Case report

Detection and reporting of *RB1* promoter hypermethylation in diagnostic screening

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ABSTRACT

Background *RB1* gene screening aids clinical management and genetic counselling in retinoblastoma families. Here we present epigenetic changes identified during routine molecular *RB1* screening of tumour and blood samples. Complications in interpreting *RB1* methylation are discussed.

Materials and Methods Screening for *RB1* promoter hypermethylation was carried out by Methylation Specific PCR (MS-PCR) after bisulphite modification of DNA. The cohort consisted of 315 tumours, and 204 blood samples, from 497 retinoblastoma patients (22 patients had both blood and tumour screened).

Results 11.4% of retinoblastoma tumours had promoter hypermethylation. It was not routinely detected in blood samples, or in tumours with two other oncogenic *RB1* changes. One blood sample had promoter hypermethylation due to an X;13 translocation. One tumour had low level methylation as well as two other oncogenic changes. Histopathological analysis of a small subset of age-matched tumours was similar regardless of promoter hypermethylation status.

Conclusions Promoter hypermethylation was detected in 11.4% of the retinoblastoma tumours and should be tested for in routine *RB1* screening programmes. Constitutional samples are not expected to display *RB1* hypermethylation. In a small proportion of cases it may not be possible to use this somatic change in patient management.

Key Words Retinoblastoma, Hypermethylation, Screening, Epigenetic

Word Count - 2715

INTRODUCTION

Retinoblastoma (RB:MIM #180200) is a childhood tumour of the developing retina that occurs with an estimated frequency of 1 in 15,000-20,000 live births. Retinal cells are fully differentiated by 2-3 years of age so that it rarely develops in older children. The time of diagnosis is important for vision preservation and survival. Early diagnosis is possible when there is a family history or predictive genetic testing, enabling targeted examination under anesthesia for at-risk children. There are two forms of RB: heritable (45-50%) and non-heritable (50-55%). All bilateral cases and 15-20% of unilateral cases are heritable. Inheritance is autosomal dominant with high penetrance. Carriers of pathogenic changes are at increased risk for non – ocular tumours such as osteosarcomas and soft tissue sarcomas.

The retinoblastoma susceptibility gene *RB1* (Genbank #L11910.1) is located on human chromosome 13q14 and its product (pRB) acts as a tumour suppressor. Non-functional pRB enables uncontrolled cell proliferation, and bi-allellic inactivation allows retinoblastoma initiation. The spectrum of *RB1* alterations is broad and widely distributed across the gene (1-3). The most common are single base substitutions (50-60%) such as missense, nonsense and splice site changes. Small length changes (30%) generating premature stop codons are associated with highly penetrant and expressive (bilateral, multifocal) retinoblastoma. Missense codons, in frame deletions/insertions, promoter and some splicing changes are associated with a low penetrance phenotype (4-6). In about 60-70% of tumours there is loss of heterozygosity (LOH). Whole genome sequencing analysis has added chromothripsis to the list of alterations affecting *RB1* in around 3% of tumours (7). A minority of sporadic, unilateral cases (around 1.5%), characterised by very early onset, can be caused by over-amplification of the *MYCN* gene (8).

The epigenetic change of promoter hypermethylation has been reported as a somatic alteration in 8-15% of retinoblastomas (2-3, 9-12). Hypermethylation inhibits the binding of transcription factors (RBF-1 and ATF) to their recognition sites in the *RB1* promoter causing reduced gene expression (11). This audit aimed to establish the percentage of retinoblastomas with promoter hypermethylation in our cohort. Histopathological analysis of a small selection of age-matched promoter hypermethylation positive and negative tumours was carried out to see if there were any differences. We also tested 204 blood samples to confirm that promoter methylation is a somatic, tumour modification which does not occur in blood samples. We discuss some individual cases with unusual results and implications for patient management.

MATERIALS AND METHODS

The methylation status of CpG islands in the *RB1* promoter (L11910.1; g.1691- g.2005) was tested by methylation specific PCR (MS-PCR) of bisulphite modified DNA. Bisulphite induced sequence differences between methylated/unmethylated DNA were detected by using primer sets for unmethylated and methylated DNA. MS-PCR was performed for 286 fresh tumours, 29 formalin fixed, paraffin embedded samples (FFPE) samples and 204 blood samples (plus another 34 blood samples taken from patients with hypermethylated tumours).

Sample collection and extraction

This audit was approved by the Barts Health Clinical Effectiveness Unit (audit no. 812). It covered the period from April 2003 to April 2014. Peripheral blood and tumour samples were collected from patients referred to the Retinoblastoma Genetic Screening Unit (Barts Health NHS Trust) for routine *RB1* screening. Patients were referred by genetic counsellors or ophthalmologists and consent was obtained from parents/guardians.

Peripheral blood was collected into EDTA tubes and genomic DNA extracted using the phenol/chloroform method, the Zymo-G midi kit (Zymo Research), or the Qiagen Midi Kit (Qiagen). DNA was extracted from fresh frozen tumour samples using the Nucleon II kit (GE Healthcare) or the Zymo-G midi kit (Zymo Research). FFPE samples were extracted as previously described (13), or with the RecoverAll Total Nucleic Acid Isolation Kit (Ambion). Kits were used according to the manufacturers' instructions.

DNA was quantified by NanoDrop analysis, and quality was also checked by amplification in triplex PCR reactions encompassing the first intron of the X-Y homologous gene Amelogenin (Xp22.31-p22.1; MIM #300391), the STR Rbi.2 in *RB1* intron 2 (D13S153), and the VNTR RB1.20 in intron 20. These markers were also used to confirm sample identity and to determine loss of heterozygosity (LOH) for those tumours where blood samples were available for comparison.

Bisulphite modification

Bisulphite modification was carried out on 400-500ng of DNA using the EZ DNA Methylation-Gold Kit (Zymo Research) according to manufacturer's instructions. A positive control of 'CpGenome' globally methylated DNA (Merk-Millipore) was included. Modified DNA was eluted in 1XTE buffer (Sigma Aldrich) and used for MS-PCR amplification. Some samples were modified by a manual method (14).

Methylation specific PCR (MS-PCR)

MS-PCR for *RB1* was optimised in house. Primers (Table 1) were designed to be specific for either modified methylated sequences, or modified unmethylated sequences. Both the sense and antisense strands were tested. Wild-type primers (specific to the sense strand) were used to check for unmodified DNA bypassing the modification process. Each MS-PCR included a

positive sample from a previous batch as a PCR control. Unmodified DNA and water samples were used as negative controls. Modified DNA from blood was included as a positive control for unmethylated samples. Amplifications were carried out using HotStar Taq (Qiagen) in a 20ul reaction mix containing 1ul modified DNA and 10 pmoles of each primer.

Agarose gel electrophoresis

Fifteen microlitres of PCR product were electrophoresed (100Volts for 1 hour) on a 1.5% agarose gel (Invitrogen) in X0.5 TBE buffer (National Diagnostics). Products were visualized using 0.5 ug/ml ethidium bromide (Sigma Aldrich) in the agarose and running buffer. All results were checked by two clinical scientists. Positive results for hypermethylation were confirmed in a different DNA aliquot. When a tumour was found to be positive then that patient's blood sample was also tested (where available).

Histopathological analysis

Tumours from eighteen age-matched (at the time of diagnosis) patients were assessed blindly and independently for histological features by two experienced pathologists. The samples were from tumours with homozygous methylation (n=3), heterozygous methylation (n=5), homozygous for other pathogenic changes (n=5) and heterozygous for other changes (n=5). Features including tumour size, focality, differentiation, invasion, necrosis and calcification were verified upon review.

RESULTS

315 tumour DNAs were screened. 11.4% (2 FFPE and 34 FT) were positive for promoter hypermethylation. We previously reported a figure of 13.9% (27/194 samples) in a smaller cohort (2). For 34 of the hypermethylated tumours, blood samples were also available. None of these cases were constitutively methylated. Additionally, 204 other blood samples (sporadic RB cases) were tested. One had promoter hypermethylation due to presence of mosaic X;13 translocation (Table 2).

2/315 tumours had no *RB1* alterations but exhibited *MYCN* amplification (15). Typical, or known, *RB1* pathogenic variants were detected in 274 of the remaining 313 samples (87.5%). However, not all tumour samples received full *RB1* screens, although all were tested for promoter hypermethylation. There was incomplete screening in 26 samples where DNA was from a FFPE tumour and/or was of poor quantity/quality (n=11), or when blood samples had been referred for screening and pathogenic germline *RB1* variants were already identified (n=15). Only thirteen fully screened tumours had pathogenic variations missing (13/287; 4.5%). Only 1 tumour displayed *RB1* promoter hypermethylation alongside two other pathogenic variants (1/274; 0.4%).

In the total tumour population with two *RB1* alterations, 166/274 (60.6%) had LOH characterised by STR analysis and/or QF-PCR. A further 27 had whole *RB1* gene deletions, which if counted as LOH would bring the number to 193/274 (70.4%). In 14/36 (38.9%) of the hypermethylated tumours there was LOH, and in a further 5 there was a whole *RB1* gene deletion which if included gives a figure of 19/36 (52.8%). In the other samples hypermethylation was accompanied by a transition/transversion, or a small deletion. Table 3 details the other *RB1* variations detected in hypermethylated tumours.

Histopathological analyses of a subset of age-matched (at diagnosis) tumours were carried out. Ten unmethylated samples were compared to eight with methylation. There were no distinguishing histological features (as used in tumour classification) between these two groups. Features assessed included the level of differentiation, extent of necrosis, calcification, choroid invasion, iris neovascularisation and synechia.

DISCUSSION

This study is an audit of the methylation status of the *RB1* promoter in 315 retinoblastomas. In 274 tumours where an adequate screen was possible, two *RB1* inactivating changes were identified. We previously reported promoter methylation in 13.9% (27/194) of tumours (2). In this larger series, 36 tumour samples (2 FFPE and 34 FT) were found to have promoter hypermethylation. This represents 11.4% of all tumours tested (36/315), or 13.0% (36/276) of tumours where all expected pathogenic changes are known (including *MYCN* amplification). Since this audit another 68 tumours have been tested. 10 were methylation positive so that our current methylation frequency remains at 12.0% (46/383).

In 19/36 (52.8%) of positive tumours, methylation was accompanied by loss of heterozygosity/del *RB1*. This figure is lower than the results of Richter *et al* who reported a figure of 88% (17). One tumour sample displaying LOH and a stop codon also showed low level methylation (case 36, Table 3). Usually, when there are two other pathogenic changes in retinoblastomas there is no promoter hypermethylation, and this exception may be due to the tumour being multifocal.

It was initially reported that *RB1* hypermethylation is primarily associated with sporadic, unilateral retinoblastoma (12,16-17), and 35/36 (97.2%) of our methylated tumours were

from such patients. We also detected promoter methylation in one familial case [c.2359 C>T, p.(R787*)] and in a multifocal tumour with a germline variant (c.2490-1 G>A) (Table 3, cases 36 and 4). Two other positive tumours were probably multifocal and may therefore be from germline mosaics (the changes were not detectable in blood). In a previous report of a hereditary retinoblastoma case with *RB1* promoter methylation, the hypermethylation was also shown to be the 'second hit' (16). Also, Joseph *et al* (2004) reported two methylated tumours from bilateral patients in India (18). Since this audit we have found tumour methylation in a bilateral case. *RB1* hypermethylation may potentially occur in any retinoblastoma tumour regardless of laterality/family history.

Hypermethylation was not routinely detected in tumours with two other oncogenic changes. It therefore does not appear to constitute a third (progression) hit and is reported as an oncogenic change for use in clinical management/counselling of patients. However, in case 36 there were two pathogenic *RB1* variants alongside a low level of promoter methylation. This was from a familial, unilateral case (not obviously multifocal) and carried a homozygous exon 23 stop codon [p.(R787*)] which was the inherited, germline variant. The tumour had LOH yet the promoter hypermethylation was heterozygous. Multiple Ligation Probe Analysis (MLPA) showed that the tumour was hemizygous for *RB1*. This result could be due to the presence of two different tumour populations; a large population with a hemizygous exon 23 stop, and a smaller population with the exon 23 stop plus hypermethylation.

Case 28 was a sporadic, unilateral patient who developed a second tumour in the same eye. The first tumour had homozygous methylation (with LOH extending beyond the 5' of *RB1* but no hemizygosity) and no other detectable change. The second tumour displayed heterozygous methylation (not counted in cohort) despite also having LOH. Again, no other

variant could be detected. We have seen two similar cases of LOH tumours having heterozygous methylation. We cannot be sure that there is a germline alteration involved here as the second tumour may have seeded from the first. If it was a seeded focus then the secondary tumour could be progressing by losing methylation and gaining other changes which drive growth more effectively. It has been suggested that a hypermethylation-mediated block on differentiation may promote initial growth, but then later inhibit other processes which contribute to progression, and that cancer hypermethylator phenotypes have better clinical prognosis (19-20).

Case 16 had a tumour with heterozygous methylation and an intron 15 splice donor variant (c.1421+18_1421+33del). This was tested at the transcription level to determine whether it homozygously expressed the predicted skipping of exon 15. It showed heterozygous skipping of exons 15 and 16, suggesting that the promoter methylation is not completely inhibiting transcription. Other changes may be required for tumour progression, especially if methylation is incomplete. Determining the positions of cytosines that are methylated could reveal if the transcription factor binding sites are hotspots for methylation. We sequenced the methylated products from this tumour and found that the majority of the CpG sites were methylated on the sense strand, whilst on the antisense strand there were more unmethylated CpG sites. Both strands appeared to have an unmethylated CpG site at the E2F binding site. Of the 5 positive tumours which were sequenced, 3 were unmethylated at the E2F site. MS-PCR of bisulphite treated DNA does not distinguish between 5-methylcytosine (5-mC) and the related base 5-hydroxymethylcytosine (5-hmC) and it is not clear how this would affect expression.

There is a weak, alternative promoter for *RB1* in intron 2. This is imprinted and can produce an alternative transcript from the unmethylated, paternal allele which inhibits the production of the full length product from that allele, leading to expression bias from the maternal allele (21). Whether a predisposing variant is maternally or paternally inherited may cause a low/high penetrance phenotype due to activity at this promoter (22). It is possible that tumours with promoter variants can initiate transcription from this region to overcome inhibition at the usual promoter. In a study of human hepatocellular carcinoma, 40% of specimens (16/40) showed hyper- or hypomethylation at the CpG island in *RB1* intron 2 (23). Loss of imprinting at this locus was considered an additional mechanism for deregulating *RB1* expression.

We accept hypermethylation as a pathogenic variation in tumours which have been fully screened. However, in FFPE tumours where a full screen is not possible, another change could have been missed and it is recommended that this result is not used in patient management. Since this audit, homozygous methylation was detected in two sporadic, unilateral tumours with LOH, and where QF-PCR indicated a double deletion of the promoter region. Although MLPA analysis showed that *RB1* had no copy number change, QF-PCR indicated that there was a double deletion from the promoter to intron 2. It is possible that small rearrangements altered primer binding sites for QF-PCR whilst still allowing the MLPA probes to bind (the probes will still bind after inversions/rearrangements so long as the probe sites themselves are still intact). Consequently, these findings cannot currently be used in patient management/counselling.

34 blood samples from methylation positive tumour patients were tested for *RB1* promoter methylation, and all were negative. This suggests that this epigenetic variation is not

transmitted through the germ line and it is somatic in origin. We identified *RB1* methylation in one blood sample from a sporadic patient with dysmorphic/congenital abnormalities. Karyotyping showed a reciprocal translocation between the long arms of one X chromosome and one chromosome 13. There were two cell lines – in one the X;13 translocation was balanced whilst the other was unbalanced. *RB1* methylation is expected to be due to X chromosome inactivation spreading to chromosome 13 and inactivating an *RB1* allele as previously reported (24).

Our MS-PCR is not quantitative and does not determine whether all CpG islands are methylated. MLPA studies would be quantitative but still do not indicate whether one or two alleles are contributing to a heterozygous result, whether all islands on all strands are affected, or if there is low level mosaicism. MLPA failed to detect the one, positive blood in this cohort, probably because MLPA is not sensitive to low level mosaics. Quantitative Analysis of Methylated Alleles-QAMA (25). would help in identification of individuals with mosaic, low level methylation.

We do not routinely screen blood samples from sporadic RB patients. We continue to test blood samples from patients with hypermethylated tumours. There is evidence that this type of epigenetic change could be heritable as in hereditary nonpolyposis colorectal cancer (26). There has also been a report suggesting a similar mechanism in a low penetrance RB family with biparental contribution to the RB phenotype, which displayed a promoter variant associated with methylation (27). As part of on-going quality checks we continue to test some tumours with two other pathogenic *RB1* variants for promoter hypermethylation, and have yet to find another sample which also has promoter hypermethylation.

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CONTRIBUTORS

EAP, RP, KK, SH and ZO performed *RB1* screening, and analysed the variants. EAP and ZO interpreted the data and drafted the article. EK and IS carried out histopathological analysis of tumours. MSS and MAR referred cases, provided clinical information and material for analysis and revised/approved the article. All authors critically read, revised and approved the final article.

FOOTNOTES

Declaration of Interest None

Patient consent Obtained

Ethics approval Barts Health Clinical Effectiveness Unit

REFERENCES

1 Lohmann DR. *RB1* gene mutations in retinoblastoma. Hum Mutat 1999;14:283-88. [http://www.d-lohmann.de/Rb/mutation.html]

2 Price EA, Price K, Kolkiewicz K, Hack S, Reddy MA, Hungerford JL, Kingston JE, Onadim Z. Spectrum of RB1 mutations identified in 403 retinoblastoma patients. J Med Genet 2014;51:208-14.

3 Dommering CJ, Mol BM, Moll AC, Burton M, Cloos J, Dorsman JC, Meijers-Heijboer H, van der Hout AH. RB1 mutation spectrum in a comprehensive nationwide cohort of retinoblastoma patients. J Med Genet 2014;51:366-74.

4 Sakai T, Ohtani N, McGee TL, Robbins PD, Dryja TP. Oncogenic germ-line mutations in Sp1 and ATF sites in the human retinoblastoma gene. Nature 1991;353:83-6.

5 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. Proc Natl Acad Sci U S A 1992;89:6177-81.

6 Lohmann DR, Brandt B, Hopping W, Passarge E, Horsthemke. Distinct RB1 gene mutations with low penetrance in hereditary retinoblastoma. Hum Genet 1994;94:349-54.

7 McEvoy J, Nagahawatte P, Finkelstein D, Richards-Yutz J, Valentine M, Ma J, Mullighan C, Song G, Chen X, Wilson M, et al. RB1 gene inactivation by chromothripsis in human retinoblastoma. Oncotarget 2014;5:438-50.

8 Rushlow DE, Mol BM, Kennett JY, Yee S, Pajovic S, Thériault BL, Prigoda-Lee NL, Spencer C, Dimaras H, Corson TW, et al. Characterisation of retinoblastomas without RB1 mutations: genomic, gene expression and clinical studies. Lancet Oncol 2013;14:327-34.

9 Greger V, Passarge E, Hopping W, Messmer E, Horsthemke B. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. Human Genet 1989;83:155-8.

10 Greger V, Debus N, Lohmann D, Höpping W, Passarge E, Horsthemke B. Frequency and parental origin of hypermethylated RB1 alleles in retinoblastoma. Hum Genet 1994;94:491-6.

11 Ohtani-Fujita N, Fujita T, Aoike A, Osifchin NE, Robbins PD, Sakai T. CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. Oncogene 1993;8:1063-7.

12 Klutz M, Horsthemke B, Lohmann DR. RB1 gene mutations in peripheral blood DNA of patients with isolated unilateral retinoblastoma. Am J Hum Genet 1999;64:666-7.

13 Onadim Z and Cowell JK. Application of PCR amplification of DNA from paraffin embedded tissue sections to linkage analysis in familial retinoblastoma. J Med Genet 1991;28:312-14.

14 Clark SJ, Harrison J, Paul CL, Frommer M. High Sensitivity Mapping of Methylated Cytosines. Nucleic Acids Res 1994;22:2990-7.

15 Lillington DM, Goff LK, Kingston JE, Onadim Z, Price E, Domizio P, Young BD High level amplification of N-MYC is not associated with adverse histology or outcome in primary retinoblastoma tumours. Br J Cancer 2002;87:779-82.

16 Ohtani-Fujita N, Dryja TP, Rapaport JM, Fujita T, Matsumura S, Ozasa K, Watanabe Y, Hayashi K, Maeda K, Kinoshita S, et al. Hypermethylation in the retinoblastoma gene is associated with unilateral, sporadic retinoblastoma. Cancer Genet Cytogenet 1977;98:43-49.

17 Richter S, Vandezande K, Chen N, Zhang K, Sutherland J, Anderson J, Han L, Panton R, Branco P, Gallie B. Sensitive and efficient detection of RB1 gene mutation enhances care for families with retinoblastoma. Am J Hum Genet 2003;72:253-269.

18 Joseph B, Mamatha G, Raman G, Shanmugam MP, Kumaramanickavel G. Methylation status of RB1 promoter in Indian retinoblastoma patients. Cancer Biol Ther 2004;3:184-7.

19 Sproul D, Meehan RR. Genomic insights into cancer-associated aberrant CpG island hypermethylation. Brief Funct Genomics 2013;12:174-90.

20 Thériault BL, Dimaras H, Gallie BL, Corson TW. The genomic landscape of retinoblastoma: a review. Clin Experiment Ophthalmol 2014;42: 33–52.

21 Kanber D, Buiting K., Roos C, Gromoll J, Kaya S, Horsthemke B, Lohmann D. The Origin of the RB1 imprint. PLoS ONE 2013;8:e81502. doi: 10.1371/journal.pone.0081502.

22 Eloy P, Dehainault C, Sefta M, Aerts I, Doz F, Cassoux N, Lumbroso le Rouic L, Stoppa-Lyonnet D, Radvanyi F, Millot GA, et al. A Parent-of-Origin Effect Impacts the Phenotype in Low Penetrance Retinoblastoma Families Segregating the c.1981C>T/p.Arg661Trp Mutation of RB1. PLoS Genet 2016;12:e1005888. doi: 10.1371/journal.pgen.1005888.

23 Anwar SL, Krech T, Hasemeier B, Schipper E, Schweitzer N, Vogel A, Kreipe H, Lehmann U. Deregulation of RB1 expression by loss of imprinting in human hepatocellular carcinoma. J Pathol 2014;233:392-401.

24 Jones C, Booth C, Rita D, Jazmines L, Brandt B, Newlan A, Horsthemke B. Bilateral retinoblastoma in a male patient with an X; 13 translocation: evidence for silencing of the RB1 gene by the spreading of X inactivation. Am J Hum Genet 1997;60:1558-62.

25 Zeschnigk M, Bohringer S, Price EA, Onadim Z, Masshofer L, Lohmann DR. A novel real-time PCR assay for quantitative analysis of methylated alleles (QAMA): analysis of the retinoblastoma locus. Nucleic Acids Res 2004;32:e125. doi: 10.1093/nar/gnh122.

26 Chan TL, Yuen ST, Kong CK, Chan YW, Chan AS, Ng WF, Tsui WY, Lo MW, Tam WY, Li VS, Leung SY. Heritable germline epimutation of MSH2 in a family with hereditary nonpolyposis colorectal cancer. Nature Genetics 2006;10:1178-83.

27 Quinonez-Silva G, Davalos-Salas M, Recillas-Targa F, Ostrosky-Wegman P, Aranda, DA, Benitez-Bribiesca L. Monoallelic germline methylation and sequence variant in the promoter of the RB1 gene: a possible constitutive epimutation in hereditary retinoblastoma. Clinical Epigenetics 2016;8:1. DOI 10.1186/s13148-015-0167-0.

Table 1 Primers used for MS-PCR of *RB1*.

***RB1* Strand**	**Primers (L11910.1 sequence)**	**Product Size/Position**
Methylated Sense	**5**'GGGTTCGGGAGTTTCGCGGACG **3**'ACGCGCGCGCACGTCGAAACA 38 cycles at 67^0C	179 bp g.1827 – 2005
Unmethylated Sense	**5**' GGGTTTGGGAGTTTTGTGGATG **3**' ACACACACACACATCAAAACA 40 cycles at 67^0C-57^0C; Touchdown	179 bp g.1827- 2005
Methylated Antisense	**5**'AAAAACACGTCCGAACCGCGCCGA **3**' AAAAATCGGACGCGTTTTTTTCGTTC 38 cycles at 63^0C	242 bp g.1691 – 1932
Unmethylated Antisense	**5**' GTTTGGTAATTGAGTGTTGTG **3**' CACATCCAAACCACACCAAAT 38 cycles at 55^0C	213 bp g.1908 – 1696
Unmodified DNA	**5**' GCACGTCCGGGCCGCGCCGG **3**' GGACGCGCCCTCCCCCGCCC 35 cycles 70^0C	230 bp g.1695 – 1924

Table 2 Results of MS-PCR testing in retinoblastoma patients.

Sample Type	Number Screened	Hypermethylated
Tumour; unilateral cases	243	36 (14.8%)
Tumour; bilateral cases	70	0 (0.0%)
*Total tumour	313	**36 (11.5%)
Blood; routine screening	204	***1 (0.5%)
Blood; matched to a positive tumour	34	0 (0.0%)
Total blood	238	***1 (0.4%)

*Excluded 2 *MYCN*^{AMP} positive tumours with no *RB1* changes.
**1 was familial, 1 was multifocal (germline) and 2 were potentially multifocal. The rest were sporadic unilateral.
*** Mosaic X;13 translocation detected by cytogenetic analysis.

Table 3 Other variants carried by tumours with *RB1* hypermethylation.
All tumours were from sporadic, unilateral patients except for case 36 who was familial, unilateral.

Case	Diagnosis age (months)	Variant g. number (L11910.1)	Variant c. number (LRG_517t1)	Putative consequence
1	9 **Multifocal?**	g.150,037C>T	c.1735C>T	p.(R579*) exon 18
2	36	g.70,332A>T	c.1215+3A>T	Int 12 splice donor
3	19	g.64348C>T	c.958C>T	p.(R320*) exon 10
4	3 **Multifocal**	g.170371G>A Germline	c.2490-1G>A	Int 23 splice acceptor
5	35	g. 76460C>T	c.1363C>T	p.(R455*) exon 14
6	137	g.78238C>T	c.1654C>T	p.(R552*) exon 17
7	61	g.162237C>T	c.2359C>T	p.(R787*) exon 23
8	22	g.156775G>A	c.2043G>A	p.(W681*) exon 20
9	24	g.64348C>T	c.958C>T	p.(R320*) exon 10
10	25	g.64348C>T	c.958C>T	p.(R320*) exon 10
11	30	g.78238C>T	c.1654C>T	p.(R552*) exon 17
12	32	g.156797C>T	c.2065C>T	p.(Q689*) exon 20
13	Unknown	g.156774G>A	c.2042G>A	p.(W681*)exon 20
14	31	g.65392delA	c.1078delA	p.(S360Vfs*7) exon 11
15	35	g.76,407_76,417del11	c.1333-13_1333-23del	Int 13 splice acceptor
16	15	g.76,938_76,953del16	c.1421+18_1421+33del	Int 15 splice donor
17	15	g.170,405_170,408del4	c.2520+3_2520+6del	Int 24 splice donor
18	27	n/a	n/a	LOH
19	14m	n/a	n/a	LOH
20	28	n/a	n/a	LOH
21	18	n/a	n/a	LOH
22	45	n/a	n/a	LOH
23	42	n/a	n/a	LOH
24	3	n/a	n/a	LOH
25	43	n/a	n/a	LOH
26	36	n/a	n/a	LOH
27	32	n/a	n/a	LOH
28	36 **Multifocal /seeding?**	n/a	n/a	LOH
29	36	n/a	n/a	LOH
30	24	n/a	n/a	LOH
31	20	n/a	n/a	LOH
32	36	n/a	n/a	del*RB1*
33	11	n/a	n/a	del*RB1*
34	18	n/a	n/a	del*RB1*
35	42	n/a	n/a	del*RB1*
36	12	g.162,237 C>T Familial/germline	c.2359C>T	p.(R787*) **and del*RB1***