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-allelic 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 \textit{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. \textit{RB1} methylation is expected to be due to X chromosome inactivation spreading to chromosome 13 and inactivating an \textit{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 \textit{RB1} variants for promoter hypermethylation, and have yet to find another sample which also has promoter hypermethylation.
ACKNOWLEDGEMENTS

The authors gratefully acknowledge the contribution of the late Dr Judith Kingston, Consultant Paediatric Oncologist, for the referral, provision of clinical information and material for analysis. We thank Mr. John Hungerford for providing material for analysis whilst he was the Consultant Ophthalmologist for retinoblastoma at Barts Health NHS Trust. We also thank the clinical nurse specialists and Barts Health Pathologists for their help in collecting fresh tumour samples. Thanks to Valerie Witt, Johanna Lowery, Simon Hack and Kelly Price for their contribution while working in our Unit. Many thanks to the Retinoblastoma Society – CHECT (Childhood Eye Cancer Trust) for their invaluable support during the set up of the RGSU.

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


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

<table>
<thead>
<tr>
<th><em>RB1</em> Strand</th>
<th>Primers (L11910.1 sequence)</th>
<th>Product Size/Position</th>
</tr>
</thead>
</table>
| Methylated Sense  | 5’GGGTTCCGGGAGTTTCGCGGACG  
3’ACGCGCGCAGCAGTCGAAACA  
38 cycles at 67°C         | 179 bp  
g.1827 – 2005              |
| Unmethylated Sense| 5’ GGGTTTGGGAGTTTTGTGGATG  
3’ ACACACACACACCATCAAAACA  
40 cycles at 67°C-57°C; Touchdown | 179 bp  
g.1827- 2005              |
| Methylated Antisense | 5’AAAAACACGTCCGAACCGCGCCGA  
3’ AAAATCGGACGCGTTTTTTTTCTTC  
38 cycles at 63°C          | 242 bp  
g.1691 – 1932              |
| Unmethylated Antisense | 5’ GTTTGGAATTGAGTGTGTTGTG  
3’ CACATCCAACACCACACCAAAT  
38 cycles at 55°C          | 213 bp  
g.1908 – 1696              |
| Unmodified DNA    | 5’ GCACGTCCGGCGCGCCGCGCGG  
3’ GGACGCGCCCTCCCCCGGCC  
35 cycles  70°C            | 230 bp  
g.1695 – 1924              |
Table 2 Results of MS-PCR testing in retinoblastoma patients.

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

*Excluded 2 MYCNAMP 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.

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis age (months)</th>
<th>Variant g. number (L11910.1)</th>
<th>Variant c. number (LRG_517t1)</th>
<th>Putative consequence</th>
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<tbody>
<tr>
<td>1</td>
<td>9 Multifocal?</td>
<td>g.150,037C&gt;T</td>
<td>c.1735C&gt;T</td>
<td>p.(R579*) exon 18</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
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<td>c.1215+3A&gt;T</td>
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<tr>
<td>3</td>
<td>19</td>
<td>g.64348C&gt;T</td>
<td>c.958C&gt;T</td>
<td>p.(R320*) exon 10</td>
</tr>
<tr>
<td>4</td>
<td>3 Multifocal</td>
<td>g.170371G&gt;A</td>
<td>c.2490-1G&gt;A</td>
<td>Int 23 splice acceptor</td>
</tr>
<tr>
<td>5</td>
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<td>p.(R455*) exon 14</td>
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<tr>
<td>6</td>
<td>137</td>
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<td>p.(R552*) exon 17</td>
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<tr>
<td>7</td>
<td>61</td>
<td>g.162237C&gt;T</td>
<td>c.2359C&gt;T</td>
<td>p.(R787*) exon 23</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
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<td>c.2043G&gt;A</td>
<td>p.(W681*) exon 20</td>
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<tr>
<td>15</td>
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<td>28</td>
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<td>n/a</td>
<td>del(RB1)</td>
</tr>
<tr>
<td>36</td>
<td>12</td>
<td>g.162,237 C&gt;T</td>
<td>c.2359C&gt;T</td>
<td>p.(R787*) and del(RB1)</td>
</tr>
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