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Original contribution

Epigenetic changes underlie the aggressiveness of histologically benign meningiomas that recur☆

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Summary Meningiomas are the most frequent primary brain tumor. Usually, they are curable by surgery, but even after seemingly complete resection, some low-grade lesions recur. Despite recent improvements, signatures having prognostic value in grade I tumors remain poorly characterized. The frequency and delicate location of these tumors suggest that the risk of recurrence might be more accurately predicted. Herein, we show an easy way to evaluate the methylation status of meningiomas and its correlation with the prognosis of the disease. A series of 120 meningiomas, including primary tumors and recurrences, were analyzed histopathologically, and 24 tumor suppressor genes (TSGs) were studied by methylation-specific multiple ligation probe amplification. Long-term follow-up was conducted to classify patients with grade I primary tumors according to their outcomes. We found that hypermethylation in at least one TSG is frequent. The number of hypermethylated TSG per case was significantly higher in recurrences than in primary tumors and in primary benign meningiomas that recurred than in tumors from patients who showed no evidence of disease during follow-up. Finally, hypermethylation in *RASSF1A*, *MLH1*, and *CDKN2B* was an independent prognostic factor associated with the time to recurrence of these benign tumors that were biologically aggressive. To our knowledge, this is one of the widest studies of primary grade I tumors of patients who developed a tumor recurrence. The frequency of epigenetic changes suggests that hypermethylation is an early event in meningiomas, whereas the accumulation of epigenetic changes is related to greater biological aggressiveness and may be a signature of potential clinical relevance.

Abbreviations: FISH, fluorescence in situ hybridization; HPFs, high-power fields; MN, meningioma; MS-MLPA, methylation-specific multiple ligation probe amplification; NED, no evidence of disease; PT, primary tumor; PTR group, primary grade I meningiomas that recurred during the follow-up; RC, recurrence; TSG, tumor suppressor gene; WHO, World Health Organization.

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1. Introduction

MNs, which arise from arachnoid cells in the meninges, are the most frequent primary brain tumor type, accounting for 30% to 35% of all central nervous system tumors. Women are more likely to be affected, with a female-to-male ratio of 2.3:1 [1,2]. Histologically, MNs are a heterogeneous group of tumors, classified according to the 2016 WHO classification as grades I to III depending on histologic features such as mitotic activity, brain invasion, high nuclear-cytoplasmic ratio, prominent nucleoli, sheeting, and foci of spontaneous necrosis [2]. RC, which affects 18% of the patients in the first 5 years after resection and as many as 25% within 10 years, is the most significant complication that influences the clinical course of the disease. Grade II and III MNs show a high incidence of RC (29%-52% and 50%-94%, respectively) [2-4]. Grade I MNs are apparently benign, although they recur in 7% to 25% of cases. Given that they represent the most frequent MN, most RCs appear within this group of MNs in absolute numbers [4-7]. To ensure adequate treatment, there is a great need to predict accurately the clinical aggressiveness of histologically benign MN.

Cytogenetic and molecular analyses strongly suggest that loss of chromosome 22 is a primary event in the development of these tumors [8,9]. Although there have been many attempts to improve the genomic profiling of MNs using high-throughput techniques, *NF2* is the only specific gene frequently involved in their early genesis [2,10-15]. The genetic features described, such as *AKT1*, *SMO*, *TRAF7*, or *KLF4* mutations, improve the characterization of the different grades, subtypes, or locations. However, these findings lack prognostic significance in benign MNs [2,3,14,15]. In addition, cytogenetics, which offers the most consensual prognostic results, has not entered the diagnostic routine, making it clear that the biological heterogeneity of these tumors requires more accurate but also cost-effective studies focused on the mechanisms underlying aggressiveness.

In recent years, the importance of epigenomics in cancer has become evident [7,16,17]. Hypermethylation of DNA was the first epigenetic alteration to be observed in cancer cells, and it is better understood than other epigenetic changes. Hypermethylation has been reported in as many as 70% of MN patients, independent of the WHO tumor grade [18,19]. Some studies have shown promising results regarding a direct relation between hypermethylation in specific TSGs and high-grade MNs, but the results have been contradictory [16,19-22]. Thus, our understanding of the epigenetic events that increase the RC probability in histologically benign MNs remains limited.

In this study, we aimed to establish the methylation status in a series of primary MNs with a cost-effective technique. We then compared our findings in PTs with those detected in tumor RCs. To study the characteristics of methylation in patients with histologically benign grade I MNs who had RCs, we compared them with grade I tumors that show truly benign behavior (NED) with extended follow-up. Finally, we looked for similarities between PTRs and RCs to determine methylation patterns related to aggressive behavior in histologically benign MNs.

2. Materials and methods

2.1. Samples and clinical study

Tumor samples (n = 120) were collected from 101 patients found to have MN at Hospital Clínico Universitario in Valencia; these lesions consisted of 92 PTs and 28 RCs. The study protocol was reviewed and approved by the Institutional Ethics Committee at the University of Valencia and Hospital Clínico Universitario in Valencia. None of the patients received chemotherapy or radiotherapy before surgery. Tumor specimens were fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Samples were categorized according to the WHO classification [2]. Mitotic index values were obtained by counting the total number of mitotic figures in 10 HPFs.

Patients were followed up by magnetic resonance imaging at 3 and 6 months, and annually after the first year without evidences of disease. This follow-up lasted between 5 and 19 years. *Progression-free survival* was defined as the time elapsed from surgery to the date an RC was detected. The long-term follow-up was critical for our statistical analysis because we went on to study the outcome of histologically benign grade I PTs, subgrouping them into patients who showed no RC during the follow-up (NED group) and those who had RCs despite their initial radical surgery to remove the tumor completely (PTR group).

2.2. Immunohistochemistry staining

The immunohistochemical study was performed on paraffin-embedded sections using the avidin-biotin peroxidase method. The Ki-67 proliferation index was evaluated by MIB-1 antibody staining (Dako, Glostrup, Denmark) and was calculated by determining the percentage of immunopositive nuclei in 2 sections, classifying them as 1 (<1% stained nuclei), 2 (1%-5% stained nuclei), 3 (5%-10% stained nuclei), and 4 (>10% stained nuclei), a technique modified by Barbera et al [8].

2.3. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) for chromosomes 1, 14, and 22 was performed using tissue microarrays that were constructed by introducing 4 selected 1-mm cores per case in a recipient paraffin block. Nonneoplastic tissues from brain controls were included in each tissue microarray. To carry out the FISH analysis, probes LSI 22q12, LSI 1p36/LSI 1q25, and t(11;14)IGH/CCND1 were used according to the manufacturer's instructions (Vysis; Abbott Scientific, Madrid, Spain). Nuclei were counterstained using diamidino-2-phenylindole dichloride. The fluorescent signals were detected using a Leica

LAS AF (Leica; Leica Biosystems Nussloch Gmbh, Nußloch, Germany) photomicroscope with appropriate filters. Signals were counted in a range of 100 to 150 nonoverlapping tumor cell nuclei per case. An interpretation of deletion was made when greater than 20% of the nuclei harbored losses on the basis of the cutoffs established in the control samples [8].

2.4. Molecular analysis

Selected areas of the paraffin blocks from each sample were used for DNA extraction with a QIAamp DNA FFPE tissue kit (Qiagen, Valencia, CA). The quality and quantity of the DNA were improved by standard ethanol precipitation in all tumor and control samples. MS-MLPA was performed to determine the extent of methylation of 24 TSGs using a SALSA MLPA kit (ME001-C2, lot 0808) following the manufacturer's instructions (MRC-Holland, Amsterdam, the Netherlands). The TSGs were selected because of their role in proliferation, angiogenesis, cell cycle control, and apoptosis as described in previous reports [18,23].

Briefly, DNA was denatured at 98°C for 5 minutes and hybridized with the appropriate probe mix at 95°C for 1 minute followed by a 60°C overnight incubation. Ligation and digestion reactions with *Hha*I were carried out at 48°C for 30 minutes followed by a step at 98°C for 5 minutes. The polymerase chain reaction was performed using the SALSA polymerase chain reaction primer mix and SALSA polymerase and consisted of 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute with a final step at 72°C for 20 minutes (all reagents from MRC-Holland). The TSGs, their chromosomic locations, and the primers for each multiplexed reaction are summarized in Supplementary Table 1. The fragments were separated by capillary electrophoresis in an ABI 310 Sequencer (Applied Biosystems, Foster City, CA) and were analyzed with Coffalyser Excel-based software (MRC-Holland). Data were intranormalized, and results greater than 20% were considered positive for promoter hypermethylation, as previously described [24]. We used blood samples from nonrelated healthy donors as negative controls.

2.5. Statistics

Statistical analysis was performed with SPSS-PASW (version 22) software (IBM, Madrid, Spain). When possible, the variables were categorized. Comparisons for categorical and quantitative variables were evaluated using the Kolmogorov-Smirnov and Levene tests; depending on their results, ANOVA or Mann-Whitney *U* tests were then carried out. Categorical variables were evaluated using the χ^2, Fisher exact, and Cramer *V* statistic tests, depending on their characteristics. We also carried out a survival analysis to evaluate the differences between time-to-RC curves using the Kaplan-Meier method. An event was defined as an RC when it was diagnosed with imaging techniques. The statistical significance of these survival curves was calculated using the log-rank (Mantel-Cox) test. Significance was accepted when the probability was less than .05.

3. Results

3.1. Clinical and histologic characteristics

We studied 120 MNs—92 PTs and 28 RCs—from 101 patients. The patients' ages ranged from 7 to 88 years, and the mean age at diagnosis was 58.1 ± 13.4 years. Our cohort included 71 women and 30 men (2.4:1 women/men). From the PTs, 70 were classified as grade I, 21 as grade II, and 1 as grade III. From the RCs, 10 were classified as grade I, 8 as grade II, and 10 as grade III. For subsequent analyses, grades II and III were evaluated together. The main clinical and histopathologic data are summarized in Table 1 and detailed in Supplementary Tables 2 and 3. The female/male ratio was 3.4:1 in grade I PTs, but it was 1.4:1 in higher grades. This difference was more marked in the RCs, with a distribution of 1:1 in grade I and 1:2.6 in higher-grade lesions. The tumor location was supratentorial in 63.0% of PTs and 57.1% of RCs. Both groups showed similar locations in every grade, and the main histologic subtypes were meningothelial and transitional MNs. The overall mitotic index was 1.0 ± 0.2 mitosis/10 HPFs in primary MNs and significantly higher, 2.4 ± 0.4 mitosis/10 HPFs, in RCs ($P = .040$). It was also higher in grade II and III tumors than in grade I lesions in both PT and RC MNs (Table 1). The Ki-67 proliferation index was 1.7 in PTs and 2.3 in RCs: it was 1.5 in grade I PTs and 2.2 in higher-grade PT MNs, whereas it was 2.0 in grade I RCs and 2.4 in higher-grade RCs (Fig. 1A-C and Table 1).

3.2. FISH analysis

Loss of chromosome 22 was the most common cytogenetic event, being present in about half of the samples. There were no significant differences among grades, either between PTs and RCs or depending on the outcome of grade I MNs (Fig. 1D). Loss of chromosome 1 was signifi-

Table 1 Summary of clinical and histopathologic data

	n	Mean age (y)	Sex (F/M)	Mitosis	Ki-67
PT					
Grade I	70	59.4	54/16	0.5 ± 0.1 [a]	1.5
Grade II, III	22	55.7	13/9	2.9 ± 0.7 [a]	2.2
RC					
Grade I	10	52.8	5/5	0.9 ± 0.2 [b]	2
Grade II, III	18	63	5/13	3.2 ± 0.45 [b]	2.4

Abbreviations: F, female; M, male; y, years.
[a] $P = .026$.
[b] $P = .031$.

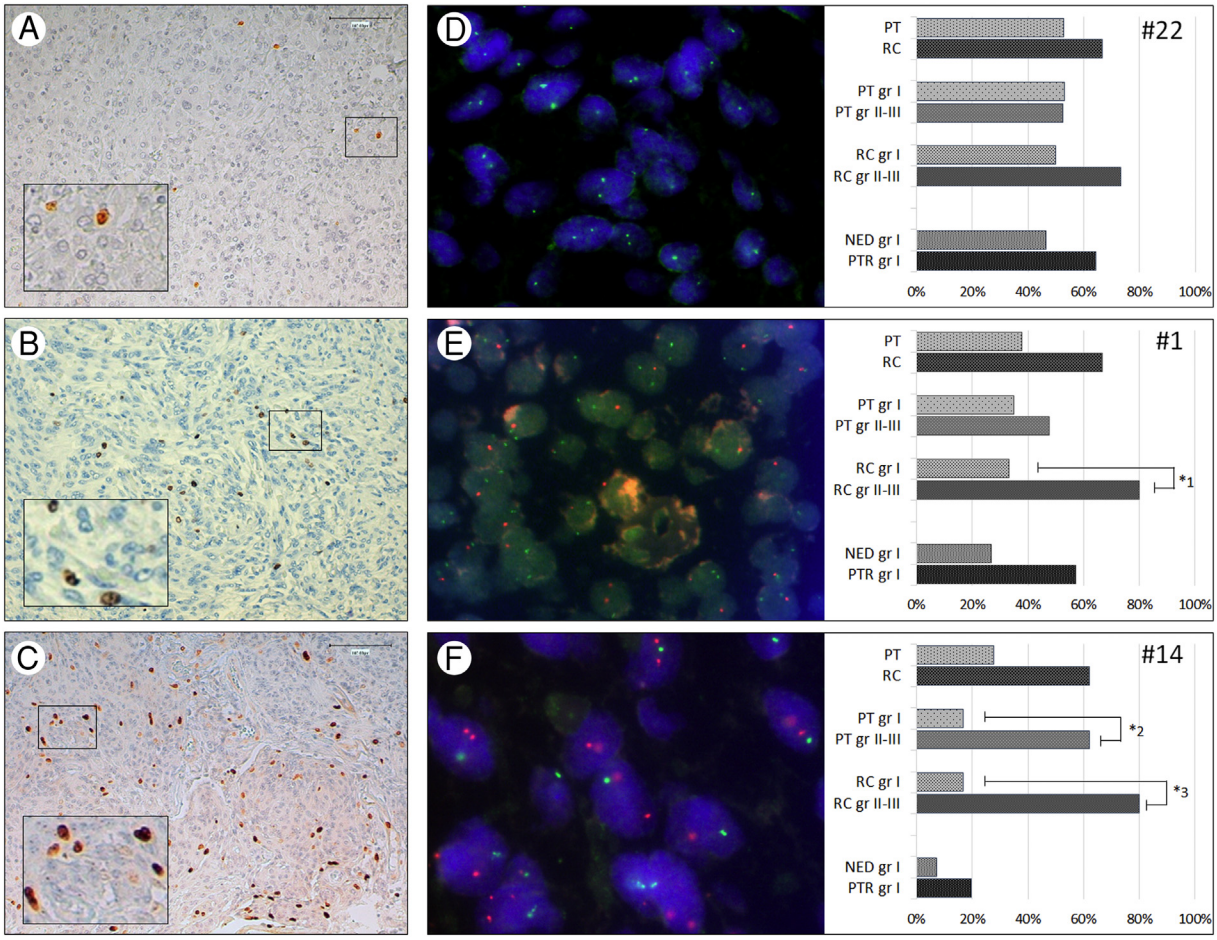

Fig. 1 Proliferation study and FISH analysis. A-C, Representative microphotographs of Ki-67 immunostaining (A, 0-1%; B, 5%-10%; C, >10%). D-F, FISH analysis. D, Interphase nuclei with 1 signal (monosomy) and 2 signals (disomy) corresponding to chromosome 22; a 3% cutoff was established compared with nonneoplastic control tissue. Bar chart shows similar distribution of losses in all groups. E, Chromosome 1 in interphase nuclei; 1p36 is marked in red and 1q25 (control) in green. Bar chart shows higher proportion of 1p losses in high-grade RCs (*1 $P = .040$). F, Interphase nuclei with signals for chromosome 11 (red) used as a control and for chromosome 14 (green). Bar chart shows higher proportion of 14q losses in high-grade meningiomas, both in primary tumors (*2 $P < .001$) and in recurrences (*3 $P = .007$). Abbreviations: PT, primary tumor; RC, recurrence; gr, grade; NED, no evidence of disease; PTR, primary tumor that recurred.

cantly more common in grade II RCs, and it was higher, although not significantly so, in the PTR group than in the NED group ($P = .056$; Fig. 1E). Loss of chromosome 14 was statistically elevated in grade II tumors in both PTs and RCs (Fig. 1F). Detailed proportions and *P* values are included in Table 2.

3.3. DNA hypermethylation in primary MNs

From the 92 analyzed primary MNs, 73.4% showed hypermethylation of at least 1 of the 24 TSGs assayed (Fig. 2A). Also, 15.2% of the cases showed hypermethylation in 3 or more genes (Fig. 2B), and the average number of genes

Table 2 Summary of FISH analysis (%)

Chr	PT			RC			Grade I PT		
	Global	Grade I	Grade II, III	Global	Grade I	Grade II, III	Global	NED	PTR
22	52.9	53.0	52.4	66.7	50.0	73.3	50.9	46.3	64.3
1	37.9	34.8	47.6	66.7	33.3 [a]	80.0 [a]	34.5	26.8	57.1
14	27.6	16.7 [b]	61.9 [b]	61.9	16.7 [c]	80.0 [c]	16.4	7.1	19.5

Abbreviation: Chr, chromosome.
[a] $P = .040$.
[b] $P = .001$.
[c] $P = .007$.

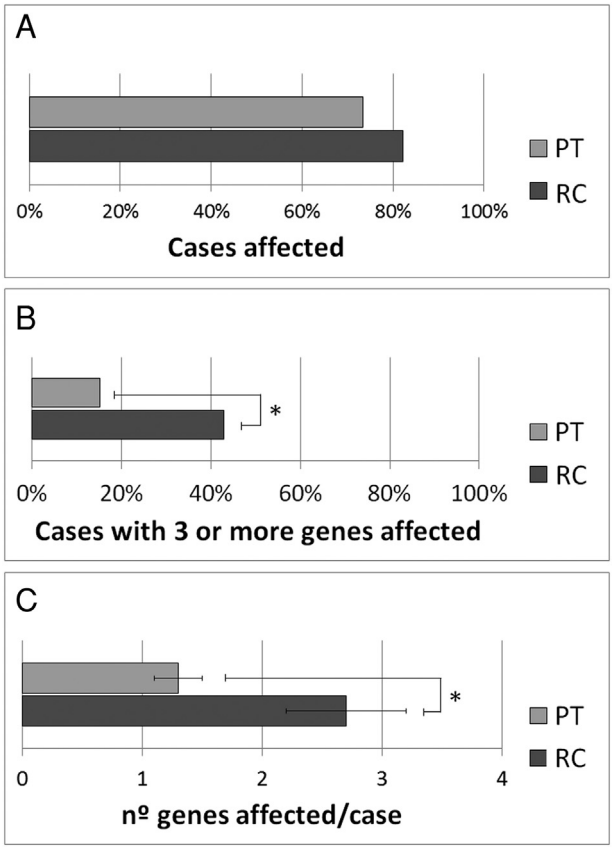

Fig. 2 Global epigenetic analysis of primary MNs and RCs. A, Percentage of cases with hypermethylation of at least one of the TSGs in primary MNs (PT) and RCs. B, Percentage of cases with hypermethylation in 3 or more of the TSGs assayed comparing PT and RC (P = .003). C, Average number of hypermethylated TSGs per case in PT compared with RC (P = .049).

affected per case was 1.3 ± 0.2 (Fig. 2C). The most frequently altered genes were *GSTP1* (22.8%) and *CDH13* (20.7%; Table 3). Statistical analysis comparing the histologic grades showed a similar incidence of hypermethylation in grade I and higher-grade MNs, with an average of 1.4 and 1.2 TSGs affected per case, respectively. The average number of hypermethylated TSGs in each case is available in Supplementary Tables 2 and 3.

3.4. Comparison of hypermethylation in primary MNs and RCs

We analyzed 28 MN RCs, comparing them with PTs; 82.1% displayed DNA hypermethylation in at least 1 of the assayed TSGs (Fig. 2A). Compared with the 15.2% found in the PT group, 42.9% of RCs showed promoter hypermethylation in 3 or more TSGs (P = .003; Fig. 2B). The average number of hypermethylated genes per case was 2.7 ± 0.5 in RCs compared with 1.3 ± 0.2 in primary MNs (P = .049; Fig. 2C). The main hypermethylated genes in these RCs were *RASSF1A* (35.7%), *GSTP1* and *CDH13* (32.1% for both), *CDKN2B* (25.0%), and *TP73* and *ESR1* (21.4% for both). Of these, *RASSF1A* and *ESR1* were significantly more altered in RCs than in PTs (P = .010 and P = .019, respectively; Table 3); 50.0% of the grade I RCs and a 38.9% of higher-grade RCs showed hypermethylation of 3 or more genes. The average numbers of genes affected per case were 1.7 ± 0.5 in grade I RCs and 3.3 ± 0.7 in higher-grade RCs (nonsignificant difference).

3.5. Epigenetic changes depending on sex

The average number of hypermethylated genes per case was 1.6 ± 0.3 versus 1.1 ± 0.2 in men and women, respectively (P = .046). In grade I MNs, we found 1.2 ± 0.2 hypermethylated TSGs per case in women and 1.4 ± 0.3 in men (P = .048). In higher-grade MNs, we found 0.7 ± 0.3 hypermethylated genes per case in women and 1.9 ± 0.4 in men (P = .036).

3.6. DNA hypermethylation in benign MNs that recurred

Histologically benign MNs can evolve aggressively, causing RCs. To improve the epigenetic characterization of such cases, we performed an in-depth analysis of hypermethylation status in the 70 patients with grade I MNs who were followed up for at least 5 years. From these cases, 47 had NED after their final follow-up, whereas 18 patients with grade I MNs had relapses despite gross total resection of the tumor (PTR group). Subtotally resected tumors (n = 5) were excluded from the analysis.

We detected hypermethylation of at least one TSG in 63.4% of the NED cases and 83.3% cases in the PTR group (P > .050). However, the patients in the PTR group had accumulated epigenetic changes: 33.3% of these tumors showed hypermethylation of 3 or more genes compared with 10.3% in the NED group (P = .043; Fig. 3A). The average number of hypermethylated TSGs per case was significantly higher in the PTR group compared with the NED group at 2.5 ± 0.7 versus 1.0 ± 0.2 affected genes, respectively (P = .023; Table 2 and Fig. 3B).

3.7. Correlations with time to RC in histologically benign MNs

A third of PTR cases showed hypermethylation in *MLH1*, whereas it was 9.8% in the NED group; *RASSF1A* and *CDKN2B* were both hypermethylated in 4.9% and 22.2% of the cases in the NED and PTR groups, respectively (Table 4). Although these differences did not reach statistical significance, they indicate that *MLH1* was 3.4-fold more likely to be hypermethylated in the PTR group than in the NED group; this figure increased to 4.5-fold for *RASSF1A* and *CDKN2B*. Interestingly, we found a significant correlation between hypermethylation and time to RC: Kaplan-Meier analysis showed that RCs were seen earlier in cases with *MLH1* (P < .001), *RASSF1A* (P = .046), or *CDKN2B* (P = .046) hypermethylation (Fig. 3C-E). Other genes also were notable, although

Table 3 Frequency of hypermethylation in primary MNs and RCs

Gene	Locus	Global		PT		RC	
		PT	RC	Grade I	Grade II-III	Grade I	Grade II-III
TP73	01p36	13.0	21.4	12.9	13.6	20.0	22.2
CASP8	02q33-q34	1.1	3.6	1.4	0	0	5.6
VHL	03p26-p25	1.1	3.6	0	4.5	0	5.6
RARB	03p24	0	0	0	0	0	0
MLH1	03p21.3	10.9	10.7	14.3	0	10.0	11.1
RASSF1A	03p21.3	13.0 [a]	35.7 [a]	11.4	18.2	20.0	44.4
FHIT	03p14.2	1.1	0	1.4	0	0	0
APC	05q21	2.2	3.6	1.4	4.5	0	5.6
ESR1	06q25.1	5.4 [b]	21.4 [b]	7.1	0	0 [c]	33.3 [c]
CDKN2A	09p21	13.0	17.9	14.3	9.1	20.0	16.7
CDKN2B	09p21	10.9	25.0	8.6	18.2	20.0	27.8
DAPK1	09q34.1	0	3.6	0	0	0	5.6
PTEN	10q23.31	9.8	14.3	11.4	4.5	10.0	16.7
CD44	11p13	1.1	0	1.4	0	0	0
GSTP1	11q13	22.8	32.1	22.9	22.7	10.0	44.4
ATM	11q22.3	1.1	3.6	1.4	0	0	5.6
IGSF4	11q23	0	3.6	0	0	10.0	0
CDKN1B	12p13.1	0	0	0	0	0	0
CHFR	12q24.33	0	0	0	0	0	0
BRCA2	13q12	1.1	0	1.4	0	0	0
CDH13	16q24.2	20.7	32.1	20.0	22.7	30.0	33.3
HIC1	17p13.3	2.2	10.7	2.9	0	0	16.7
BRCA1	17q21	0	3.6	0	0	0	5.6
TIMP3	22q12.3	2.2	10.7	2.9	0	10.0	11.1
≥3 hypermethylated		15.2 [d]	42.9 [d]	14.3	18.2	50.0	38.9
Average TSG affected per case		1.3 ± 0.2 [e]	2.7 ± 0.5 [e]	1.4 ± 0.2	1.2 ± 0.3	1.7 ± 0.5	3.3 ± 0.7

NOTE. Data are expressed as percentages (%), unless otherwise indicated.

[a] $P = .010$.

[b] $P = .019$.

[c] $P = .019$.

[d] $P = .03$.

[e] $P = .049$.

the differences did not reach statistical significance: *ESR1*, *PTEN*, and *CDKN2A* were hypermethylated 3.4-, 3.0-, and 2.8-fold more, respectively, in the PTR group than in the NED group.

3.8. Epigenetic landscape of benign primary MNs that recurred and RCs

The methylation pattern of the PTR group shared more similarities to the RCs studied than with samples from the NED group; there were 2.5 ± 0.7, 2.7 ± 0.5, and 1.0 ± 0.2 hypermethylated TSGs per case in the PTR, RC, and NED groups, respectively (PTR versus NED, $P = .023$; RC versus NED, $P = .002$; Fig. 3F). In addition, the frequency of alterations in *RASSF1A*, *CDKN2B*, *ESR1*, and *CDKN2A* was more similar in the PTR and the RC groups than in the PTR and the NED groups. Statistical analysis revealed that *RASSF1A* and *CDKN2B* hypermethylation was significantly lower in the NED group (Fig. 3G).

4. Discussion

MNs are usually benign tumors curable by surgery, but their delicate location and high incidence in adult populations warrant further study to improve the classification of their RC risk [6,25,26]. Patients with grade II and III MNs have a higher probability of RC [2], but present clinical management of these cases is adequate to their risk. Grade I MNs are the most common, and so, in terms of absolute numbers, they cause the majority of RCs, even when they are removed by radical surgery [5-7].

The results of our proliferation analysis agreed with classical reports, confirming the usefulness of Ki-67 and the mitotic index, as both measures increase with tumor grade [2,4]. However, neither Ki-67 nor the mitotic index is able to identify aggressive grade I tumors accurately, as, according to the WHO, their proliferation rate generally is low [2]. Our FISH results confirm many well-known facts [9,18,22,23]. In the first place, loss of chromosome 22 is the main cytogenetic initiating event in MN. Second, loss of chromosome 14 is characteristic of

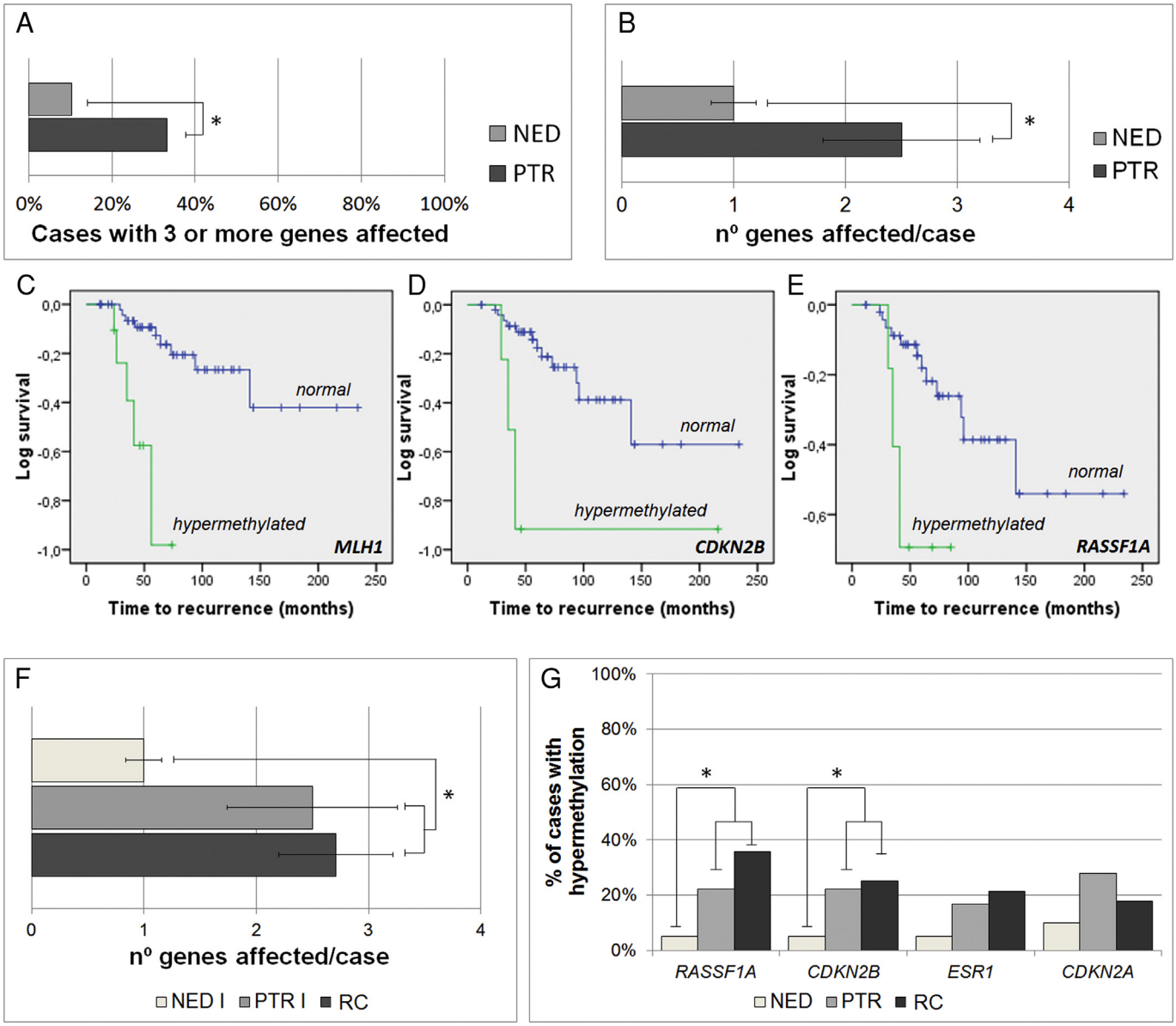

Fig. 3 Hypermethylation of TSGs in benign MNs depending on outcome. The figure shows various comparisons of epigenetics between MNs that recurred during 5-year follow-up (PTR) and MNs that did not show any evidence of disease (NED). A, Percentage of cases with hypermethylation in 3 or more TSGs. B, Average number of hypermethylated TSGs per case. C-E, Time-to-RC curves, as estimated by the Kaplan-Meier method. The *y*-axis represents the logarithm of the cumulative survival time in terms of probability: it ranges from 0 (100% of cases) to −1 (0 cases). The *x*-axis shows the time to RC, expressed in months. Blue line plots cases with no hypermethylation in the TSG in question; green curve plots cases with hypermethylation of this TSG; every vertical drop indicates an RC. C, Time to RC for *MLH1* hypermethylation. D, Time to RC for *CDKN2B* hypermethylation. E, Time to RC for *RASSF1A* hypermethylation. F and G, Epigenetic landscape of the PTR, NED, and RC groups. F, Average number of hypermethylated genes per case in different groups ($P = .023$ and $P = .002$). G, TSGs that were more similarly affected in PTRs and RCs than in NED cases. *RASSF1A* (NED versus PTR, $P = .046$; NED versus RC, $P = .026$) and *CKDN2B* (NED versus PTR; $P = .046$; NED versus RC; $P = .042$) are most likely to be associated with RC.

progression: it is high in grade II tumors and in RCs. Third, chromosome 1 also is associated with grade II RCs. However, although losses of 1p are increased in grade I MNs that recur, this change was not statistically significant in PTRs.

Different research groups have provided interesting results on MN genetics using high-throughput techniques, but these data sometimes are difficult to understand and to interpret accurately [18,21,27]. In the present work, we show a semi-guided genetic analysis of TSGs previously implicated in MN [18,23,24,28,29]. Our analyses provide evidence that TSG hypermethylation affecting any regulatory gene is an early event in the development of MN, whereas accumulation

Table 4 Frequency of hypermethylation in primary grade I MNs depending on their outcome

Gene	Locus	NED	PTR
TP73	01p36	7.3	16.7
CASP8	02q33-q34	0	0
VHL	03p26-p25	0	0
RARB	03p24	0	0
MLH1	03p21.3	9.8 [a]	33.3 [a]
RASSF1A	03p21.3	4.9 [b]	22.2 [b]
FHIT	03p14.2	2.4	0
APC	05q21	0	5.6
ESR1	06q25.1	4.9	16.7
CDKN2A	09p21	9.8	27.8
CDKN2B	09p21	4.9 [c]	22.2 [c]
DAPK1	09q34.1	0	0
PTEN	10q23.31	7.3	22.2
CD44	11p13	0	5.6
GSTP1	11q13	24.4	33.3
ATM	11q22.3	0	5.6
IGSF4	11q23	0	0
CDKN1B	12p13.1	0	0
CHFR	12q24.33	0	0
BRCA2	13q12	0	5.6
CDH13	16q24.2	17.1	27.8
HIC1	17p13.3	2.4	5.6
BRCA1	17q21	0	0
TIMP3	22q12.3	2.4	5.6
≥3 hypermethylated		10.3 [d]	33.3 [d]
Average TSG affected per case		1.0 ± 0.2 [e]	2.5 ± 0.7 [e]

NOTE. Data are expressed as percentages (%), unless otherwise indicated.
[a] $P = .001$.
[b] $P = .046$.
[c] $P = .046$
[d] $P = .043$.
[e] $P = .023$.

of hypermethylated genes happens during the evolution of the tumor and is a hallmark of aggressiveness.

The elevated frequency of TSG hypermethylation observed in PTs is high and consistent with data in previous reports [16,18,22]. Also, similar to previous work, we found no statistically significant global differences between grade I and higher-grade PTs [6,28,29]. Taken together, these data indicate that hypermethylation in at least one TSG likely occurs early in the genesis of MN. As none of the patients received chemotherapy or radiotherapy before surgery, there was no effect on the observed epigenetic landscape related to the treatment. In this study, the most frequently altered TSGs in primary MNs were *GSTP1*, involved in DNA damage protection and previously detected in cancer-precursor lesions in many tumor types [30], and *CDH13*, one of the TSGs most commonly inactivated epigenetically in human tumors. Similar to our results, *CDH13* methylation in breast and lung cancers did not have prognostic implications [31,32]. The early occurrence of the epigenetic changes identified in this study agrees with the hypothesis of Kishida et al [19], which proposes that hypermethylation precedes histologic changes in MN and acts as a mechanism for the acquisition of aggressiveness.

We found a significant increase in the average number of genes affected per case in the RC group, supporting the idea that the accumulation of epigenetic modifications increases during tumor life. Other authors have reached similar conclusions [6,25]. In our series, accumulation of epigenetic damage also correlated with sex, being higher in men than in women. Although previous studies do indicate that men show more genetic changes and a worse prognosis compared with women [4,6], this is the first time epigenetics and sex have been correlated in MN.

To the best of our knowledge, this article and recent reports from Sahm et al [25] and Olar et al [6] represent the most extensive work done on primary histologically benign tumors in patients who later had a tumor RC. The first study focused on developing a new grouping system to classify MNs independent of the grade. As grade II and III MNs generally are more aggressive than grade I lesions, we focused on a better stratification of these grade I MNs. The second one distinguished 2 groups of MN patients having significant differences in RC-free survival, including examination of grade I tumors and providing proof of concept that a methylation signature could be used in addition to the current predictors to identify patients prone to tumor RC. However, exploration of similar classifications made by cost-effective techniques, as we show here, is necessary to get them included in the diagnostic routine.

Our extended follow-up and detailed clinical analysis allowed us to detect changes in primary benign MNs that influence the development of RCs in this theoretically benign tumor. As is known, the main factor that predisposes a tumor to RC is the extent of the surgery, so herein, we evaluated only the outcome of patients who had undergone gross total resection. Our data suggest a significant correlation between the accumulation of epigenetic changes and RC in patients with benign MNs. Other works, such as the one from Kishida et al [19], found that the aggressive behavior in grade II and III MNs is associated with a methylator phenotype, similar to our idea of accumulation of epigenetic changes, but they were unable to predict which grade I MNs would recur with significant confidence. Previous reports, describing smaller series, found only differences in the landscape between benign and anaplastic tumors [29] or were focused on the methylation profile of atypical MNs [28].

In our series, the presence of 3 or more hypermethylated TSGs was a useful biomarker for risk stratification in MN. Moreover, in this study, alterations in *RASSF1A*, *CDKN2B*, *MLH1*, and *ESR1* were more than 3-fold more common in the PTR group than in the NED group, showing a significant correlation with the time to RC. Several authors have associated *RASSF1A* hypermethylation with both MN histologic grade and complex karyotypes, although none of them proved to play a role in the RC tendency [18,22,33]. However, similar findings are described in other cancer types [34].

Hypermethylation of *MLH1* has been described in several neoplasms, although not in MN. Most references to genetic alterations in *CDKN2B* relate it to high-grade MN, but only one report concerns its epigenetic status in MN, finding a frequency of alterations similar to that in the present work [35].

While analyzing the epigenetic landscape of MNs, we noticed a striking resemblance between the PTR group and the RCs. In both cases, epigenetic alterations were detected in genes involved in the cell cycle, proliferation, and angiogenesis. Moreover, the similarity in the accumulation of hypermethylated TSGs and the frequency of alterations in *RASSF1A* and *CDKN2B* were statistically significant. This finding provides evidence that epigenetic alterations are relevant, not only in the early onset of MNs but also in their progression and aggressiveness. Further studies in larger series assessing alterations in these single genes could provide new insights.

In conclusion, although a concrete methylation marker that can identify aggressive MN subtypes at early stages of the disease remains elusive, our work suggests that the occurrence of epigenetic changes in at least one TSG is an early event in MN tumorigenesis. The most outstanding result is that we show the potential clinical value of detecting accumulated epigenetic damage to predict tumor RCs in an easy, cost-effective way. Further research is required to confirm and extend these findings.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2018.07.035.

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