

## Genotype-Phenotype Associations in Hereditary Retinoblastoma: Complex Aspects of a Simple Monogenic Trait



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**Abstract :** Retinoblastoma (Rb) is a malignant tumor of the eye that originates from developing retinal cells. Diagnosis is based on clinical signs and symptoms and is usually made in children under age of five years. Tumor formation starts from cells with mutational loss of both normal alleles of the RB1, a tumor suppressor gene that is located on 13q14. In most patients with sporadic unilateral Rb, both mutations have occurred in somatic cells and are not passed to offspring (non-hereditary Rb). Almost all patients with sporadic bilateral and virtually all patients with familial Rb are heterozygous for an oncogenic RB1 mutation and transmit Rb predisposition as an autosomal dominant trait (hereditary Rb). Penetrance of hereditary Rb depends on the functional consequence of the predisposing RB1 mutation. Mutational mosaicism, which is relatively frequent in patients with sporadic Rb is also a cause of milder phenotypic expression. Additional genetic factors can modify genotype-phenotype associations. Known modifiers include parental origin of the mutant RB1 allele and genetic variation linked to RB1.

**Key Words :** Retinoblastoma, genotype-phenotype associations, genetic modification, genetic counselling, molecular risk prediction

### Introduction :

#### Clinical Aspects : Diagnosis of Retinoblastoma (Rb)

The typical first presenting sign is a white pupillary reflex (leukocoria). Strabismus is the second most common sign and may accompany or precede leukocoria. Usually, diagnosis of Rb is established by examination of the fundus of the eye using indirect ophthalmoscopy. Additional diagnostic tools such as computer tomography (CT), magnetic resonance imaging (MRI), and ultrasonography may be required for differential diagnosis and staging. If tumor material was obtained, histopathology can confirm diagnosis of Rb. Patients can develop Rb in one eye or in both eyes (unilateral and bilateral Rb, respectively). Most children

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with bilateral Rb are diagnosed within the first year of their life. In children with unilateral Rb the tumour is usually diagnosed later but usually before age of 5 years.

### **Presentation and Family History in Patients with Rb**

Most patients (60%) have unilateral Rb. Occasionally, multiple tumor foci can be found (unilateral multifocal Rb). Vitreous seeding can mimic multifocal disease. Children with bilateral Rb represent 40% of patients. More than 90% of patients with unilateral Rb have sporadic disease, i.e. no other case of retinoblastoma has been noted in their family. About 75% of patients with bilateral Rb are also sporadic, in the remainder (25%) there is a positive family history (familial Rb). Examination of the fundus of the eye in all first degree relatives of children with Rb is required to identify retinal scars or quiescent tumours (retinomas). The presence of such lesions in a relative indicates familial disease.

### **Therapy and Prognosis of Patients with Rb**

Treatment of Rb depends on tumour stage, the number of tumour foci (unifocal, unilateral multifocal or bilateral disease), localization and size of the tumour(s) within the eye, presence of vitreous seeding, and the age of the child. Treatment options include enucleation, external-beam radiation, cryotherapy, photocoagulation, brachytherapy with episcleral plaques. After successful treatment, children require frequent follow-up examinations for early detection of new intraocular tumours in the same of in the fellow eye. For a favourable prognosis it is important that the tumour(s) have not invaded extraocular tissues. Metastasizing Rb is most often fatal.

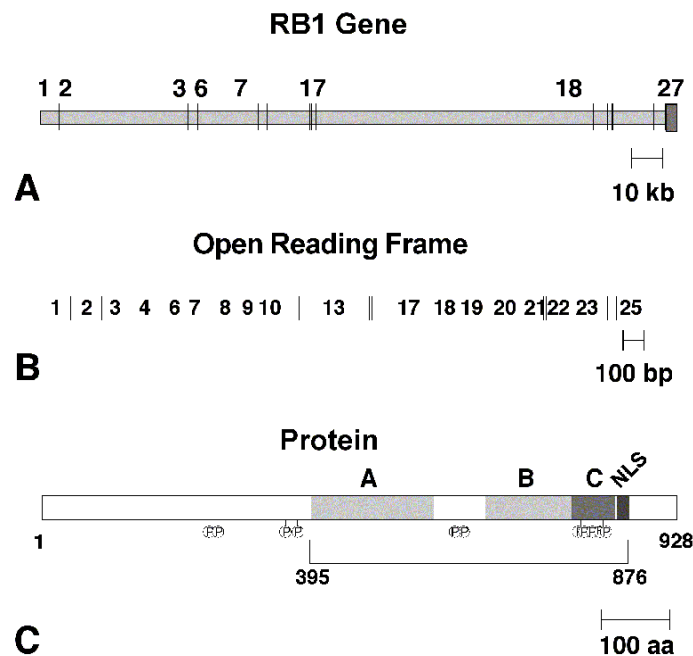
### **Second Non-ocular Tumours**

Patients bilateral or familial Rb have an increased risk of specific neoplasms outside of the eye (second tumors). The spectrum of malignant second tumors includes osteogenic sarcoma, soft tissue sarcoma, malignant melanoma, lung cancer and bladder cancer (Eng *et al.*, 1993; Fletcher *et al.*, 2004). Sarcomas are preferentially observed in patients that have received external beam radiation for treatment of bilateral Rb. Epidemiologic data suggest, that patients with hereditary Rb who are tobacco smokers have a higher relative risk to cancer compared to other tobacco smokers (Fletcher *et al.*, 2004).

## Molecular Genetics

### Rb is Caused by Two Mutations that Affect the RB1 Locus

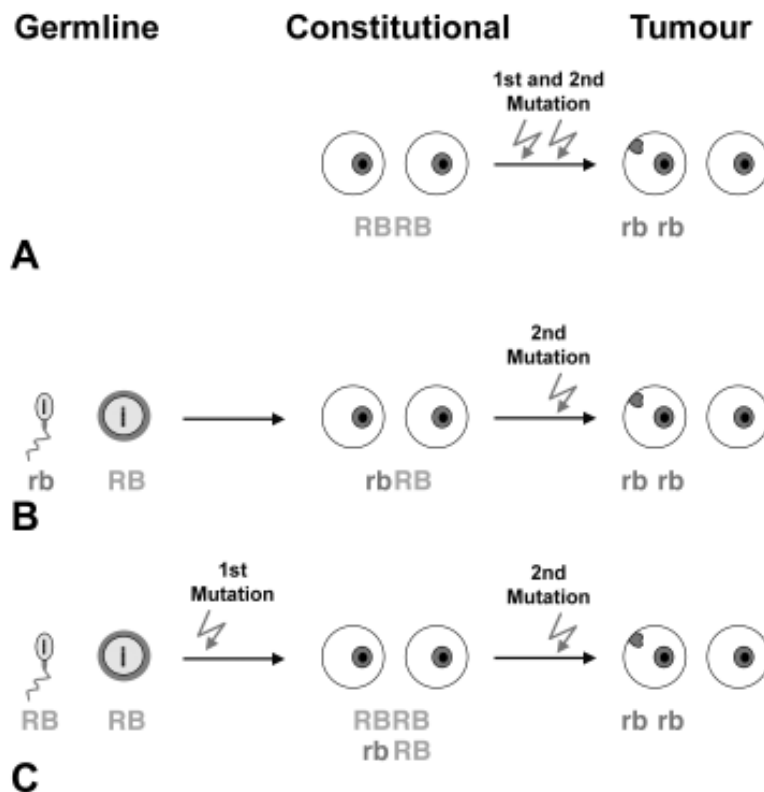
In 1971 Knudson proposed the two-hit hypothesis as model to explain the genetic events underlying non-hereditary and hereditary Rb (Knudson, 1971). He hypothesized that two mutational events are a prerequisite for Rb development. Later it was found that these two mutations affect both copies of one gene, the retinoblastoma gene, RB1 (Fig. 1) (Cavenee *et al.*, 1983; Friend *et al.*, 1986). The non-hereditary and hereditary forms of retinoblastoma are distinguished by the timing of the first mutation:



**Fig. 1 :** Genomic organization of the RB1 gene and functional domains of the encoded protein, pRb. The RB1 is composed of 27 exons that are scattered over 180 kb of genomic sequence (A). All 27 exons contribute to the 2.7kb open reading frame (B), which is translated in a 928 amino acid protein (C). The protein is subject to cell cycle dependent phosphorylation at several sites (denoted by an encircled P). Domains A and B of underphosphorylated pRb can form a pocket that mediates binding to several target proteins. For a recent review of pRb function see (Mittnacht, 2005).

- In most patients with sporadic unilateral Rb, the two mutations that are necessary to alter both copies of the RB1 gene occur in somatic cells (Fig 2 A). These patients have non-hereditary Rb.

- In most patients with sporadic bilateral Rb and in patients with familial Rb, the first mutation has occurred de-novo in the germline of one of the parents or has been transmitted via the germ line from a heterozygous parent. All non-tumorous cells (constitutional cells) are heterozygous for the mutant RB1 allele. Tumour development is proceeded by a second mutation that occurs in a somatic cell and inactivates the remaining normal RB1 allele (Fig. 2 B). Multiple tumour foci form in consequence of independent second somatic mutations.



**Fig. 2 :** Genetic basis of hereditary and non-hereditary Rb. Mutational inactivation of both alleles of the RB1 are required for development of Rb. (A) In patients with non-hereditary Rb the first and the second mutations are somatic events. (B) In patients with hereditary Rb, one RB1 mutation has occurred de-novo in the germline of one of the parents or has been transmitted from a parent that is an affected or non-penetrant mutation carrier. (C) In a few patients, the first mutation occurs during early embryonic development thus resulting in a mutational mosaicism. RB, normal RB1 allele; rb, mutant RB1 allele.

### **Some Patients with Rb Show Mutational Mosaicism**

Recently, molecular analyses have shown that in addition to the classical non-hereditary and hereditary forms of retinoblastoma, which were hypothesized by Knudson in 1971, there is a third form. In some patients with sporadic Rb, the first mutation has occurred de-novo during embryonal development (Carlson and Desnick, 1979; Lohmann *et al.*, 1997; Sippel *et al.*, 1998). This causes mutational mosaicism, *i.e.* some non-tumourous cells are heterozygous for the mutant RB1 allele (mutant sector) while other cells only have two normal RB1 alleles. Tumour development is only initiated if a second mutation inactivates the normal allele in a cell that is part of the mutant sector (Fig 2 C).

### **Spectrum of RB1 gene mutations**

#### **Cytogenetic deletions**

Using conventional cytogenetic analysis of peripheral blood lymphocytes, deletions involving 13q14 can be found in 4% of patients with retinoblastoma (Albrecht, in press). Children with cytogenetic deletions often show facial dysmorphism and delay of motor and speech development. These problems are thought to be caused by haploinsufficiency for genes located in the deleted chromosomal region adjacent to the RB1.

#### **Gross deletions**

Submicroscopic deletions larger than 100 bp (gross deletions) account for 8% of constitutional RB1 mutations in patients with Rb (Albrecht, in press). There are no regions within the RB1 that are frequently involved in deletion formation. Possibly, the region of the RB1 does not contain low copy repeat sequences that can serve as a target for recurrent nonhomologous recombination. However, scaffold/matrix attachment (S/MAR)-associated regions, which mark the position of recombinogenic DNA structures (Bode *et al.*, 2000), have been identified in two deletion breakpoint clusters in introns 23 and 24 of the RB1 gene (Albrecht *et al.*, 2004).

#### **Single Base Substitutions**

More than 50% of the mutations detected in peripheral blood DNA from patients with hereditary retinoblastoma are single base substitutions (database of RB1 gene mutations: [www.d-lohmann.de/Rb/mutations.html](http://www.d-lohmann.de/Rb/mutations.html)). About 70% of these changes are nonsense mutations. CpG-transitions are frequently observed at 12 of the 15 CGA codons within the open reading

frame of the RB1. Recurrent mutations at these sites account for the high relative frequency of mutations in exons 8, 10, 11, 14, 15, 17, 18, and 23 of the RB1 (Fig. 3). Analysis of some of these CGA codons has confirmed the presence of 5- methylcytosine (Mancini *et al.*, 1997). Most probably, the propensity for deamination, which is inherent to this base, is the cause for recurrent transitions at these sites. This is in accordance with the observation that no mutation was reported at the CGA codon which is contained in the unmethylated CpG-island at the 5'-end of the RB1 gene. Two other CGA codons without reported mutations are located at the very 3'-end of the open reading frame. Possibly, nonsense mutations at these sites are not oncogenic because they will not trigger decay of the mutant transcript via the nonsense-mediated decay pathway (Hentze and Kulozik, 1999). This may also account for the low relative frequency of mutations in exons 25, 26, and 27 of the RB1 (Fig. 2). The low frequency of mutations in exons 5, 7, and 9 is unexplained yet. Single base substitutions that change conserved nucleotides in 5' and 3' splice sites account for about 20% of oncogenic single base

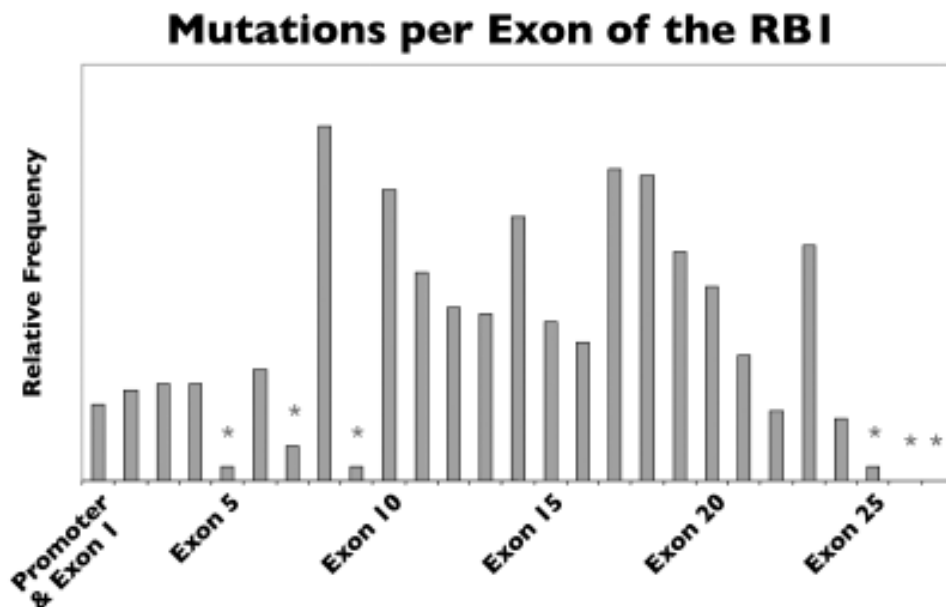
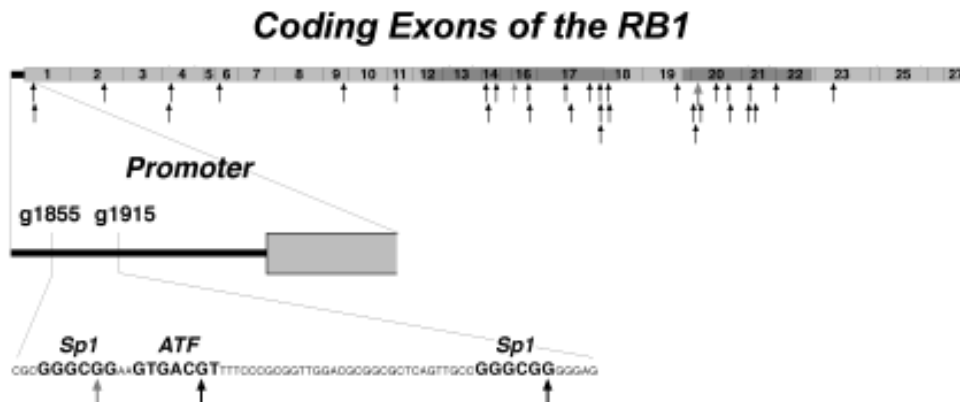


Fig. 3 : Relative frequency of point mutations in exons 1 to 27 of the RB1 and its promoter. The asterisks mark exons with low mutation frequency (exons 5, 7, 9, 25, 26, and 27).

substitution. Recurrent splice mutations are observed at the CpG-dinucleotide that is contained in the 5' splice site of intron 12 (AACgta to AACata) and at the 5' splice site of intron 6 (Klutz *et al.*, 2002). Single base substitutions that result in missense mutations represent only 10% of known oncogenic changes. Most missense mutations affect regions that code for amino acids that form structures (A/B pocket domains) that are essential for proper function of the retinoblastoma protein (Fig. 1, 4). A recurrent missense alteration, again a CpG-transition (CGG to TGG) is observed at codon 661 in exon 20 (R661W). A few mutations have been observed within the promoter region of the RB1 gene (Fig. 4).



**Fig. 4 :** Upper panel, distribution of missense mutations and small in-frame length mutations in the coding regions of the RB1. Lower panel, oncogenic point mutations in the promoter region of the RB1. Mutation data see (Lohmann, 1999).

### Small Length Mutations

Almost 30% of oncogenic RB1 mutations are small length mutations with a length of the deleted or inserted sequence ranging from -100 to +100 bp. More than 80% of the small length mutations within the open reading frame result in a frameshift and premature termination. Some 15% of small length mutations disrupt splice signals. Specifically, the splice donor sites of intron 19 and 24, which both contain repetitive sequence motives, are a recurrent target of small length mutations (Lohmann *et al.*, 1996). In-frame deletions are rare (less than 5%).

### Complex Mutations

A few mutations are complex sequence changes that can be described as a combination of a deletion, insertion, or base substitution. It is possible

that this type of mutation is underreported because it is difficult to correctly characterize complex sequences changes in the heterozygous state (for example in peripheral blood DNA of patients with hereditary retinoblastoma).

### **Loss of Heterozygosity in Tumours**

In about 70% of tumours, loss of one normal RB1 allele is accompanied by loss of constitutional heterozygosity (LOH) at polymorphic loci located on chromosome 13. LOH can result from deletions and several chromosomal mechanisms such as mitotic recombination and nondisjunction (Zhu *et al.*, 1992; Hagstrom and Dryja, 1999).

### **Epigenetic Silencing of Transcription**

Another class of mutation peculiar to tumours is hypermethylation of the CpG-rich island at the 5'-end of the RB1 gene, which is normally unmethylated. Hypermethylation is observed in about 10% of retinoblastomas and results in reduction or loss of transcriptional activity of the gene (Greger *et al.*, 1994).

### **Genotype-Phenotype Associations**

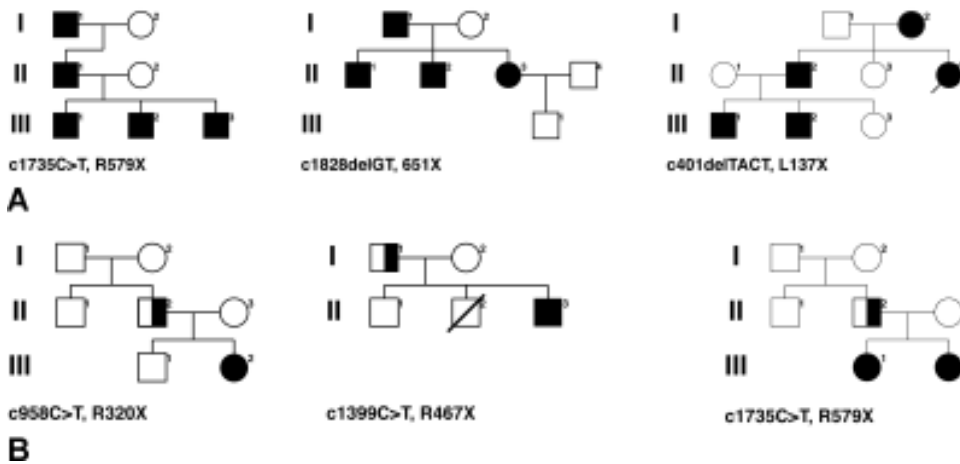
#### **Variation of Phenotypic Expression**

Heterozygous carriers of oncogenic RB1 gene mutations show variable phenotypic expression. For example, there is variation in the number of Rb foci. Most patients have multiple tumour foci in both eyes. However, some patients show milder phenotypic expression – i.e. fewer tumours – and may show unilateral Rb only. At the extreme end of this distribution are carriers of an oncogenic RB1 gene mutation who develop no Rb 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 (Fig 1). However, analysis of families with retinoblastoma shows that penetrance and expressivity are not randomly distributed but can vary between families (Fig. 5).

#### **Phenotypic Expression of Rb is Dependent on the Functional Consequence of the Predisposing RB1 Mutation**

Variation of phenotypic expression between families with distinct RB1 mutations suggests that the nature of the predisposing mutation is an important determinant of penetrance and expressivity:

- RB1 gene mutations that cause premature termination codons almost invariably result in bilateral tumours in heterozygous carriers (complete penetrance). Most carriers develop multiple tumours in both eyes. Nonsense mutations and frameshift length mutations, which represent the majority of oncogenic mutations in patients with Rb, belong to this class of mutation (Fig 5A).
- Distinct missense changes, promoter mutations, and in-frame deletions are associated with incomplete penetrance and milder expressivity, i.e. preponderance of unilateral Rb (Lohmann *et al.*, 1994; Sakai *et al.*, 1991) (Fig5 B).
- Most splice site mutations that result in out-of-frame exon skipping or intron retention show complete penetrance. However, the effect of some splice mutations is incomplete, that is, a fraction of the mutant allele is processed into a normal transcript (leaky splice mutations). Leaky splice mutations are associated with incomplete penetrance and milder expressivity.
- Patients with deletions of the whole RB1 gene show milder expressivity: about half of individuals who carry such deletions have Rb in one eye only (Albrecht, in press).



**Fig. 5 :** Families with bilateral and unilateral retinoblastoma (fully and half filled symbols, respectively). Non-penetrant mutation carriers are indicated by a dot. Upper panel, families with complete penetrance. Lower panel, families with incomplete penetrance.

## **Genetic Factors can Modify Genotype-Phenotype Associations Interfamilial Variation**

Most of the phenotypic variation that is observed between families with retinoblastoma can be explained by mutational heterogeneity. However, there are unrelated families that segregate identical mutations but show striking differences of phenotypic expression. For example, two unrelated retinoblastoma families have been reported that both segregate an identical splice mutation, which results in in-frame loss of exon 13 (Scheffer *et al.*, 2000; Genuardi *et al.*, 2001). In both families there are several mutation carriers unaffected by Rb. Interestingly, in only one of the two family, mutation carriers show multiple subcutaneous lipoma. It is known that lipomata, which are benign neoplasms of adipose tissue, occur more frequently in adult patients with hereditary Rb (Li *et al.*, 1997). Obviously, predisposition to lipoma is not caused by the splice mutation, which is the same in both families. Linkage of the lipoma trait to the RB1 suggests that a heritable modifying factor that is present in the lipoma/Rb family determines lipoma predisposition.

## **Intrafamilial variation of phenotypic expression**

Another 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 out of frame loss of exon 6. Contrary to expectations, these families do not show complete penetrance. Rather, some mutation carriers remain unaffected. However, this is only true for mutation carriers who have received the mutant allele via the maternal germ line whereas most of the mutation carriers that have received the mutant allele via the paternal germ line have developed retinoblastoma. Intriguingly, RNA analysis in these families has shown that the level of nonsense transcript is reduced in carriers of paternally inherited mutant alleles whereas carriers of maternally inherited mutant alleles show a balanced ratio of normal and mutant mRNA. The biologic mechanisms underlying this parent-of-origin effect have not been elucidated yet. However, this example shows that some aspects of the genetic mechanisms that cause retinoblastoma have not elucidated yet.

## Application of Genetic Knowledge: Genetic Counselling and Roadmap for Molecular Testing

### Risks to Family Members

Relatives of all patients with RB are at an increased risk to carry a predisposing RB1-gene mutation and, consequently, tumour development. Risk figures in Table 1 are based on empirical analyses (Draper *et al.*, 1992). Molecular testing can provide data that enable a more precise risk estimation. In many cases, accurate risk prediction in relatives depends on identification of the mutation that caused Rb in the index patient (Table 2).

**Table 1 : Risk for Rb in family members.**

Clinical Presentation Index Case	Risk to Siblings	Risk to of Offspring
sporadic unilateral Rb	= 1%	6%
sporadic bilateral Rb	= 2%	close to 50%
familial bilateral Rb (one parent affected)	close to 50%	50%
familial Rb, incomplete penetrance type	< 40%	< 40%

### Strategy for Predictive Testing

- In patients with familial or bilateral retinoblastoma molecular genetic testing is first performed on peripheral blood DNA. In view of the predominance of point mutations in patients with Rb, a screening of all coding parts and flanking splice sites is usually the first step of analysis. Various screening techniques have been employed, including single strand DNA conformation polymorphism (SSCP, (Lohmann *et al.*, 1996)), heteroduplex analysis (HDA, (Lohmann *et al.*, 1996; Van Orsouw *et al.*, 1996)), and denaturing high performance liquid chromatography (DHPLC, (Houdayer *et al.*, 2004)). Recently, an optimized strategy based on quantitative multiplex PCR and sequencing of all exons of the RB1 was developed by Richter *et al* (Richter *et al.*, 2003). Quantitative multiplex PCR can also detect the presence of gross deletions, which are not detected by most of the methods that are employed for point mutation analysis.

- In a few patients with bilateral retinoblastoma and no family history, no oncogenic mutation is identified in peripheral blood. In such cases, tumour DNA has to be investigated. If tumor DNA demonstrates two mutations then peripheral blood can be tested for the presence of one of the two mutations identified in analysis of the tumour. If neither disease-causing mutation is found in DNA from blood cells, the patient most has mutational mosaicism (Lohmann *et al.*, 1997; Sippel *et al.*, 1998). Although this finding lowers the probability that a germline mutation is present, there is still a considerable recurrence risk in offspring and, therefore, molecular testing in children is required for precise risk assessment.
- In patients with unilateral sporadic retinoblastoma, molecular genetic testing is first performed on tumour tissue. After the causative mutations that resulted in inactivation of the two RB1 alleles have been identified, peripheral blood DNA must be checked. In about 15% of such patients (see Table 2) an oncogenic RB1 mutation is also identified in peripheral blood. It must be noted that in some of these children the mutation is present in a mosaic state. Therefore, mutation detection methods used must provide the sensitivity that is needed to identify the presence of a mutant allele on a strong background of normal signals.

In about 5 to 10 % of patients current methods used for mutation detection fail to identify the genetic alteration that has caused Rb in a patient. In order to exclude an increased risk in family members, segregation analysis of linked polymorphic markers, e.g . RB1.20 and RBi2 (Brandt *et al.*, 1992; Toguchida *et al.*, 1993; Yandell and Dryja 1989), may be used. However, it is to be hoped that with further development of mutation detection technologies, the proportion of patients without known mutation can be lowered. Promising candidates in this respect are strategies that use analysis of RNA to identify mutations.

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**Table 2. Laboratory Testing used for predictive testing in relatives of patients with Rb**

<b>Clinical presentation</b>	<b>Genetic Mechanism</b>	<b>Genetic analyses</b>
Sporadic Unilateral Rb	In >90% of patients, tumour development results from somatic mutations. <10% of patients have inherited a predisposing mutation.	Mutation identification in DNA from tumour. Two mutations have to be identified. In 15% of the patients, an oncogenic RB1 mutation is detected in peripheral blood DNA
Sporadic Bilateral Rb	>90% of patients are heterozygous for an oncogenic RB1 allele that originated from a new mutation in the parental germ line. <10% of patients are mutational mosaics because the predisposing mutation has occurred during embryonal development.	Identification of the predisposing mutation in DNA from peripheral blood or from tumour. Because of mutational mosaicism in some patients, analysis of tumour DNA may be required to identify the predisposing RB1 mutation.
Familial Rb	Patients have inherited an oncogenic RB1 gene mutation.	Mutation identification in DNA from peripheral blood of patients that have inherited a mutant allele. If mutation detection fails, genotyping of linked polymorphic loci may be used to identify cosegregating marker alleles.

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