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A PCR test for the detection of hypermethylated alleles at the retinoblastoma locus

EDITOR—The development of retinoblastoma, a childhood malignancy of the eye, is initiated by mutations in both alleles of the retinoblastoma gene (*RB1*). Mutations changing the nucleotide sequence at this locus and chromosomal mechanisms resulting in loss of heterozygosity (LOH) are the most common events responsible for gene inactivation.[1] However, in the *RB1* gene, as well as in other tumour suppressor genes including *BRCA1*, *p15*, and *p16*, hypermethylation of 5' regulatory regions may also cause gene inactivation and, consequently, tumour development.[2-6] Methylation of the CpG rich island at the 5' end of the *RB1* gene is observed in some 10% of unilateral sporadic retinoblastomas.[3 4 7] Recently, the methylation pattern of tumours with hypermethylated *RB1* alleles was analysed in detail by the bisulphite genomic sequencing technique.[8] In most of these tumours, most CpG sites were methylated (75-100%). In one of seven tumours only, the density of methylated CpG sites was more variable, ranging from 25% to 62.5%.

Mutation analysis in tumours from patients with sporadic unilateral retinoblastoma is required for accurate risk prediction in relatives.[1] Consequently, we have devised a methylation specific PCR assay (MSP)[5 9] for rapid and reliable detection of methylation at the *RB1* promoter. Bisulphite treatment of denatured DNA converts all unmethylated cytosines to uracil leaving methylated cytosines in CpG dinucleotides unaltered. After bisulphite treatment, a methylated allele differs from the unmethylated allele in nucleotide sequence at all CpG positions. The downstream PCR primer (RBcom) was designed to bind to the bisulphite treated sense strand irrespective of the methylation status (fig 1). Binding of RBcom to a methylated or unmethylated allele results in different complementary strands in the first round of the PCR. Upstream primers are designed to bind to the DNA strand synthesised in

Figure 1 Relevant part of the RB1 promoter. For sequence see T'Ang et al.[10] The positions of the MSP primers RBmet, RBunmet, and RBcom are indicated by arrows. Recognition sites for transcription factors SP1 and E2F as well as for methylation sensitive restriction enzymes BssHII and SmaI are shown. Positions of CpG dinucleotides are given by vertical lines. BssHII restriction site is located 64 bp upstream of the initiation codon. Primer sequences: RBcom: 5'-CCTACCCCRACTCCCRTTACAAAAATAATTTCAAC-3'; RBmet: 5'-GCGTTTTAGTTCGCGTATCGATTAGCGTTTTAG-3'; RBunmet: 5'-TGGTGGGTTTGGGAGTTTTGTGGATGTGATGTT-3'. R=adenine and guanine were added during synthesis of primer RBcom in equal amounts to avoid preferentially binding to methylated or unmethylated template.

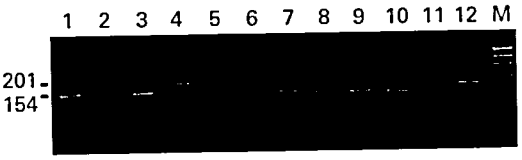

Figure 2 MSP analysis of hypermethylated (lanes 2, 4, 7, 11, and 12) and unmethylated (lanes 1, 3, 5, 8, 9, and 10) retinoblastomas; PCR failure (lane 6); marker(M) = MspI digested pUC19 DNA. The positions of the 201 bp PCR product representing the methylated allele and the 154 bp PCR product representing the unmethylated allele are indicated; 5 µg genomic DNA was bisulphite treated as previously described.[9 11] Amplifications were performed in a reaction volume of 25 µl containing 2 µl of bisulphite treated DNA (Perkin Elmer, PE9600). Conditions: 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.2 µmol/l of each dNTP, 0.25 µmol/l of primer RBcom, 0.3 µmol/l of primer RBmet, 0.05 µmol/l of primer RBunmet, and 0.5 units AmpliTaq (Perkin Elmer). Cycling conditions: 94°C for five minutes for one cycle; 95°C for 15 seconds, 64°C for 15 seconds, and 72°C for 30 seconds for 35 cycles, followed by 72°C for five minutes. PCR products were separated on 3% agarose gels.

the first round of PCR. Primer RBmet binds to the strand derived from the methylated allele, whereas primer RBunmet will only bind to the DNA strand generated from the unmethylated *RB1* allele. The methylated allele results in a 201 bp PCR product, whereas the unmethylated allele results in a 154 bp PCR product. As unmodified genomic DNA is not amplified, incomplete modification of genomic DNA in the bisulphite reaction will not yield specific PCR products (not shown). However, mutations affecting the primer binding sites might result in diminished or absent amplification of the corresponding PCR product and thus false negative results.

To establish the MSP at the *RB1* promoter, 20 tumour DNA samples, which had previously been characterised in detail,[1] were used. Characterisation included genotyping with intragenic markers, DNA sequencing, and analysis of the methylation status by Southern blot hybridisation of genomic DNA digested with methylation sensitive restriction enzyme *Bss*HII.[3] All tumours showed loss of heterozygosity (LOH) at the intragenic polymorphic loci RBi2 and RB1.20, and an inactivating mutation was identified in the remaining allele. In eight tumours a methylated allele was identified by Southern blot hybridisation. In each of these samples, a 201 bp PCR product was clearly visible when tested in the MSP assay (fig 2, lanes 2, 4, 7, 11, and 12). As expected, DNA from tumour and blood cells not containing a methylated *RB1* allele produced a 154 bp PCR product only (fig 2, lanes 1, 3, 5, 8, 9, and 10). No PCR product was obtained from unmodified genomic DNA under the conditions used here (data not shown). Trace amounts of the 154 bp PCR product were also detectable in tumours with LOH at intragenic loci. This finding might be the result of an unhomogeneous cell population consisting of cells with varying degrees of hypermethylation and non-tumour cells. Therefore, the presence of a 154 bp PCR product in the *RB1*-MSP assay does not necessarily indicate the presence of an unmethylated *RB1* allele in the tumour. A 201 bp PCR product specific for the

methylated allele, however, was detected only in tumours that also showed a methylated allele by Southern blot hybridisation.

To show that the *RB1*-MSP is applicable in routine diagnosis, a second set of 20 randomly selected tumour DNA samples with unknown methylation status was tested by the *RB1*-MSP. All tumours had been investigated for LOH at RBi2 and RB1.20. In two of these samples, a methylated *RB1* promoter region was identified by the MSP assay and verified by Southern blot analysis. Although this is a small number of tumours, the finding of methylated *RB1* alleles in 10% of unilateral sporadic retinoblastomas is in agreement with previous estimates on the frequency of hypermethylated *RB1* alleles.[4] An additional 154 bp PCR product representing an unmethylated allele was also obtained in these two tumours, which was to be expected as they did not show LOH at the intragenic polymorphic loci RBi2 and RB1.20.

In summary, analysis of 40 samples has shown that our MSP reliably identifies tumours with hypermethylated *RB1* alleles as detected by Southern hybridisation. Compared to Southern blot analysis, however, MSP is faster, can be performed with small amounts of genomic DNA, and does not need radioactive components. Thus, MSP facilitates identification of *RB1* gene hypermethylation in RB and other tumours.[12]

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Frequency and predictive value of 22q11 deletion

EDITOR—Malformations resulting from the adverse effects of 22q11 deletion are now recognised to be of one of the most important diagnostic categories in dysmorphology. The predictive value of this common deletion remains to be fully elucidated since even large reviews[1] are by their nature subject to ascertainment bias. In our original report of familial heart disease associated with submicroscopic 22q11 deletion, which predated the routine availability of fluorescence in situ hybridisation (FISH), the carrier mother was dysmorphic but had a normal heart.[2] We and others have drawn attention to the marked variability of the phenotype and the need to be alert to the possibility of subtle features in a parent.[3–5] These observations raise the possibility of subclinical deletion being more common than has been recognised. This would have significance in genetic counselling when 22q11 deletion is detected in the first trimester as occurred in the case described below.

Following the recognition of a deletion 22q11 in III.2 (fig 1) diagnosed clinically as DiGeorge syndrome with complex cardiac malformations including pulmonary atresia, double outlets of the right ventricle, subaortic interventricular septal defect, mitral atresia, restrictive intra-atrial communication, hypoplastic left ventricle, patent ductus arteriosus, and right aortic arch leading to neonatal death, the parents were invited to undergo chromosome analysis. The father (II.4) (fig 2) was found to carry a del22q11.1-11.2 karyotype and displayed minor facial features, mild learning difficulties, and was on the 10th centile for height.[4] The couple went on to have three subsequent pregnancies. In their third pregnancy, early amniocentesis showed a 22q11 deletion but no evidence of

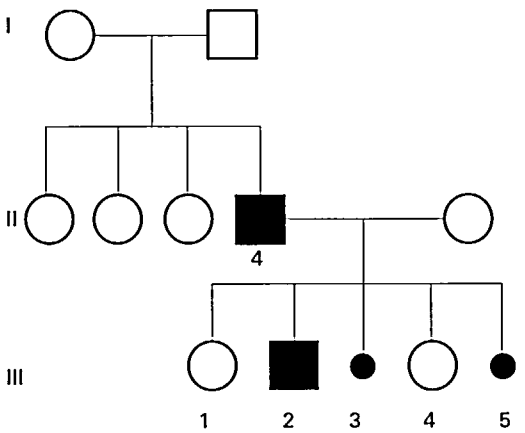

Figure 1 22q11 deletion in two generations with marked variation in phenotype.

structural abnormality was detected on an anomaly scan and fetal echocardiography at the 17th and 18th week. A further examination at 19 weeks showed a major structural heart defect. At necropsy the aborted fetus (III.3) was found to have slightly abnormal facial features, pulmonary atresia, perimembraneous ventricular septal defect, secundum atrial septal defect, retro-oesophageal right subclavian artery, a small thymus, and one ectopic parathyroid. The couple's fourth pregnancy resulted in a healthy daughter (III.4) while their fifth (III.5) was a severely affected fetus with 22q11 deletion who died in utero.

There have been eight reports of prenatal diagnosis based on a 22q11 deletion but the growing recognition of this disorder in clinical practice means this will become a more common event. In order to improve the predictive value of detection of 22q11 deletion in pregnancy, we undertook an investigation of 22q11 deletion in an unselected population.