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RESEARCH

Epigenetic and Copy Number Variation Analysis in Retinoblastoma by MS-MLPA

Gabriella Livide · Maria Carmela Epistolato · Mariangela Amenduni · Vittoria Disciglio · Annabella Marozza · Maria Antonietta Mencarelli · Paolo Toti · Stefano Lazzi · Theodora Hadjistilianou · Sonia De Francesco · Alfonso D'Ambrosio · Alessandra Renieri · Francesca Ariani

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Abstract Retinoblastoma is the most common primary intraocular malignancy in children. Two step inactivation of *RB1* (M1-M2) represents the key event in the pathogenesis of retinoblastoma but additional genetic and epigenetic events (M3-Mn) are required for tumor development. In the present study, we employed Methylation Specific Multiplex Ligation Probe Assay to investigate methylation status and copy number changes of 25 and 39 oncosuppressor genes, respectively. This technique was applied to analyse 12 retinoblastomas (5 bilateral and 7 unilateral) and results were compared to corresponding normal retina. We identified hypermethylation in seven new genes: *MSH6* (50%), *CD44* (42%), *PAX5* (42%), *GATA5* (25%), *TP53* (8%), *VHL* (8%) and *GSTP1* (8%) and we confirmed the previously reported hypermethylation of *MGMT* (58%), *RB1* (17%) and *CDKN2* (8%). These genes belong to key pathways including DNA repair, pRB and p53 signalling, transcriptional regulation, protein degradation, cell-cell interaction, cellular adhesion and migration. In the same group of retinoblastomas, a total of 29 copy number changes (19 duplications and 10 deletions) have been identified. Interestingly, we found deletions of the following oncosuppressor genes that might contribute to drive retinoblastoma tumorigenesis: *TP53*, *CDH13*, *GATA5*, *CHFR*, *TP73* and *IGSF4*. The present data highlight the importance of epigenetic changes in retinoblastoma and indicate seven hypermethylated oncosuppressors never associated before to retinoblastoma pathogenesis. This study also confirms the presence of copy number variations in retinoblastoma, expecially in unilateral cases (mean 3 ±1.3) where these changes were found more frequently respect to bilateral cases (mean 1.4±1.1).

Keywords Retinoblastoma · MS-MLPA · Epigenetics · Copy number changes

G. Livide · M. C. Epistolato · M. Amenduni · V. Disciglio · A. Marozza · M. A. Mencarelli · A. Renieri · F. Ariani
Medical Genetics, Department of Biotechnology, University of Siena, Siena, Italy

M. A. Mencarelli · A. Renieri
Medical Genetics, Azienda Ospedaliera Universitaria Senese, Siena, Italy

P. Toti · S. Lazzi
Department of Human Pathology and Oncology, Section of Pathology, University of Siena, Siena, Italy

T. Hadjistilianou · S. De Francesco
Ophthalmological Science and Neuroscience, Azienda Ospedaliera Universitaria Senese, Siena, Italy

A. D'Ambrosio
Pediatrics Department, University of Siena, Siena, Italy

A. Renieri (✉)
Medical Genetics Unit, Azienda Ospedaliera Universitaria Senese, University of Siena, viale Bracci 2, 53100 Siena, Italy
e-mail: renieri@unisi.it

Introduction

Retinoblastoma (RB, OMIM#180200) is a childhood malignant tumor of the developing retina with an incidence of one case in 14,000–22,000 live births [1]. Recent findings provide support for a cone precursor origin of RB [2]. It is caused by

biallelic inactivation (M1-M2) of the *RB1* tumor suppressor gene within chromosome bands 13q14.2 [3, 4]. In non-hereditary RB (60%), both inactivating events occur in the retinal cell leading to unilateral sporadic tumors [5]. In the hereditary forms (40%), germline mutation of one *RB1* allele is associated to RB predisposition and is transmitted as an autosomal-dominant trait with 90% penetrance [5, 6]. Inactivation of the second allele occurs in the retinal cells and generally results in multiple and often bilateral tumors.

In 1999, Gallie *et al*. introduced a model for retinoblastoma development in which it was assumed that the two-step inactivation of *RB1* is necessary but not sufficient for the exponential expansion of RB and that further events (M3-Mn) are required [7]. This hypothesis was supported by the observation that RB tumors generally show additional recurrent genetic alterations [8]. In particular, studies performed by standard karyotype, CGH and array-CGH identified RB recurrent genomic rearrangements including gain of 1q, 2p, 6p and 13q, and loss of 16q [8, 9]. Additional events associated with tumor onset and progression comprehend promoter hypermethylation of CpG islands that results in transcriptional silencing of tumor suppressor genes. Promoter hypermethylation has been demonstrated to be an important mechanism in the pathogenesis of various human cancers including ovarian cancer, renal carcinoma, bladder cancer, colorectal cancer and pediatric tumors [10–15]. Using bisulfite sequencing, methylation-specific PCR and quantitative PCR assays the following genes have been found hypermethylated in RB: *MGMT*, *RASSF1A*, *CASP8*, *MLH1*, *RBL2*, *NEUROG1*, *DAP-kinase*, *RUNX3* and *CACNA1G* [15–21].

In this study, for the first time, we employed Methylation-Specific Multiplex Ligation Probe Amplification (MS-MLPA) technique to investigate RB epigenetic and copy number changes of 25 and 39 onco-suppressor genes, respectively. In particular, we analysed RB eye tissues from 12 patients, 5 bilateral and 7 unilateral, and we compared results with the corresponding normal retina.

Material and Methods

Tissue Sample Collection

We collected 12 formalin-fixed paraffin-embedded eye samples from enucleated RB patients archived in the Department of Human Pathology and Oncology of the University of Siena. After surgery, enucleated eyes were immersion-fixed in buffered formalin for 48 h. After fixation, sampling, paraffin embedding, and cutting were carried out according to the usual pathological methods. The group of samples included 5 bilateral cases and 7 unilateral cases. For each patient, the corresponding DNA sample extracted from blood was available in the Italian Retinoblastoma Biobank (http://www.biobank.unisi.it). A germline mutation in *RB1* was identified in all bilateral tumors (p.R455X in RB#263, p.R467X in RB#190, p.V144fsX155 in RB#185, p.R787X in RB#225 and p.687fsX690 in RB#167) (Table 1). No germline mutations

Table 1 Clinical and pathologic features of RB patients. For laterality: U = unilateral, B = bilateral; A/D: Alive/dead; for histology: Und = undifferentiated, Dif = differentiated; for foci: Uni: unifocal; Multi: multifocal; for therapy: JET = Carboplatinum in combination with Etoposide

Case number	Laterality	*RB1* germline mutation	Age at diagnosis (months)	A/D	TNM Classification	Histology	Foci	Vitreous Seeding	Relapses	Metastasis	Therapy
RB#263	B	p.Arg455X	4	A	pT2a	Und	Multi	No	No	No	JET post-enucleation (6 cycles)
RB#190	B	p.Arg467X	20	A	pT3a	Dif	Multi	No	No	No	JET post-enucleation (6 cycles)
RB#185	B	p.Val144fsX155	10	A	pT2	Dif	Multi	No	No	No	JET post-enucleation (6 cycles)
RB#225	B	p.Arg787X	1	A	pT1	Dif	Multi	No	No	No	Focal therapy post-enucleation
RB#167	B	p.Pro687fsX690	13	A	pT1	Dif	Multi	No	No	No	JET and focal therapy post-enucleation (4 cycles)
RB#253	U	_	48	A	pT2a	Und	Uni	Yes	No	No	No therapy
RB#313	U	_	6	D	pT2a	Dif	Uni	No	No	Yes (brain)	JET post-enucleation (10 cycles)
RB#206	U	_	44	A	pT2b	Dif	Uni	No	Yes	No	JET and focal therapy pre-enucleation (10 cycles)
RB#76	U	_	30	A	pT3a	Dif	Uni	No	No	No	No therapy
RB#268	U	_	23	A	pT2a	Und	Multi	No	No	No	No therapy
RB#297	U	_	20	A	pT2a	Dif	Multi	No	No	No	No therapy
RB#79	U	_	5	A	pT3a	Dif	Uni	No	No	No	No therapy

were detected in unilateral cases. Mutational screening was carried out by DHPLC and sequencing analysis (point mutations) and by MLPA (large rearrangements).

Laser-Capture Microdissection and DNA Extraction

Normal retina and RB tissues were identified in hematoxylin–eosin-stained sections. Sections 5 μm thick were deparaffinized, rehydrated, and stained with Mayer hematoxylin and yellow eosin, then dehydrated with xylene. Slides were observed through an inverse microscope. Cells of the two different tissues were isolated by laser-capture microdissection (Arcturus PixCell II; MWG-Biotech). Selected cells were immediately transferred into a standard microcentrifuge tube containing digestion buffer and 20 μg/mL proteinase K (Qiagen). DNA was extracted using QIAmp DNA Micro Kit according to the manufacturer's protocol. The Hoechst dye-binding assay was used on a DyNA Quant 200 Fluorometer (GE Healthcare) to determine the appropriate DNA concentration.

MS-MLPA Assay

To perform methylation specific (MS) multiplex ligation probe amplification analysis (MLPA; MRC Holland, Amsterdam, The Netherlands) we used the ME002 Tumor Suppressor-2 kit (http://www.mlpa.com). Using this kit a total of 25 tumor suppressor genes can be analysed for aberrant promoter methylation and 39 genes for copy number changes. Experimental procedures were carried out according to manufacturer's instructions. Briefly, a total of 100 ng of DNA was diluted with TE buffer and denaturated in a thermocycler. SALSA MLPA buffer and MS-MLPA probes were added and hybridized to their specific targets for 16 h at 60°C. After hybridization, samples were split equally into two vials, each containing the same amount of DNA. Ligase-65 mix (Ligase 65 buffer, Ligase 65 enzyme and water) was added to the first vial, and Ligase-Digestion Mix (Ligase 65 buffer, Ligase 65 enzyme, *Hha*I enzyme and water) to the second vial. Samples were incubated at 49°C for 30 min. The ligase enzyme was inactivated by heating at 98°C for 5 min. PCR was performed as described by the manufacturer (MRC-Holland). Subsequently PCR reaction fragments were separated and visualized on an automated sequencer (ABI PRISM 310, Applied Biosystems). Normal retina was used as control.

MS-MLPA Data Analysis

Promoter methylation and copy number changes were analysed using Coffalyser software (MRC-Holland). Methylation values were obtained by a first step of normalization to compensate for differences in PCR efficiency of the individual samples: the fraction of each peak is calculated by dividing the peak area of each probe amplification product by the combined value of the control probes within the sample. This "relative peak value" of the digested sample is divided by the "relative peak value" of the corresponding undigested sample, generating the "methylation ratio". Aberrant methylation was scored when the calculated methylation ratio was >25%. Any methylation percentage below this level was considered as background. As previously reported, ratios were interpreted as: mild hypermethylation (25%–50%), moderate hypermethylation (50%–75%) and extensive hypermethylation (>75%) (Table 2) [22]. Copy number analysis was performed using MLPA results from undigested samples. The "relative peak value" was divided by the "mean probe fraction" of this fragment within the included reference DNAs, generating the "copy number ratio". Results obtained from an experiment performed on DNA isolated from 20 normal retina samples gave threshold values to determine aberrant copy number.

Statistical Analysis

Mann-Whitney U-test was used to compare promoter hypermethylation and copy number changes between unilateral and bilateral samples. Chi square analysis in contingency tables was conducted to estimate the relationship between *MGMT* and *MSH6* hypermethylation and tumor phenotype. P-values ≤ 0.05 were considered significant.

Results

Detection of Promoter Hypermethylation

By using MS-MLPA probe set ME002 (MRC Holland) we analyzed epigenetic changes in 12 RB FFPE tissue samples (7 unilateral and 5 bilateral) and we compared results to those obtained in corresponding normal retina samples (Fig. 1). Patients' characteristics are summarised in Table 1. A total of 25 known oncosuppressor genes were analysed for aberrant methylation (Table 2). MS-MLPA analysis was executed in duplicate for all samples producing reproducible ratios (data not shown). A total of 34 hypermethylation events were identified (Table 2). Only three tumor samples (3/12; 25%) did not exhibit gene hypermethylation (RB#79, RB#225 and RB#167) (Table 2). Seven tumors (7/12; 58%) had three or more hypermethylated genes (Table 2).

Promoter hypermethylation in more than one sample was detected for the following genes: *MGMT* (7/12; 58%); *MSH6* (6/12; 50%); *CD44* (5/12; 42%); *PAX5A* (5/12; 42%); *GATA5* (3/12; 25%); and *RB1* (2/12; 17%) (Fig. 1) (Table 2). Hypermethylation in only one sample was detected in *TP53*, *IGSF4*, *VHL*, *GSTP1*, *CDKN2A* (Table 2).

Table 2 Aberrant methylation and copy number changes in 12 RB cases, 5 bilateral (B) and 7 unilateral (U). Met indicates methylation. Gains >1.3 and losses <0.7

GENE	Chr. region	RB 263 B	RB 190 B	RB 185 B	RB 225 B	RB 167 B	RB 253 U	RB 313 U	RB 206 U	RB 76 U	RB 268 U	RB 297 U	RB 79 U
TP73	1p36								Loss (0.6)				
MSH6	2p16			Met (49%)			Met (41%)	Met (68%)	Met (100%)	Met (42%)	Met (33%)		
VHL	3p25.3								Met (60%)				
APC	5q22							Gain (1.4)					
TNXB	6p21.3						Gain (2.3)			Gain (3.3)	Gain (1.9)	Gain (3.2)	Gain (1.8)
ESR	6q25.1										Gain (1.6)		
PARK2	6q26										Gain (1.4)		
PAX5	9p13		Met (51%)						Met (100%)	Met (35%)	Met (47%)	Met (33%)	
CDKN2	9p21										Met (33%)		
PTCH	9q22.3										Gain (4.9)		
MGMT	10q26	Met (46%)	Met (42%)	Met (35%)				Met (54%)	Met (38%)	Met (68%)	Met (50%)		
CD44	11p13		Met (100%)	Met (100%)			Met (42%)	Met (78%)			Met (58%)		
PAX6	11p13	Gain (1.7)			Gain (2.5)								
WT1	11p13		Gain (1.7)						Gain (1.7)	Gain (1.8)			
GSTP1	11q13								Met (36%)				
IGSF4	11q23	Met (33%)							Loss (0.6)				
CHFR	12q24.33					Gain (1.4)		Loss (0.5)	Loss (0.5)				
BRCA2	13q12.3												Loss (0.6)
RB1 (a)	13q14.2							Met (30%)		Loss (0.5)	Met (100%)		Loss (0.5)
RB1 (b)	13q14.2							Met (26%)		Loss (0.5)	Met (100%)		Loss (0.5)
MLH3	14q24.3		Gain (1.4)					Gain (1.3)	Gain (1.7)				
PYCARD	16p12	Gain (1.4)											
CDH13	16q24.2						Loss (0.5)						
TP53	17p13.1	Met (32%)					Loss (0.4)						
GATA5	20q13.33		Loss (0.6)					Met (67%)	Met (38%)		Met (70%)		

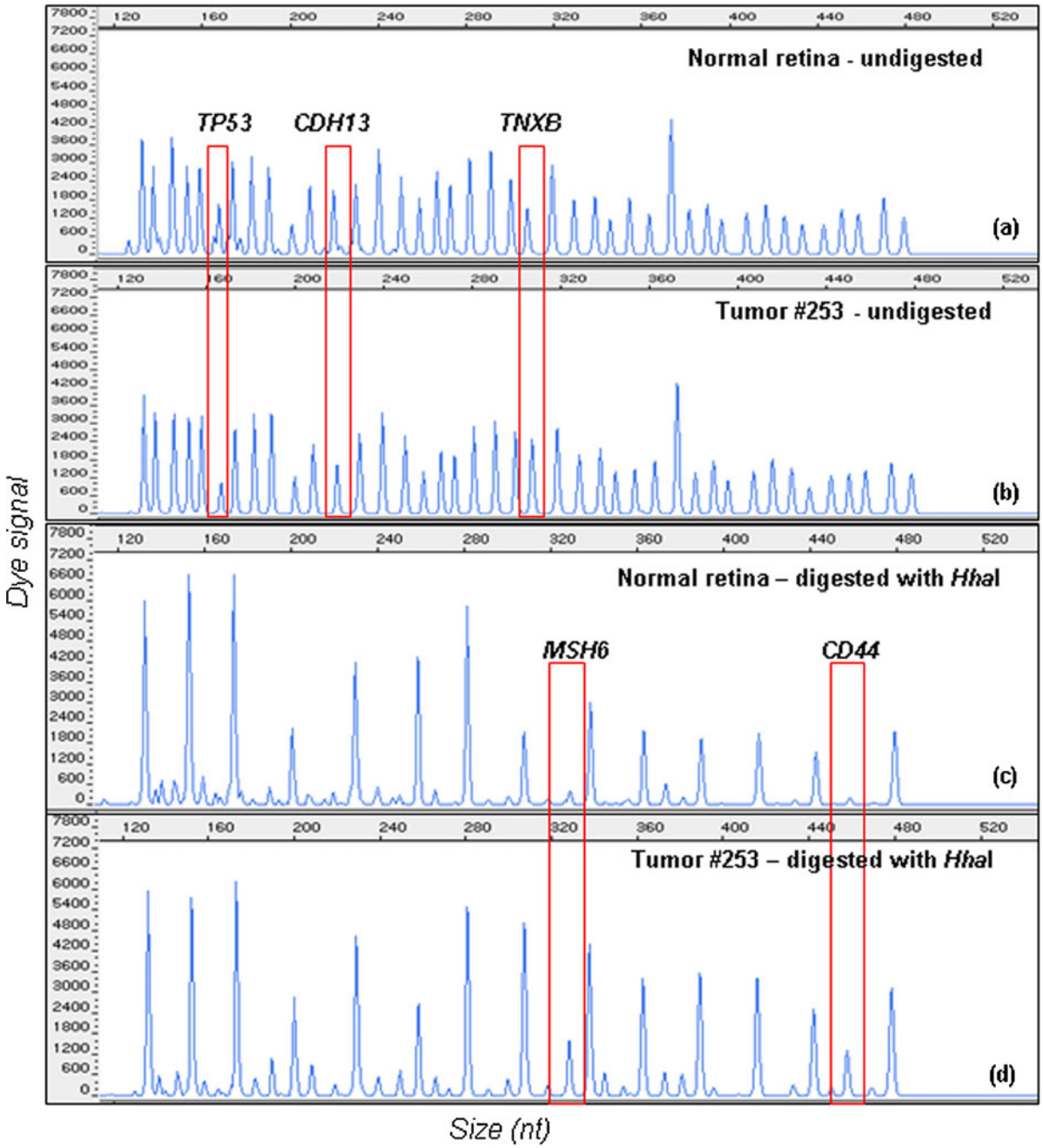

Fig. 1 Detection of copy number variations and aberrant methylation in tumor #253 respect to corresponding retina. MS-MLPA analysis without *Hha*I enzyme treatment in normal retina **a** and tumor **b**. Note *TP53* and *CDH13* losses (copy number ratio: 0.41 and 0.54, respectively) and *TNXB* gain (copy number ratio: 1.9) (*red box*). MS-MLPA analysis with *Hha*I enzyme treatment in normal retina **c** and tumor **d**. Note aberrant methylation of *MSH6* (methylation ratio: 41%) and *CD44* (methylation ratio: 42%) (*red box*)

Concerning RB phenotype, promoter hypermethylation of at least one gene was detected in six out of seven (86%) unilateral samples and in three out of five (60%) bilateral samples (Table 2). Average number of hypermethylation events was 3.6 (± 2.9) in unilateral samples and 1.8 (± 1.6) in bilateral cases (p=0.39). Hypermethylation of *MGMT* was found at approximately the same frequency in unilateral and bilateral samples (p=0.62) (Table 2). *MSH6* hypermethylation was more frequent in unilateral cases (5/7; 71%) (Table 2) (p=0.08).

Detection of Copy Number Variations

By MS-MLPA, a total of 39 genes were analysed for copy number gains/losses. On the basis of an experiment performed on DNA isolated from 20 normal retina samples thresholds to

detect gains and losses were set at 1.3 and 0.7, respectively. Overall, copy number variations were detected in all samples analysed, except for a bilateral sample (RB#185) (Table 2). In total, we found 29 copy number changes (Table 2). Concerning the phenotype, variations were found more frequently in unilateral (mean 3 ±1.3) respect to bilateral (mean 1.4±1.1) tumors (p=0.053). In particular, we found 19 gains (66%) and 10 losses (34%). On average, we detected 1.7 gains (±0.8) in unilateral samples and 1.2 (±0.8) gains in bilateral samples. Losses were identified less often with average number being 1.3 (±1.1) and 0.2 (±0.4) in unilateral and bilateral samples, respectively.

The most frequently affected MLPA probe was one targeting the *TNXB* gene, found duplicated in 5 unilateral samples (42%) (Table 2). Other frequent gains involved *MLH3* (3/12; 25%), *WT1* (3/12; 25%), and *PAX6* (2/12; 17%) (Table 2). Deletions detected in two samples involved *RB1* and *CHFR* (Table 2). Eleven genes showed copy number variations in only one sample: *TP53, CDH13, PYCARD, GATA5, APC, TP73, IGSF4, PARK2, PTCH, ESR* and *BRCA2* (Table 2).

Discussion

The advancement acquired in knowledge of gene expression regulation by epigenetic changes is improving our learning of tumor onset and development [23–25]. In 1999, Jones and Laird proposed the "expanded two hit model" to include epigenetic gene silencing as an inactivating mechanism of tumorigenesis [26]. Concerning retinoblastoma, methylation of *RB1* promoter has been reported to account for 8–13% of somatic mutations [8, 27]. In addition, the following genes have been reported as aberrantly methylated in RB: *MGMT*, *RASSF1A*, *CASP8*, *MLH1*, *NEUROG1*, *DAP-kinase*, *RUNX3* and *CACNA1G* [15–18, 20, 21]. In the present study, for the first time, we employed Methylation Specific-MLPA technique to investigate methylation profile of 25 tumor suppressor genes in 12 RB eye tissues (7 unilateral and 5 bilateral). MS-MLPA has been the method of choice since it has been demonstrated that this technique can be applied successfully to DNA derived from paraffin-embedded tissues [28]. We identified aberrant methylation in the promoter of the following 10 genes: *MGMT* (7/12; 58%), *MSH6* (6/12; 50%), *CD44* (5/12; 42%), *PAX5* (5/12; 42%), *GATA5* (3/12; 25%), *RB1* (2/12; 17%), *CDKN2* (1/12; 8%), *TP53* (1/12; 8%), *VHL* (1/12; 8%) and *GSTP1* (1/12; 8%).

The most frequent aberrant methylation was found in *MGMT* (methylation range: 35–68%) (Table 2). This is in accordance with previous studies by Choy et al, even if we reported a higher percentage (58% vs 15–35%) [16, 18]. Furthermore, in the present study, *MGMT* hypermethylation showed approximately the same frequency in bilateral (3/5; 60%) and unilateral (4/7; 57%) cases, while Choy et al. reported a higher frequency in bilateral cases [16]. Our findings are therefore in contrast with the Choy et al. hypothesis of *MGMT* hypermethylation being associated with an inherited disease genotype [16]. *MGMT* encodes the DNA repair enzyme Methylguanine-DNA Methyltransferase that removes alkylating lesions at O6 of guanine to protect against mutagenesis and malignant transformation [29]. Its evolutionary conservation suggests a fundamental role in cell physiology and genome maintenance [30]. Animal studies showed association between *MGMT* level of activity and tumorigenesis [31, 32]. While *MGMT* deletions/mutations are rarely observed, *MGMT* hypermethylation has been found in many types of cancer including breast and prostate cancer, lymphomas, gliomas, lung carcinomas, colorectal tumors and epithelial ovarian cancer [33–37]. *MGMT* epigenetic silencing leads to a mutator pathway in human cancer, because the O6-methylguanine adducts produce C:G to A:T transitions in other genes such as *K-ras* and *TP53* [38–41]. However, *MGMT* epigenetic silencing has been also described as a "predictive friend" since there is a strong and positive correlation between *MGMT* hypermethylation and increased tumor sensitivity to alkylating agents such as platinum compounds that are commonly used for RB treatment [42, 43]. On the contrary, patient RB#206 showed two relapses after JET (10 cycles) and focal therapy (Table 1). Moreover, patient RB#313 showed brain metastasis and died at 4 years and 5 months after 10 cycles of JET therapy. Unfortunately, we could not collect metastasis biopsy and MS-MLPA analysis could not be performed.

For the first time, our results indicate *MSH6* aberrant methylation (methylation range: 33–100%) in RB samples (6/12; 50%), mainly among unilateral cases (5 unilateral and 1 bilateral) (p=0.08) (Table 2). *MSH6* is an important factor of safeguarding genetic stability during replication [44, 45]. It is part of the mismatch repair (MMR) system that corrects errors of DNA polymerases that escape their 3′>5′exonucleolytic proofreading activity. It has also been implicated in the cellular DNA damage response, activating cell cycle checkpoint and apoptosis, and thus, alterations in this system can have wide-ranging biological consequences [46, 47]. MMR-defective cell lines are more resistant to cell death induced by several DNA-damaging agents [48]. Genetic alterations of the *MSH6* gene have been found in many cancer types such as colorectal and endometrial cancer [49–53]. Interestingly, we found that most *MSH6* promoter methylated cases (5/6; 83%) were also methylated in the *MGMT* gene (Table 2). This might be due to a positive selection for cellular clones bearing the two inactivation events, accelerating the pathway driving to cancer development. Since mutations in MMR genes are usually associated with a microsatellite instability (MSI) phenotype, this might also be the case in RB. Previous studies found MSI in a subset of RB samples, but this phenotype was not significantly associated with promoter hypermethylation

of another MMR gene, namely *MLH1* [17, 54]. This point would require further investigation in RB samples.

MS-MLPA also detected epigenetic changes in a transcriptional factor, *PAX5,* whose hypermethylation was never reported before in RB (Table 2). In particular, *PAX5* promoter hypermethylation (methylation range: 33–100%) was found in five RB samples (5/12; 42%), 4 unilateral and 1 bilateral. Previous reports have demonstrated *PAX5* involvement in human acute B-cell leukemia and lymphoma [55, 56], but other studies have highlighted the importance of *PAX5* also in solid cancer such as breast and lung tumors and hepatocellular carcinoma [57]. *PAX5* is frequently inactivated by hypermethylation in tumors and acts as functional tumor suppressor through direct regulation of the p53 signalling pathway [57]. Notably, an association between *PAX5* and the underphosphorylated form of pRB has been shown by Sato et al. [58]. It is therefore possible to hypothesize that *PAX5* hypermethylation might represent an inactivating event of pRB signalling that contribute to RB tumorigenesis.

The *CD44* gene was hypermethylated (methylation range: 42–100%) in a significant fraction of RB samples (5/12; 42%), 3 bilateral and 2 unilateral. *CD44* encodes a cell-surface glycoprotein that may be associated with metastases and therefore may be useful in the early detection of metastatic potential in surgical biopsy samples and early detection of recurrence in tumors [59, 60]. Among *CD44* hypermethylated samples, RB#313 displayed brain metastases (Table 1).

Three unilateral samples showed hypermethylation of *GATA5* (methylation range: 28–70%), a gene encoding a zinc finger transcriptional regulator that has been demonstrated to be inactivated in many cancer types such as lung, esophageal, pancreatic, colorectal and gastric cancer (Table 2) [61–65]. Hypermethylation in the promoter regions of *TP53*, *VHL* and *CDKN2* was found in only one RB sample (Table 2). *TP53* inactivating mutations have never been found in RB primary tumors and this is the first study reporting a mild *TP53* epigenetic inactivation in RB [66]. Epigenetic inactivation of *VHL*, a suppressor gene responsible for both hereditary and sporadic cancer forms, has been never reported in RB and might have important consequences in senescence induction in a pRb-dependent manner [67]. Hypermethylation of *GSTP1*, encoding one of the enzymes of the glutathione Stransferases superfamily, might result in DNA damage and mutations as already hypothesized in prostatic carcinogenesis [68]. Aberrant promoter methylation of *CDKN2* (also known as *p16INK4A),* a key cell cycle regulator of the pRb pathway, has been already reported in RB patients by Indovina et al. [69].

Furthermore, we found a total of 29 copy number variations, mainly gains (19 duplications and 10 deletions) (Table 2). Interestingly, copy number changes occurred more frequently in unilateral cases respect to bilateral cases even if the small sample size did not allow to reach statistical significance (p= 0.053). This is in accordance with previous data that showed a higher chromosomal instability in unilateral cases, suggesting that other molecular mechanisms could be implicated in hereditary RB [8, 70].

Our data showed a gain of *TNXB* in five unilateral cases (Table 2). In contrast with these results, it has been reported that TNXB deficiency promoted tumor invasion and metastasis in mice [71] and that TNXB downregulation was present in NF1-associated tumors [72]. However, in previous study, using array-CGH, we demonstrated that this duplication involves the entire p arm of chromosome 6, a frequent rearrangement characterising RB [70]. This rearrangement included the three known oncogenes *IRF4*, *DEK* and *PIM1* and the two members of the pRB pathway *E2F3* and *CCND3*, whose overexpression could be rather one of the driving events of RB development [70].

Deletions involve the following tumor suppressor genes: *TP53*, *CDH13*, *GATA5*, *CHFR*, *TP73*, *IGSF4* and *BRCA2*. In total, *TP53* has been found inactivated in two out of twelve RB samples (17%), indicating that *TP53* direct inactivation is not a frequent event in RB and supporting the notion that subsequent amplification and increased expression of *MDMX* likely suppress the p53 response in RB [73]. Differently, inactivation of *GATA5* appeared to be a frequent event (3 methylated and one deleted sample) (Table 2). *CDH13* is an interesting candidate gene within 16q loss, a frequent rearrangement in RB [74]. Its downregulation has been associated with diffuse vitreous seeding [75] and with poorer prognosis in various cancers [76]. Actually, patient RB#253 is the only one that showed vitreous seeding (Table 1). *CHFR* copy number changes (2 losses and 1 gain) might alter its function of mitotic checkpoint control and chromosomal stability maintenance [77, 78]. Notably, Chkraborty et al. by microarray analysis demonstrated *CHFR* downregulation in RB tissues [79]. *TP73* has been found to be transcriptionally silenced in some lymphoblastic leukemias and lymphomas due to hypermethylation [80, 81]. *IGSF4* expression has been found downregulated in non-small-cell lung cancer, hepatocellular carcinoma and pancreatic cancer cell lines [82]. *BRCA2,* involved in DNA-damage response, has been found differently expressed in RB tissues [83].

In conclusion, MS-MPLA technique allowed us to perform a study of epigenetic events and copy number variations in RB tissues. Our data highlighted the importance of epigenetic changes in RB and identified seven oncosuppressor genes never associated before with the pathogenesis of RB: *MSH6*, *CD44*, *PAX5*, *GATA5*, *TP53*, *VHL* and *GSTP1*. Since epigenetic mechanisms are potentially reversible these findings could provide new hints for the design of therapeutic strategies in RB. Copy number variations have been found in almost all samples but the genes involved often belong to larger genomic rearrangements so that it is difficult to identify factors actually driving RB tumorigenesis. Finally, in

accordance with our previous study, copy number changes have been identified more frequently in unilateral cases, suggesting that other mechanisms could be involved in hereditary RB [9].

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