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Best Practice Guidelines for Molecular Analysis of Retinoblastoma

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1. Molecular genetics of retinoblastoma

Retinoblastoma (OMIM: 180200) is a malignant childhood tumour of the eye with an estimated incidence between 1 in 15,000 and 1 in 20,000 live births. Mutations in both alleles of the retinoblastoma susceptibility gene (*RB1*, accession No L11910) are a prerequisite for the development this neoplasm. In about 60% of the children, both mutational events occur in somatic cells and cannot be passed to the offspring (non-hereditary retinoblastoma). With only rare exceptions, patients with non-hereditary disease have retinoblastoma in one eye only (unilateral retinoblastoma). In the remaining 40% of patients, one of the mutant alleles (i.e. the predisposing mutation) is present in germ line cells and thus can be transmitted to offspring (hereditary retinoblastoma). It is not unusual to find somatic and/or germ line mosaicism in founders. Mosaicism is associated with fewer tumours and can also affect the observed segregation ratio. Retinoblastoma shows variable penetrance and expressivity. Much of this variation is due to different germ line RB1-gene mutations: mutant alleles that result in premature termination codons most often are associated with almost complete penetrance and bilateral retinoblastoma whereas incomplete penetrance and reduced expressivity is usually found in families with missense alterations, substitutions in the promoter region, and some splice site mutations.

2. Clinical indication for DNA Analysis

If retinoblastoma is newly diagnosed in a family, examination of the retina is required in all near relatives to exclude the presence of retinal anomalies that may be present in clinically unaffected mutation carriers. Genetic counselling is required to identify relatives with an increased risk (table 1). If relatives at risk are still in early childhood age, repeated eye examinations under anaesthesia are required. The primary goal of molecular testing is to exclude an increased risk at a level of certainty that justifies cancelling these eye examinations.

3. Spectrum of Mutations

3.1 Oncogenic mutations in constitutional cells of patients with retinoblastoma

Most often, knowledge of the oncogenic RB1 gene mutation that is present in constitutional cells of the index patient (i.e. the predisposing *RB1* gene mutation) is a prerequisite for accurate risk prediction. The spectrum of predisposing RB1-gene mutations is heterogeneous.

3.1.1 Cytogenetic Aberrations

Conventional cytogenetic analysis of peripheral blood lymphocytes shows deletions and rearrangements involving 13q14 in 8% of patients with bilateral and in 1 to 5% of patients with sporadic unilateral retinoblastoma (Ejima et al. 1988; Bunin et al. 1989). Especially in the latter group of patients, it is not uncommon to find mutational mosaicism. Submicroscopic deletions have been detected by fluorescent in situ hybridization (FISH).

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3.1.2 Large Deletions and Rearrangements

Using Southern blot hybridization with cDNA and genomic clones, subcytogenetic deletions are identified in about 10% of patients with bilateral or familial retinoblastoma (Kloss et al. 1991; Blanquet et al. 1991). Because of the size and complexity of the RB1-gene, deletions of one or few exons may go undetected by Southern blot hybridization. Therefore, procedures for relative quantification based on comparative multiplex PCR have been developed and reveal changes in the copy number of exons in 17% of RB1 mutant alleles (Gallie, in preparation; (Du and Gallie 1999)). In addition to whole-exon-deletions, comparative multiplex PCR can also detect changes in the size of exons caused by small insertions or deletions. Consequently, about 37%of identified mutations can be identified using this approach.

3.1.3 Small Mutations

The majority of mutations that predispose to retinoblastoma are single base substitutions (40-50%) and small length mutations (25-30%). Mutations have been identified in all but the last and penultimate exon of the RB1-gene (Lohmann 1999). The distribution of mutational events is not uniform. CGA codons in internal exons are frequently hit by CpG-transitions and thus represent relative mutation hot spots. The frequency of mutations also varies among those exons that do not contain hypermutable CpG-sites. However, the ranking of exons according to mutation density is not final.

3.2 Oncogenic mutations in tumour cells of patients with retinoblastoma

The spectrum mutations in retinoblastoma is, as far as the first mutation is concerned, similar to the spectrum of oncogenic mutations in constitutional cells. However, the second mutational event, which results in biallelic inactivation of the RB1-gene and thus initiates tumour development, is frequently (65%) caused by a chromosomal mechanism and is accompanied by loss of constitutional heterozygosity (LOH) at polymorphic loci located on chromosome 13 (Cavenee et al. 1983; Zhu et al. 1992; Hagstrom and Dryja 1999). Therefore, in order to specify both RB1-gene mutations in a tumour, genotyping of linked polymorphic loci is useful. Another mutational mechanism practically unique to tumours is hypermethylation of the CpG-rich island at the 5´-end of the RB1 gene, which is normally unmethylated (Greger et al. 1989). Hypermerthylation, which is observed in about 10% of retinoblastomas (Klutz et al. 1999; Ohtani-Fujita et al. 1997), may be detected by Southern Blot analysis using methylation sensitive restriction enzymes or analysis of bisulfite treated DNA (Zeschnigk et al. 1999).

4. Strategy, and Material required

4.1 Direct testing

4.1.1 Methods

Mutational analysis is used to identify the predisposing RB1 gene mutation in peripheral blood DNA from patients with familial or bilateral retinoblastoma (table 2). As most patients with isolated unilateral retinoblastoma are not heterozygous for an oncogenic mutation, tumour material is required to identify the relevant RB1 gene mutations. Several technical procedures for mutation identification are required to cover the whole spectrum of oncogenic RB1 gene mutations (table 3).

4.1.2 Interpretation

In most cases, inferring the oncogenic nature of alterations identified in the course of mutational analysis is straightforward. The majority of mutations are large deletions, frameshift length mutations, nonsense mutations or splice mutations that affect the invariable nucleotides of splice sites. Mutations that result in premature termination codons are usually associated with almost complete penetrance and, therefore, are rarely found in unaffected family members. Nevertheless, it is always advisable to investigate peripheral blood DNA from parents to check if the mutation has occurred de novo. More than a quarter of new mutations are recurrent, i.e. have been identified in other, unrelated patients with retinoblastoma. There are no polymorphic variants (frequency of the rare allele > 0.01) within the coding regions of the RB1 gene. Nevertheless, rare variants have been found in some families and, therefore, further analyses may be required to obtain sufficient evidence that a sequence alteration in the coding regions or in an intron is likely to be oncogenic or a neutral variant. In some cases, RT-PCR from peripheral blood has helped to verify that a mutation ultimately results in a premature termination codon and thus can be considered oncogenic.

4.2 Indirect testing

Several polymorphic short tandem repeat (STR) loci within and closely linked to the RB1 gene can be used for indirect testing (table 4). Identification of linkage phase is possible in most multigenerational families, but these are rare. Indirect testing in two-generation families with an affected parent and an affected child may result in false positive results for siblings if the parent is germinal mosaic. In isolated cases, indirect testing may used to determine if a relative at risk and the index patient share an RB1 allele that is identical by descent. Analysis of loss of heterozygosity in DNA from tumour can be used to infer linkage phase.

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4.3 Typical settings

4.3.1 Isolated bilateral retinoblastoma

Most patients with isolated bilateral retinoblastoma are heterozygous for an oncogenic mutation in the RB1 gene. Therefore, mutation analysis in DNA from peripheral blood will be successful in most cases. However, in some patients with isolated bilateral retinoblastoma the predisposing RB1 gene mutation has occurred during embryonal development. In these patients, the mutation is present in only some constitutional cells (mutational mosaicism) and may be undetectable in DNA from peripheral blood. Therefore, DNA from tumour should be investigated for mutation in those patients, where mutation analysis was not successful in DNA from peripheral blood. It is important to note that in DNA from tumour, two mutations have to be identified (two sequence mutations, or one sequence mutation or promoter hypermethylation and LOH) unless DNA form peripheral blood shows one of these mutations.

To determine if a predisposing mutation has occurred de novo, DNA from peripheral blood of parents is investigated. Even if none of the parents is a carrier of the predisposing mutation that was identified in the index patient, recurrence risk in siblings is still increased. Therefore, DNA from peripheral blood of siblings has to be tested for the mutation of the index patient. Of course, own children of the patient have to be tested for the predisposing mutation identified in his peripheral blood DNA.

If neither of the mutations that were identified in the tumour can be detected in DNA from peripheral blood, DNA from other tissues (e.g. mouth wash) can be tested. If ever possible, methods that can identify trace amounts of mutant sequence can be employed. If all results indicate that the patient is a mutational mosaic, the predisposing mutation is of postzygotic origin. Consequently, there is no increased risk in siblings. However, if no mutation was identified in any non-tumorous tissue investigated, own children of the patient have to be tested for both mutations that had been identified in DNA from tumour.

4.3.2 Isolated unilateral retinoblastoma

Mutational analysis in DNA from tumour should result in the identification of mutations of both alleles of the RB1 gene. Oncogenic alterations include sequence mutations, promoter hypermethylation, and LOH. In some 10 to 15% of patients, one of these mutations is also detectable present in DNA from peripheral blood. Only if both RB1 gene mutations are not present in DNA from peripheral blood of the patient, is an increased risk for siblings excluded. It is important to note that eye examinations in the patient may be reduced in frequency and not involve examination under anaesthetic, but not be cancelled. This is because mutational mosaicism can not be excluded and, therefore, tumours may develop in the contralateral eye. To exclude an increased recurrence risk in offspring, children have to be tested for the both mutations identified in tumour of the parent that was affected by retinoblastoma.

5. Recommendations for reporting

The “Best practice guidelines for reporting” give important information regarding the form and contents of reports (http://www.emqn.org/guidelines/REPORT.pdf). Some points more specific for retinoblastoma are:

- For an unequivocal nomenclature of small mutations, the reference sequence must be indicated in the report. The complete genomic sequence, accession No L11910, should be used as a reference if possible. The nomenclature system described in Antonarakis et al. (1998) should be followed.
- The interpretation of the test results should clearly state if the mutation identified in the patient is an oncogenic alteration that causes predisposition to retinoblastoma. An additional statement should be included if a mutation was identified that is known to be associated with incomplete penetrance.
- The report should include estimates for expected recurrence risk to siblings and own offspring of a patient. It should be noted if more precise risk figures can be obtained by genetic testing.

It is to be anticipated that the results of molecular testing of children with retinoblastoma will be needed if the child is planning his/her own reproductive options. Therefore, a copy the report should be issued to the family. The contents of the report should be sufficiently detailed to enable molecular testing even if primary data or samples (tumour DNA) are no longer available.

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Table 1. Risk for retinoblastoma development in family members.

clinical presentation	risk to siblings	offspring
isolated unilateral retinoblastoma	1%[a]	2-6%
isolated bilateral retinoblastoma	2% [a]	close to 50%
familial bilateral retinoblastoma (affected child of a parent with isolated retinoblastoma)	close to 50%	50%
familial low-penetrance retinoblastoma (family with one or more unaffected heterozygous carriers and/or most affected carriers have unilateral retinoblastoma)	< 40%	< 40%

[a] if there is no unaffected sibling (Draper et al. 1992)

Table 2. Strategies for molecular testing of predisposition to retinoblastoma

Clinical presentation	% of Patients	Genetic Mechanism	Test Type	samples required
familial retinoblastoma	10 %	patients have inherited a mutant RB1 allele.	Genotyping of linked polymorphic loci to identify cosegregating marker alleles	DNA from peripheral blood
			Mutational analysis	DNA from peripheral blood
			FISH or karyotype if no mutation found	Peripheral blood
isolated bilateral retinoblastoma	30 %	most patients carry a predisposing RB1 allele that originated from a new mutation in the paternal (>90%) or maternal (<10%) germ line. Some patients show mutational mosaicism (mutation has occurred during embryonal development)	Mutational analysis in DNA from peripheral blood (constitutional DNA). DNA from tumour is used if no mutation is identified in constitutional DNA .	DNA from peripheral blood. DNA from tumour if enucleation is performed
			FISH or karyotype if no mutation found	Peripheral blood
isolated unilateral retinoblastoma	60 %	In most patients, tumour development results from somatic mutations in both alleles of the RB1 gene. Some 10% of the patients can transmit retinoblastoma predisposition to their offspring.	Mutational analysis in DNA from tumour. Constitutional DNA is checked for the mutations identified in DNA from tumour	DNA from peripheral blood DNA from tumour

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Table 3. Methods for identification of oncogenic RB1 gene mutations

Technical procedure	Types of mutation detected	Sample required	Proportion of predisposing RB1 gene mutations detected
conventional cytogentic analysis	large deletions, translocations	peripheral blood	8%
FISH	large deletions, (translocations, if appropriate probes are used)	peripheral blood	>8% [a]
comparative genotyping of polymorphic loci in DNA from patient and parents	large deletions	peripheral blood DNA from patient and parents	8% [b]
Southern blot hybridisation	deletions, insertions, rearrangements	peripheral blood DNA, tumour DNA	16%
quantitative multiplex PCR and high resolution fragment length analysis	deletions, insertions (including small length mutations)	peripheral blood DNA, tumour DNA	37% [c]
Exon-by-exon point mutation screening	small length mutations, base substitutions	peripheral blood DNA, tumour DNA	70% [c]
Sequencing	small length mutations, base substitutions	peripheral blood DNA, tumour DNA	75%
Methylation specific PCR	promoter hypermethylation	tumour DNA	6% of oncogenic RB1 gene mutations in tumours
comparative genotyping of polymorphic loci in DNA from blood and tumour	mutations that result in loss of heterozygosity (LOH)	peripheral blood DNA and tumour DNA	65% of second oncogenic RB1 gene mutations in tumours

[a] no figures available; [b] markers outside of the deletion have to investigated to control for uniparental isodisomy; [c] the exact nature of point mutations has to be determined by sequencing

Table 4 Polymorphic STRs within and linked to the RB1 gene

marker name	location[a]	accession
D13S161	centromeric to RB1 (1.1 Mb)	Z16802
D13S287	centromeric to RB1 (1 Mb)	Z24331
D13S164	centromeric to RB1 (0.05 Mb)	Z16858
D13S153	RB1: intron 2	Z16494
RB1.20	RB1: intron 20	
D13S1307	telomeric to RB1 (0.25 Mb)	Z51671
D13S165	telomeric to RB1 (0.9 Mb)	Z16900
D13S273	telomeric to RB1 (1.25 Mb)	Z23383

[a] based on genetic (Bhattacharyya et al. 2000) and physical data (NT_009799)

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