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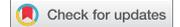
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CASE REPORT

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 tumor 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 tumors, and 204 blood samples, from 497 retinoblastoma patients (22 patients had both blood and tumor screened).

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

Conclusions: Promoter hypermethylation was detected in 11.4% of the retinoblastoma tumors 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.

Introduction

Retinoblastoma (RB:MIM #180,200) is a childhood tumor 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 tumors 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 tumor 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 tumors there is loss of heterozygosity (LOH). Whole genome sequencing analysis has added chromothripsis to the list of alterations affecting *RB1* in around 3% of tumors (7). A minority of sporadic, unilateral cases (around 1.5%), characterized 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 tumors was carried out to see if there were any differences. We also tested 204 blood samples to confirm that promoter methylation is a somatic, tumor 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 using primer sets for unmethylated and methylated DNA. MS-PCR was performed for 286 fresh tumors (FT), 29 formalin fixed, paraffin embedded samples (FFPE) samples, and 204 blood samples (plus another 34 blood samples taken from patients with hypermethylated tumors).

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 tumor 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 for testing 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 tumor 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 #300,391), 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 tumors where blood samples were available for comparison.

Bisulphite modification

Bisulphite modification was carried out on 200-500 ng of DNA using the EZ DNA Methylation-Gold Kit (Zymo Research) according to the 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

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°C	179 bp g.1827 – 2005
Unmethylated Sense	5' GGGTTTGGGAGTTTTGTGGATG 3' ACACACACACACATCAAAACA 40 cycles at 67°C-57°C; Touchdown	179 bp g.1827 – 2005
Methylated Antisense	5'AAAAACACGTCCGAACCGCGCCGA 3' AAAAATCGGACGCGTTTTTTTTCGTTC 38 cycles at 63°C	242 bp g.1691 – 1932
Unmethylated Antisense	5' GTTTGGTAATTGAGTGTTGTG 3' CACATCCAAACCACACCAAAT 38 cycles at 55°C	213 bp g.1908 – 1696
Unmodified DNA	5' GCACGTCCGGGCCGCGCCGG 3' GGACGCGCCCTCCCCCGCCC 35 cycles 70°C	230 bp g.1695 – 1924

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 20 μL reaction mix containing 1 μL modified DNA and 10 pmol of each primer.

Agarose gel electrophoresis

Fifteen microlitres of PCR product were electrophoresed (100 V for 1 h) 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 tumor was found to be positive then that patient's blood sample was also tested (where available).

Histopathological analysis

Tumors from 18 age-matched (at the time of diagnosis) patients were assessed blindly and independently for histological features by two experienced pathologists. The samples were from tumors 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 tumor size, focality, differentiation, invasion, necrosis, and calcification were verified upon review.

Results

315 tumor 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 tumors, 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

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

Sample Type	Number Screened	Hypermethylated
Tumor; unilateral cases	243	36 (14.8%)
Tumor; bilateral cases	70	0 (0.0%)
[a]Total tumor	313	[b]36 (11.5%)
Blood; routine screening	204	[c]1 (0.5%)
Blood; matched to a positive tumor	34	0 (0.0%)
Total blood	238	[c]1 (0.4%)

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

hypermethylation due to the presence of a mosaic X;13 translocation (Table 2).

2/315 tumors 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 tumor 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 tumor 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 13 fully screened tumors had pathogenic variations missing (13/287; 4.5%). Only 1 tumor displayed RB1 promoter hypermethylation alongside two other pathogenic variants (1/274; 0.4%).

In the total tumor population with two RB1 alterations, 166/274 (60.6%) had LOH characterized 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 tumors 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 tumors.

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

Discussion

This study is an audit of the methylation status of the RB1 promoter in 315 retinoblastomas. In 274 tumors where an adequate screen was possible, two RB1 inactivating changes

Table 3. Other variants carried by tumors with RB1 hypermethylation. All tumors 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	delRB1
33	11	n/a	n/a	delRB1
34	18	n/a	n/a	delRB1
35	42	n/a	n/a	delRB1
36	12	g.162,237 C > T Familial/germline	c.2359C>T	p.(R787*) **and delRB1**

were identified. We previously reported promoter methylation in 13.9% (27/194) of tumors (2). In this larger series, 36 tumor samples (2 FFPE and 34 FT) were found to have promoter hypermethylation. This represents 11.4% of all tumors tested (36/315), or 13.0% (36/276) of tumors where all expected pathogenic changes are known (including *MYCN* amplification). Since this audit another 68 tumors have been tested. Ten were methylation positive so that our current methylation frequency remains at 12.0% (46/383).

In 19/36 (52.8%) of positive tumors, 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% (16). One tumor 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 tumor 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 tumors were from such patients. We also detected promoter methylation in one familial case [c.2359 C > T, p.(R787*)] and in a multifocal tumor with a germline variant (c.2490–1 G > A) (Table 3, cases 36 and 4). Two other positive tumors 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" (17). Also, Joseph *et al.* (2004) reported two methylated tumors from bilateral patients in India (18). Since this audit we have found tumor methylation in a bilateral case. *RB1* hypermethylation may potentially occur in any retinoblastoma tumor regardless of laterality/family history.

Hypermethylation was not routinely detected in tumors 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 tumor had LOH yet the promoter hypermethylation was heterozygous. Multiple Ligation Probe Analysis (MLPA) showed that the tumor was hemizygous for *RB1*. This result could be due to the presence of two different tumor 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 tumor in the same eye. The first tumor had homozygous methylation (with LOH extending beyond the 5′ of *RB1* but no hemizygosity) and no other detectable change. The second tumor 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 tumors having heterozygous methylation. We cannot be sure that there is a germline alteration involved here as the second tumor may have seeded from the first. If it was a seeded focus then the secondary tumor 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 tumor 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 tumor 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 tumor and found that the majority of the CpG sites were methylated on the sense strand, while 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 tumors 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 tumors 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 tumors which have been fully screened. However, in FFPE tumors 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 tumors 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 while 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 tumor 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 while 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 tumors. There is evidence that this type of epigenetic change could be heritable as in hereditary non-polyposis 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 tumors with two other pathogenic *RB1* variants for promoter hypermethylation, and have yet to find another sample which also has promoter hypermethylation.

Acknowledgments

Declaration of interest

The authors declare no conflicts of interest.

Ethics approval

Barts Health Clinical Effectiveness Unit

Notes on contributors

EAP, RP, KK, SH, and ZO performed *RB1* screening, and analyzed the variants. EAP and ZO interpreted the data and drafted the article. EK and IS carried out histopathological analysis of tumors. 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.

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