind to members of the E2F family of transcription factors and other nuclear proteins that contain the LxCxE peptide motif (such as histone deacetylases 1 and 2). The C-terminal region of pRB contains a nuclear localization signal and a cyclin-cdk interaction motif that enables it to be recognized and phosphorylated by cyclin-cdk complexes. The C-terminal region can also bind to the nuclear c-Abl tyrosine kinase and to MDM2, which are proteins with oncogenic properties.

A prominent role of pRb is its function as a gatekeeper that negatively regulates progression through the G1 phase of the cell cycle. During the G1 phase of the cell cycle, pRb is hypophosphorylated. Hypophosphorylated pRb can bind E2F and causes a repression of E2F-mediated transcription. Beginning in late G1 and continuing to the M phase, pRB is phosphorylated by cyclin-dependent kinases (for review see Mittnacht [1998]). Upon phosphorylation of pRb, E2F is released and promotes the expression of genes that are required for cell division. Consequently, pRB controls cell-cycle phase transition by transcriptional repression. Besides cell-cycle regulation, pRb has roles in apoptosis and differentiation (for review see

DiCiommo et al. [2000] and Classon and Harlow [2002]).

It is intriguing that the phenotypic consequences of mutations in a gene whose function is required in many different cell types are seemingly restricted to predisposition of neoplastic transformation of retinal precursor cells. *RB1* gene mutations have been reported in almost 1,000 patients with retinoblastoma (for review see Lohmann [1999] and Richter et al. [2003]). Almost all types of mutations have been identified, including translocations, deletions, insertions, point mutations, and epigenetic mutations (hypermethylation of the CpG-island in the promoter region of the gene). In this article, we investigate the genotype–phenotype associations that have been observed in retinoblastoma. This knowledge is important for genetic counseling and for management of patients with retinoblastoma. Moreover, analysis of genotype–phenotype associations will help to identify the genetic factors that modify phenotypic expression in retinoblastoma in individual mutation carriers.

RB1 GENE MUTATIONS AND VARIATION OF PHENOTYPIC EXPRESSION

Heterozygous carriers of oncogenic *RB1* gene mutations show variable phenotypic expression. Patients may develop tumors in both eyes or in one eye only (variable expressivity). Some carriers show no retinoblastoma at all (incomplete penetrance). According to the two-hit hypothesis, variation of phenotypic expression is to be expected, because the development of an individual tumor focus depends on the chance occurrence of a second somatic mutation. However, a quantitative analysis of phenotypic variation in families with retinoblastoma shows that stochastic effects can account for only a part of the observed differences. It is now well established that penetrance and expressivity of hereditary retinoblastoma can vary with the nature of the predisposing mutation.

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Phenotypic Expression in Carriers of Mutations That Result in Premature Termination Codons

The majority of germline mutations that have been identified in patients with hereditary retinoblastoma are nonsense or frameshift mutations. These mutations are located in exons 1–25 of the *RB1* gene. With rare exceptions, nonsense or frameshift mutations in internal exons (2–25) are associated with bilateral retinoblastoma. In some genetic diseases, mutant alleles with nonsense and frameshift mutations are associated with distinct phenotypic expression, depending on the location of the premature stop codon within the causative gene. Genotype-phenotype variations of this kind have not been observed in hereditary retinoblastoma, in which the site of the internal premature stop codon within the *RB1* gene seems to have little or no effect on phenotypic expression. Possibly, this is because transcripts of *RB1* alleles with internal premature stop codons are recognized by nonsense-mediated decay, which is a posttranscriptional surveillance mechanism that causes degradation of mutant transcripts [Frischmeyer and Dietz, 1999]. Consequently, in cells heterozygous for a mutation that triggers nonsense-mediated decay, only transcripts of the normal allele remain. Interestingly, no mutation has been identified in the two terminal exons of the *RB1* gene (exons 26 and 27), although this region contains two CGA codons, which are potential hot spots of nonsense mutations [Cooper and Krawczak, 1990]. However, according to what is known from other genes, premature stop codons in these regions will not trigger nonsense-mediated decay [Hentze and Kulozik, 1999]. This suggests that proteins resulting from these mutant *RB1* alleles, pRBs with late C-terminal truncation, may have sufficient tumor suppressive activity to prevent development of retinoblastoma.

Mutational Mosaicism

As a rule, carriers of RB1 alleles with nonsense or frameshift mutations in internal exons develop bilateral retinoblastoma.

As a rule, carriers of *RB1* alleles with nonsense or frameshift mutations in internal exons develop bilateral retinoblastoma. Occasionally, such a mutation is identified in a patient with isolated unilateral retinoblastoma or in a unilaterally affected parent of a child with bilateral retinoblastoma [Lohmann et al., 1997; Sippel et al., 1998]. However, in some of these patients the mutation is present in a mosaic state. This parallels findings in several other disorders with dominant inheritance, in which, compared to the phenotype in heterozygous mutation carriers, mosaicism is associated with milder phenotypic expression [Hall, 1988].

Phenotypic Expression in Carriers of *RB1* Gene Mutations That Result in Aberrant Splicing

The second important class of oncogenic alterations of the *RB1* gene are point mutations in intronic or exonic sequences that cause aberrant splicing. Many splicing errors result in premature termination codons due to out-of-frame exon skipping and, as is expected from the genotype–phenotype associations outlined above, these splice mutations are typically associated with complete penetrance. However, this is only valid for point mutations that alter invariable splice signals. Mutations that affect splice

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signals in exons, or in less conserved intronic splice signals, are more likely to be associated with unilateral retinoblastoma or incomplete penetrance. One reasonable explanation is that the effect of these mutations on splicing is "leaky," i.e., a fraction of the mutant transcript is processed into a normally-spliced mRNA [Boerkoel et al., 1995]. As a consequence, dosage of normal transcript is higher compared to cells that are heterozygous for a null mutation. The milder expression seen in carriers of such mutations corresponds to findings in other genes in which leaky splice mutations also tend to be associated with milder phenotypes [Boerkoel et al., 1995; Mautner et al., 1996; Svenson et al., 2001]. Another class of mutations that does not result in complete loss of function are alterations of promoter sequences. Thus, it is no surprise that such mutations have been identified in several families with incomplete penetrance and milder expressivity of retinoblastoma.

Phenotypic Expression in Carriers of Missense and In-Frame *RB1* Gene Mutations

Missense and small in-frame–length alterations do not result in premature termination codons; therefore, the transcripts expressed from these alleles are not recognized by posttranscriptional surveillance mechanisms. However, it is important to investigate the effect of supposed missense and in-frame mutations on the RNA level. It has been shown that mutations in exons may result in premature termination because of altered splicing [Cartegni et al., 2002]. For most reported missense and in-frame mutations in the *RB1* gene, such data are not available. Despite the uncertainty about the effect of individual mutations, the phenotypic expression that is associated with missense and in-frame mutations is often well distinguished from that of alleles with premature termination codons. Many

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missense and in-frame mutations are associated with incomplete penetrance or milder expressivity. The amino acids that are substituted, deleted, or inserted as a consequence of these mutations are most often part of the A/B pocket domain of pRB. A few in-frame mutations have been identified outside of regions that code for the A/B pocket, including deletions of exon 4 [Dryja et al., 1993] and of exons 24–25 [Bremner et al., 1997]. Functional studies have shown that mutant pRB expressed from alleles that are associated with incomplete penetrance show only a partial loss of normal function [Bremner et al., 1997; Otterson et al., 1997].

GENETIC MODIFICATION OF PHENOTYPIC EXPRESSION

Interfamilial Variation

Interestingly, families with identical mutations can show nonrandom differences of phenotypic expression.

Interestingly, families with identical mutations can show nonrandom differences of phenotypic expression. An example of interfamilial variation is obvious in two unrelated retinoblastoma families that both segregate an identical splice mutation, which results in skipping of exon 13 [Scheffer et al., 2000; Genuardi et al., 2001]. Both families show incomplete penetrance of retinoblastoma, but in one family, mutation carriers have developed multiple subcutaneous lipomas. Lipomas are benign neoplasms of adipose tissue that, compared to the general population, occur more frequently in adult patients with hereditary retinoblastoma [Li et al., 1997]. Interestingly, in the family with the lipoma/retinoblastoma phenotype, penetrance of lipoma is almost complete, whereas in the other family, none of the carriers of the identical mutation has developed lipoma. This shows that predisposition to lipoma is not caused by the splice mutation per se, but is due to a heritable modifying effect. As the lipoma phenotype was found to be linked to the mutant *RB1* allele, the modifying effect is most likely due to a genetic factor in cis relative to the mutant *RB1* allele. It is conceivable that predisposition to second cancers, which are an important health problem in patients with retinoblastoma, is also subject to modifier effects.

Intrafamilial Variation of Phenotypic Expression

A distinct modifier effect has been identified in two families that have the same base substitution in intron 6 of the *RB1* gene [Klutz et al., 2002]. This mutation results in a premature termination codon because of exon skipping. Contrary to what is expected from the genotype–phenotype associations outlined above, both families show incomplete penetrance. Intriguingly, most of the mutation carriers that have received the mutant allele via the maternal germline are unaffected, whereas almost all mutation carriers that have received the mutant allele via the paternal germline have developed retinoblastoma. RNA analysis in these families has shown that the level of nonsense transcript compared to that of the normal transcript is reduced only in carriers of paternally-inherited mutant alleles. Carriers of maternally-inherited mutant alleles, however, show a balanced ratio of

normal and mutant mRNA. The biologic mechanisms underlying this parent-of-origin effect have not yet been elucidated. Possibly, milder phenotypic expression is due to residual function of the protein expressed from the nonsense transcripts.

CONCLUSIONS

Mutation analysis in retinoblastoma has confirmed that mutations in both alleles of the *RB1* gene are a prerequisite for this tumor to occur. The correlations between genotype and phenotype suggest that phenotypic expression of retinoblastoma, i.e., the number of tumors, can vary with the functional consequence of the predisposing mutation and is also subject to modification by other genetic factors. How do we reconcile this with the two-hit hypothesis, according to which the number of tumor foci depends on the occurrence of inactivating second somatic mutations? It was suggested that second somatic mutations that result in loss of the normal allele and reduplication of a weak predisposing allele will not trigger tumor formation because sufficient residual tumor suppressive activity is left [Sakai et al., 1991]. Homozygosity for the mutant allele, which can result from chromosomal nondisjunction or mitotic recombination, is observed in about 60% of retinoblastomas [Zhu et al., 1992; Hagstrom and Dryja, 1999]. According to this explanation, carriers of weak alleles develop fewer tumors because the types of second mutations that can trigger tumor formation is restricted. From this explanation, one might expect that phenotypic expression is similar in all carriers of weak mutant *RB1* alleles. However, penetrance and expressivity can vary significantly even between families with identical weak mutations. This suggests that the development of a retinoblastoma focus in a heterozygous patient is not an immediate consequence of a mutation of the second allele. If residual pRB function is left—as in carriers of weak mutations—the probability of progression towards retinoblastoma may be reduced, thus resulting in milder phenotypic expression. This model of retinoblastoma development can also integrate modifying genetic factors. It is important to identify the genetic factors that modify genotype–phenotype relationships in retinoblastoma, because this knowledge will not only help us to arrive at a more precise prognosis for individual mutation carriers, but may also point out mechanisms that can be used to reduce the risk of tumor development in children that have inherited an oncogenic *RB1* allele.

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