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Evidence of predisposing epimutation in Retinoblastoma.

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Abstract

Retinoblastoma (RB), which represents the most common childhood eye cancer, is caused by biallelic inactivation of *RB1* gene. Promoter hypermethylation is quite frequent in RB tissues but conclusive evidence of soma-wide predisposing epimutations is currently scant. Here, 50 patients who tested negative for *RB1* germline sequence alterations were screened for aberrant promoter methylation using methylation-specific MLPA. The assay, performed on blood, identified a sporadic patient with methylation of CpG106, absent in parents' DNA. Bisulfite pyrosequencing accurately quantified CpG methylation in blood DNA (mean ~49%)

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and also confirmed the aberration in DNA isolated from oral mucosa although at lower levels (mean ~34%). Using a tag-SNP, methylation was demonstrated to affect the maternal allele. Real-Time qPCR demonstrated *RB1* transcriptional silencing. In conclusion, we documented that promoter methylation can act as the first 'hit' in Knudson's model. This mosaic epimutation mimics the effect of an inactivating mutation and phenocopies RB onset.

Keywords: retinoblastoma, promoter methylation, epimutation, gene silencing, mosaicism.

Retinoblastoma (RB; MIM# 180200), with an incidence ranging from 1 in 15,000 to 1 in 18,000 live births, represents the most common intraocular pediatric tumor (Bishop & Madson, 1975). According to Knudson's two-hit hypothesis, it is caused by the biallelic inactivation of *RB1* (RB transcriptional corepressor 1) tumor suppressor gene (Knudson, 1971). In hereditary cases, the first event occurs in one of the parent's germ cells and the second one in the developing retina, typically leading to early onset bilateral and/or multifocal tumors (median age 11 months) (Vogel, 1979). Patients with hereditary RB can transmit tumor predisposition as an autosomal dominant trait with high penetrance (90%) and are at increased risk of developing extraocular primary tumors. In non-hereditary cases both inactivating events occur in a single retinal cell, leading to unilateral tumors with later onset (median age 22 months) (Vogel, 1979). More recently, it has been demonstrated that RB is associated with a high incidence of mosaicism, making more complex to predict disease transmission and phenotypic manifestations (Rushlow et al., 2009)(Amitrano et al., 2015).

Epimutations are epigenetic errors resulting in aberrant gene silencing/activation (Hesson, Hitchins, & Ward, 2010). Alterations in the epigenotype determining aberrant gene expression are a hallmark of cancer cells and represent important "driver" events of early tumorigenesis and malignant progression. Indeed sporadic cancers frequently show epigenetic inactivation of tumor suppressor genes through methylation of promoter CpG sites (Hesson, Hitchins, & Ward, 2010). Constitutional epimutations predisposing to cancer are more rarely reported and have been well documented in a limited number of conditions such as Lynch syndrome and Wilms tumor (Hitchins, 2016) (Moulton et al., 1994). Depending on the mechanism by which constitutional epimutations are originated, they can be distributed

throughout all normal tissues (soma-wide) or show mosaicism (Cropley, Martin, & Suter, 2008). In RB tissues, methylation of a CpG island (CpG106) encompassing the promoter region of *RB1* is a quite frequent mechanism inactivating one copy of the gene (13%) but the same event in patients' non-tumor cells has been rarely described (Greger et al., 1994). In 1997, a first report provided evidence that RB can develop in the context of a X;13 translocation spreading X inactivation to chromosome 13 and producing functional monosomy for genes on proximal 13q including *RB1* (Jones et al., 1997). A more recent study reported a possible constitutional methylation of the *RB1* promoter as cause of hereditary RB; however such results have been questioned by Sloane and colleagues, in a subsequent letter on the same journal, given the lack of all the criteria necessary to demonstrate the presence of an epimutation (Quinonez-Silva et al., 2016; Sloane, Ward, & Hesson, 2016).

In the present study, a series of 50 RB patients (45 unilateral and 5 bilateral), tested by deep sequencing (Ion Torrent PGM, Life technologies) and negative for *RB1* germline pathogenic variants, were analyzed for methylation pattern abnormalities (CpG106 and CpG85) by Methyilation Specific Multiplex ligation-dependent probe amplification (MS-MLPA). Study patients were selected from an initial cohort of 476 RB cases diagnosed over a period of 17 years at the Retinoblastoma Referral Centre of Siena (Ophthalmology Department, AOUS). Families underwent genetic counseling, provided and signed a written informed consent at the Medical Genetics Unit of Azienda Ospedaliera Universitaria Senese, (Siena, Italy) for the use of their DNA samples for diagnostic purposes. Blood samples were collected in EDTA-containing tubes and sent to the Unit of Medical Genetics of Siena for *RB1* mutational analysis. Genomic DNA was extracted from EDTA peripheral blood samples

using MagCore HF16 (Diatech Lab Line). The LRG Reference Sequence for *RB1* gene analyzed with Human GRCh37/hg19 is LRG_517. For *RB1* copy number and methylation analysis of DNA isolated from family members (probands and parents) and 100 healthy controls, the methylation-specific Salsa MS-MLPA Kit P047-RB1 (MRC Holland, Amsterdam, The Netherlands) was used according to the manufacturer's instructions. This kit contains methylation-sensitive probes for the promoter region (CpG106) and for the imprinted locus (CpG85). Analysis and interpretation were performed with the Coffalyser software (MRC Holland, Amsterdam, The Netherlands). Three control samples were included in each experiment. The study was approved by the Local Ethics Committee. In one case, the assay, revealed a certain degree of amplification of all the probes annealing at CpG106 (extending from 5'UTR up to intron 1) spanning the promoter of the gene (Fig. 1A). CpG85, responsible for *RB1* imprinting, was normally methylated (~50%) (Fig. 1A). MS-MLPA, did not detect any amplification for CpG106 probes in parents' DNA. The index case was a child of a couple of healthy non-consanguineous parents (mother 33 years old and father 36 years old), clinically evaluated when he was 19 months old. The proband was born at term and a normal perinatal period was reported. His neuromotor development was unremarkable. At the age of 19 months during investigation for a sudden onset of strabismus in the right eye, the diagnosis of unilateral retinoblastoma was made. The ophtalmoscopic evaluation identified a cavitary retinoblastoma with multiple calcified areas, group C - ABC classification, and group III - Reese classification. Intrarterious chemotherapy was then started (melphalan + etoposide for three cycles) resulting in tumor regression. At physical examination he showed normal growth parameters (head circumference of 48.7 cm; 50° percentile), mild frontal bossing, and haemangioma of the glabella extending to the upper left eyelid and uplifted ear lobes.

Since the *core* promoter (from c.-208 to c.-179, NG_009009.1) is the region responsible for the binding with factors such as ATF and SP-1 required for full gene expression, we specifically investigated whether this specific sequence was included in the aberration. Proband's DNA was treated with sodium bisulfite, which converts every unmethylated cytosine into uracil leaving unchanged methylated cytosines (Gill et al., 1994). 500ng of DNA were treated using EZ DNA Methylation-Gold™ Kit following the manufacturer's instructions. After sodium bisulfite treatment, sequencing with primers specific for the methylated strand demonstrated that the *core* promoter was involved in the epigenetic aberration (Fig. 1B) (Supp. Table S1). Bisulfite-converted DNA of the proband was then analysed by pyrosequencing (Pyromark Q96, Qiagen) to confirm and accurately quantify methylation levels with an independent method. Two tissues of different embryonic origin, blood and oral mucosa, were analysed. DNA from buccal swab was isolated with ChargeSwitch™ gDNA Buccal Cell Kit (ThermoFisher Scientific) then, after PCR amplification of bisulfite-converted DNAs (from blood, oral mucosa and control samples), pyrosequencing was performed as previously described (Anwar & Lehmann, 2018). The assay quantified the average methylation at 49% in patient's blood DNA and at 34% in DNA from oral mucosa, while healthy control samples showed very low percentages, comparable to background noise (Fig. 2A). The identification of the aberration in tissues derived from different germ layers (mesoderm and endoderm) suggests that methylation occurred during early embryogenesis. In order to investigate whether aberrant methylation was mono/biallelic we took advantage of a tag-SNP (NC_000013.10:g.48878292G>A: rs2252544) located within CpG106 that was found in heterozygous state in the proband (Fig. 2B). Specific sequencing of the methylated strand in the proband showed only the presence of adenosine, demonstrating that methylation is allele-specific (Fig. 2B). Analysis in parent's DNA

revealed that SNP genotype was homozygous (A/A) in the mother and heterozygous (G/A) in the father, indicating that aberrant methylation affects the maternal allele (Fig. 2B); specific sequencing of the un-methylated strand revealed the presence of both polymorphic alleles, as expected for a mosaic alteration (Supp Figure S1). Real-Time qPCR with *RB1* Taqman gene expression assay was then employed to assess methylation impact on gene transcription. RNA was isolated from whole blood stabilized in PAXgene Blood RNA tubes (PreAnalytiX®, Qiagen) following the manufacturer's instructions. RNA quality was evaluated by NanoDrop 2000 Spectrophotometer (Thermo Scientific). 1 µg of RNA was reverse transcribed into cDNA using a dedicated Qiagen kit (QuantiTect® Reverse Transcription Kit, Qiagen). Real-time qPCR was performed using RB1 TaqMan® Gene Expression Assay (Assay ID Hs01078066_m1, ThermoFisher Scientific); Glucuronidase beta (*GUSB*) was used as housekeeping gene (Assay ID Hs00939627_m1, ThermoFisher Scientific). Experiments were performed in triplicate in a final volume of 20ul with 25ng of cDNA and 25nM of each primer, following the SYBR Green protocol (Roche). Standard thermal cycling conditions were: 2 min at 50°C and 10 min at 95°C; 40 cycles at 95 °C for 15s and 60°C for 1 min. Data were analyzed using the comparative Ct method (Livak, 1997). T-test was used to assess whether *RB1* expression was statistically different in the methylated sample respect to six healthy controls. The expression of total *RB1* resulted decreased (mean±SD, 0.65% ±0.14) in comparison to control samples, at a level comparable to that of a patient with a pathogenic promoter variant (NG_009009.1:g.4969G>A; rs387906521) (mean±SD, 0.73±0.10) and that of a patient with a heterozygous *RB1* deletion involving the promoter,and the first two exons of the gene (rsa 13q14.2(RB1 exons1-2- intr2)x1; rsa 13q14.2(ITM2B exon5)x1; rsa 13p14.2(MED4exon3)x1) (mean±SD, 0.63±0.10) (Fig. 2C). Since ocular tissues of the proband were not available, we do not have information on *RB1*

promoter methylation status, mRNA or protein expression in the tumor. However, previous studies reporting mosaic constitutional epimutations already showed an association between blood and tumor methylation, supporting a causative role for the aberration in cancer predisposition (Snell et al., 2008) (Wong et al., 2011). The identified pathogenic epimutation was submitted to the LOVD database (http://rb1-lsdb.d-lohmann.de) with the following ID: RB1_02119.

In the index patient, high-depth NGS performed on *RB1* untranslated/coding regions and MLPA CNVs analysis did not identify any DNA aberration underlying the epimutation. These data support the classification of the aberration as primary epimutation but a genetic cis-acting alteration in regulatory elements distant from *RB1* cannot be ruled out. Since primary epimutations may not be inherited in a Mendelian fashion, these findings raise complex question relating to the heritability of epimutation (Greger, Passarge, Hopping, Messmer, & Horsthemke, 1989; Sloane et al., 2015). However, since parental epigenetic marks in somatic cells are erased and re-established with each subsequent generation, it is very unlikely that transgenerational inheritance will occur. Moreover, in this case, the risk of heritability of the epimutation is further reduced by mosaicism. However, an appropriate surveillance is required given a higher risk for the patient to develop second primary malignancies. The mosaic pattern of the identified epigenetic alteration could also be transient over the time in the same tissue thus giving reason of the multiple calcified areas early detected by the opthalmoscopic evaluation and probably indicative of a spontaneous regression (Greger, Passarge, Hopping, Messmer, & Horsthemke, 1989). Importantly, this observation underlines the reversible nature of epimutations leading to new encouraging opportunities for targeted therapeutic intervention.

In conclusion, we provided evidence supporting the identification of a constitutional epimutation acting as the first "hit" in the Knudson model of RB development. Our findings suggest that epimutations do not represent a frequent cause of RB predisposition but this is an understudied etiological phenomenon and, beside promoter methylation, other untested epigenetic events may reduce gene expression, phenocopying RB onset.

Author's contribution

Elisa Gelli has performed the experiments and has drafted the manuscript. Alessandra Renieri and Francesca Ariani have made substantial contributions to conception and design of the study and reviewed the manuscript. Anna Maria Pinto has made important contributions in interpretation of molecular results and clinical data collection. Serena Somma, Valentina Imperatore, Marta Giulia Cannone and Mirella Bruttini have contributed to the experiments and data acquisition. Theodora Hadijstilianou and Sonia De Francesco are ophthalmologists that have made RB diagnosis and treated the patient. Daniela Galimberti is the paediatrician who referred us the patient. Francesca Mari and Aurora Currò have conducted the genetic counselling to the family. All authors have given the approval of the final version of the manuscript to be published. They ensure that all aspects of the work are investigated and resolved with accuracy and integrity.

Competing interests

None declared.

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FIGURE LEGENDS

Figure 1. *RB1* promoter methylation detection and quantification. A) MS-MLPA of *RB1* gene in blood DNA of the proband (indicated by the arrow) and healthy parents. Circles highlight the five probes related to the CpG106 island spanning the promoter region. All CpG106 probes are amplified in proband's DNA while no signal is detected in parents' DNA. **B)** Schematic representation of the *RB1* promoter with enlargement of the "*core*" region (from c.-208 to c.-179, NG_009009.1 (LRG_517)), containing the binding sites for SP-1 and ATF transcription factors (upper panel); methylation-specific sequencing of the "*core*" region after DNA bisulfite treatment indicating the methylated cytosines.

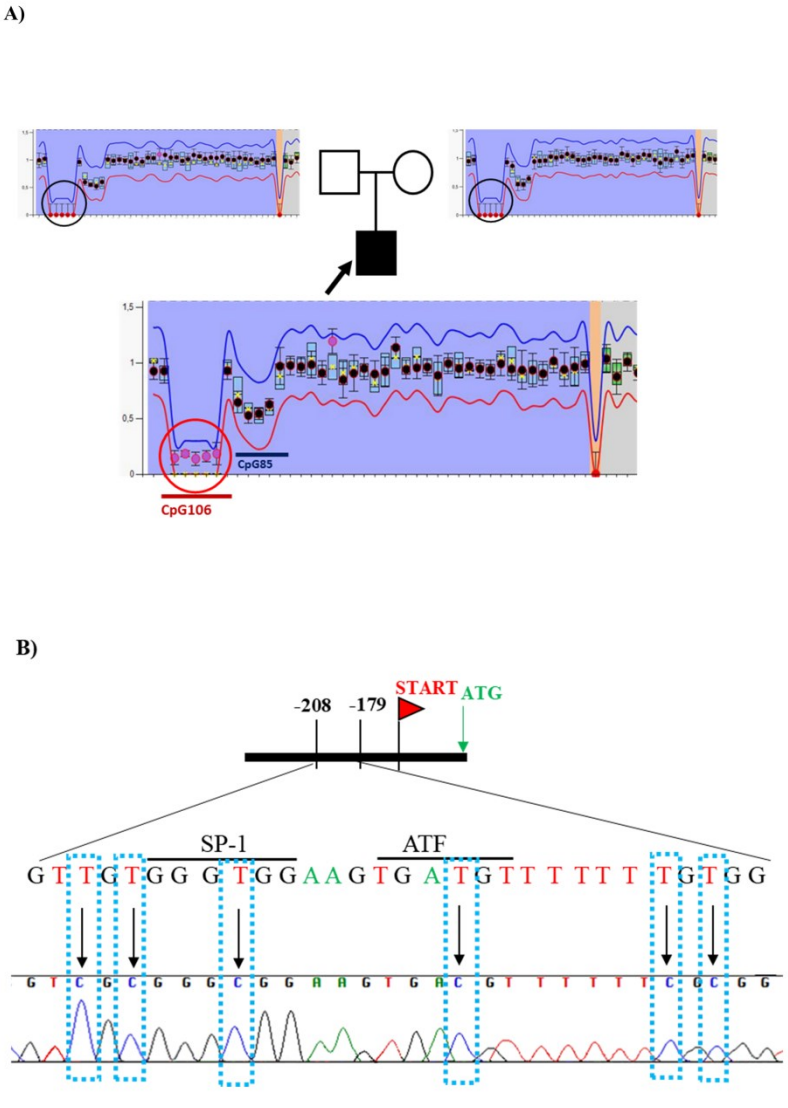
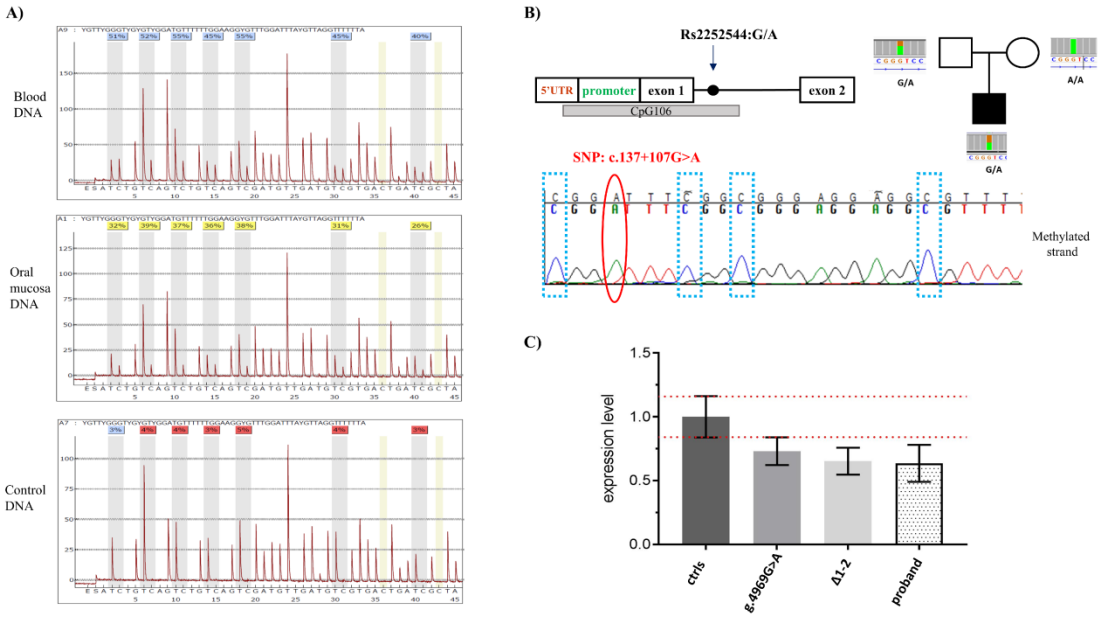


Figure 2. *RB1* promoter methylation characterization and impact on gene expression. A) Pyrograms of CpG sites within CpG106 of *RB1* gene; Y-axis denotes methylation intensities (in a.u. arbitrary unit), on the X-axis the nucleotide dispensation order. DNA methylation of three samples, patient' blood (upper panel), patient' oral mucosa (middle panel) and control sample (lower panel) was quantified in a single pyrosequencing run. CpG sites are highlighted in grey and the percentage of methylation is shown on the top; conversion controls are represented by the thin earl-grey bars. **B)** Schematic representation of CpG106 island and the tag-SNP (rs2252544) (NC_000013.10:g.48878292G>A) (upper panel, on the left); segregation analysis of the tag-SNP in the family showing heterozygosity (G/A) in both the proband and the father and homozygosity (A/A) in the mother (upper panel, on the right). Specific amplification of the methylated strand in proband's DNA showing the presence of only the adenosine (lower panel). **C)** Graph showing the results of Real-Time quantitative PCR analysis performed on blood RNA isolated from the proband, one patient with an heterozygous pathogenic variant in the promoter (NG_009009.1:g.4969G>A; rs387906521), one patient with an heterozygous gene deletion (Δ promoter and exons 1-2 and upstream genes *ITM2B* and *MED4*) and six healthy control samples (ctrls). Bars are representative of the *RB1* expression level; dotted lines indicate the normal range. *RB1* expression levels in the proband are decreased (0.63±0.14) respect to controls and comparable with those of mutated patients (p<0,01, T-test). Data represent mean and SD.



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