DNA methylation-induced E-cadherin silencing is correlated with the clinicopathological features of melanoma

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Abstract. E-cadherin, a calcium-dependent cell-cell adhesion molecule, has an important role in epithelial cell function, maintenance of tissue architecture and cancer suppression. Loss of E-cadherin promotes tumor metastatic dissemination and predicts poor prognosis. The present study investigated the clinicopathological significance of E-cadherin expression in cutaneous, mucosal and uveal melanoma related to epigenetic mechanisms that may contribute to *E-cadherin* silencing. E-cadherin expression was reduced in 55/130 cutaneous (42.3%), 49/82 mucosal (59.7%) and 36/64 uveal (56.2%) melanoma samples as compared to normal skin controls and was inversely associated with promoter methylation. Of the 10 different CpG sites studied (nt 863, 865, 873, 879, 887, 892, 901, 918, 920 and 940), two sites (nt 892 and 940) were 90-100% methylated in all the melanoma specimens examined and the other ones were partially methylated (range, 53-86%). In contrast, the methylation rate of the E-cadherin gene was low in normal tissues (range, 5-24%). In all the three types of melanoma studied, a significant correlation was found between reduced levels of E-cadherin and reduced survival, high mitotic index and metastasis, accounting for the predilection of lymph nodal localization. In cutaneous and mucosal melanoma, low E-cadherin expression was positively correlated also with head/neck localization and ulceration. A high frequency of reduced E-cadherin levels occurred in choroid melanomas. *In vitro* experiments showed that E-cadherin transcription was restored following 5-aza-2'-deoxycytidine (5-aza-dC) treatment or DNMT1 silencing and was negatively correlated with the invasive potential of melanoma cells. The significant relationship between E-cadherin silencing and several poor prognostic factors indicates that this adhesion molecule may play an important role in melanomagenesis. Therefore, the inverse association of E-cadherin expression with promoter methylation raises the intriguing possibility that reactivation of E-cadherin expression through promoter demethylation may represent a potential therapeutic strategy for the treatment of melanoma.

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Abbreviations: FFPE, formalin-fixed paraffin-embedded; NHEMs, normal human epidermal melanocytes; qPCR, quantitative real-time PCR; MSP, methylation-specific PCR; HPF, high-power field; TSS, transcriptional start site

Key words: E-cadherin, gene expression, DNA methylation, clinicopathological parameters, melanoma

Introduction

In recent years, the incidence and mortality rate of melanoma have considerably increased (1), whereby the need to discover new therapeutic strategies has become increasingly urgent (2-6). E-cadherin has been shown to play a key role in cell adhesion, growth and differentiation (7), frequently overexpressed in melanoma. Several studies have revealed that E-cadherin is expressed in the normal epidermis and lost in invasive and metastatic melanoma cells (8). Depletion of functional E-cadherin induces the escape of melanocytes from keratinocyte-mediated growth and phenotypic control, thus allowing invasion and migration (9).

Although reduced expression of E-cadherin is known to be caused by promoter hypermethylation in several types of tumors (10), such an epigenetic event has been only partially investigated in melanoma. Moreover, the existence of a relationship between epigenetic changes, E-cadherin expression and clinicopathological features has not yet been evaluated in melanoma.

In the present study, we aimed to investigate whether E-cadherin expression is epigenetically regulated in a cohort of patients affected by cutaneous, mucosal and uveal melanoma and is associated with specific clinicopathological parameters. Findings from the present study revealed a strong association between *E-cadherin* promoter methylation and several significant pathological characteristics, whereby they may provide insights into the biology of this cancer.

Materials and methods

Cell cultures. Normal human epidermal melanocyte (NHEM) cells were grown in Melanocyte Medium plus Bullet kit (both from Lonza, Walkersville, MD, USA). Cutaneous (G361, WM-115 and WM-266-4), mucosal (MMel-1 and MMel-2) and uveal (OCM-1, OCM-3 and 92.1) melanoma cells were grown as previously described (11,12).

Treatment with the DNA demethylating agent 5-aza-dC. Cells were treated with 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma Chemical Co.) by addition of fresh medium containing 5-aza-dC (10 μmol/l) every day for three consecutive days.

Tissue specimens. Formalin-fixed paraffin-embedded (FFPE) tissue sections of 130 cutaneous, 82 mucosal and 64 uveal melanomas, and 65 normal skin specimens were collected from the Department of Human Pathology, University of Messina, Messina, Italy. The data regarding patients are shown in Table I. The investigation adhered to the Declaration of Helsinki and was approved by the Ethics Committee of the University Hospital of Messina. Informed consent was provided by the patients.

Determination of mitotic index. Mitotic index was determined by counting the number of mitoses in 10 consecutive non-overlapping high power fields (HPFs) with commencement in an area of high mitotic activity using a magnification of x400.

DNA and RNA extraction. Total RNA and DNA extraction from cells was performed using TRIzol reagent (Invitrogen), and Recover All Total Nucleic Acid Isolation kit (Ambion Inc., Austin, TX, USA) was used for extractin from FFPE samples.

Reverse transcription and qPCR. Total RNA was reverse-transcribed with IMProm-II™ Reverse Transcriptase kit (Promega, Milan, Italy). qPCR was performed using the ABI Prism 7500 Real-Time PCR system (Applied Biosystems, Milan, Italy). Primers and probes were previously described (11). The mRNA levels of E-cadherin were normalized to endogenous β-actin (Applied Biosystems). The control was represented by NHEM cells (described in the paragraph 'Cell culture'). Their expression was considered as 1.

Bisulfite modification and MSP. Bisulfite-modified DNA obtained using the EpiTect Bisulfite kit (Qiagen, Milan, Italy) was amplified using the following primers as previously reported (13): methylated DNA-specific primers: forward primer, 5'-TTAGGTTAGAGGGTTATCGCGT-3' and reverse primer, 5'-TAACTAAAAATTCACCTACCGAC-3' (115 bp; genomic position relative to TSS, -176/-61); unmethylated DNA-specific primers: forward primer, 5'-TAATTTTAGGTT AGAGGGTTATTGT-3' and reverse primer, 5'-CACAACCAA TCAACAACACA-3' (97 bp; genomic position relative to TSS, -181/-84). PCR products were separated by 2% agarose gel containing ethidium bromide.

Bisulfite genomic sequencing. Bisulfite-treated DNA was amplified by PCR with primers that were specific for modified DNA but did not contain any CpG sites in their sequence.

Table I. Patient characteristics.

Characteristics	Cutaneous melanoma (n=130) n (%)	Mucosal melanoma (n=82) n (%)	Uveal melanoma (n=64) n (%)
Males	78 (60)	40 (48.8)	31 (48.4)
Females	52 (40)	42 (51.2)	33 (51.6)
AJCC stage			
I	34 (26.2)	23 (28.0)	14 (21.9)
II	38 (29.2)	19 (23.2)	14 (21.9)
III	30 (23.1)	24 (29.3)	18 (28.1)
IV	28 (21.5)	16 (19.5)	18 (28.1)
Site			
Extremities	39 (30)		
Trunk	26 (20)		
Head/neck	65 (50)		
Vulvovaginal		28 (34.1)	
Anorectal		22 (26.9)	
Head/neck		32 (39.0)	
Choroid			34 (53.1)
Ciliary body			17 (26.6)
Iris			13 (20.3)
Ulceration			
Absent	83 (63.8)	34 (41.5)	64 (100)
Present	47 (36.2)	48 (58.5)	0 (0)
Breslow thickness (mm)			
≤1.00	41 (31.5)	23 (28.0)	24 (37.5)
1.01-4.00	42 (32.4)	25 (30.5)	19 (29.7)
>4.01	47 (36.1)	34 (41.5)	21 (32.8)
Mitotic index (per 10 HPFs)			
1-5 mitoses	29 (22.3)	19 (23.2)	12 (18.8)
6-10 mitoses	43 (33.1)	15 (18.3)	18 (28.1)
11-15 mitoses	19 (14.6)	18 (21.9)	15 (23.4)
16-20 mitoses	39 (30.0)	30 (36.6)	19 (29.7)
Metastatic lesions			
Present	64 (49.2)	47 (57.3)	37 (57.8)
Absent	66 (50.8)	35 (42.7)	27 (42.2)

AJCC, American Joint Committee on Cancer; HPFs, high power fields.

The primers were: S1 (TTT AGT AAT TTT AGG TTA GAG GGT T, upstream, nt 836-861; GeneBank accession no. L34545) and S2 (CTA ATT AAC TAA AAA TTC ACC TAC C, downstream, sequence position nt 965-940) (14). The PCR conditions were 94˚C for 2 min; 35 cycles of 94˚C for 20 sec, 48˚C for 20 sec and 72˚C for 30 sec; and a final extension at 72˚C for 5 min. The PCR product was extracted from the gel with the QIAquick Gel Extraction kit (Qiagen). The purified DNA samples were sequenced with the CEQ DTCS Quick Start kit, and with an automated DNA sequencer (Beckman Coulter CEQ 2000 analysis system) (both from Beckman Coulter, S.p.A.).

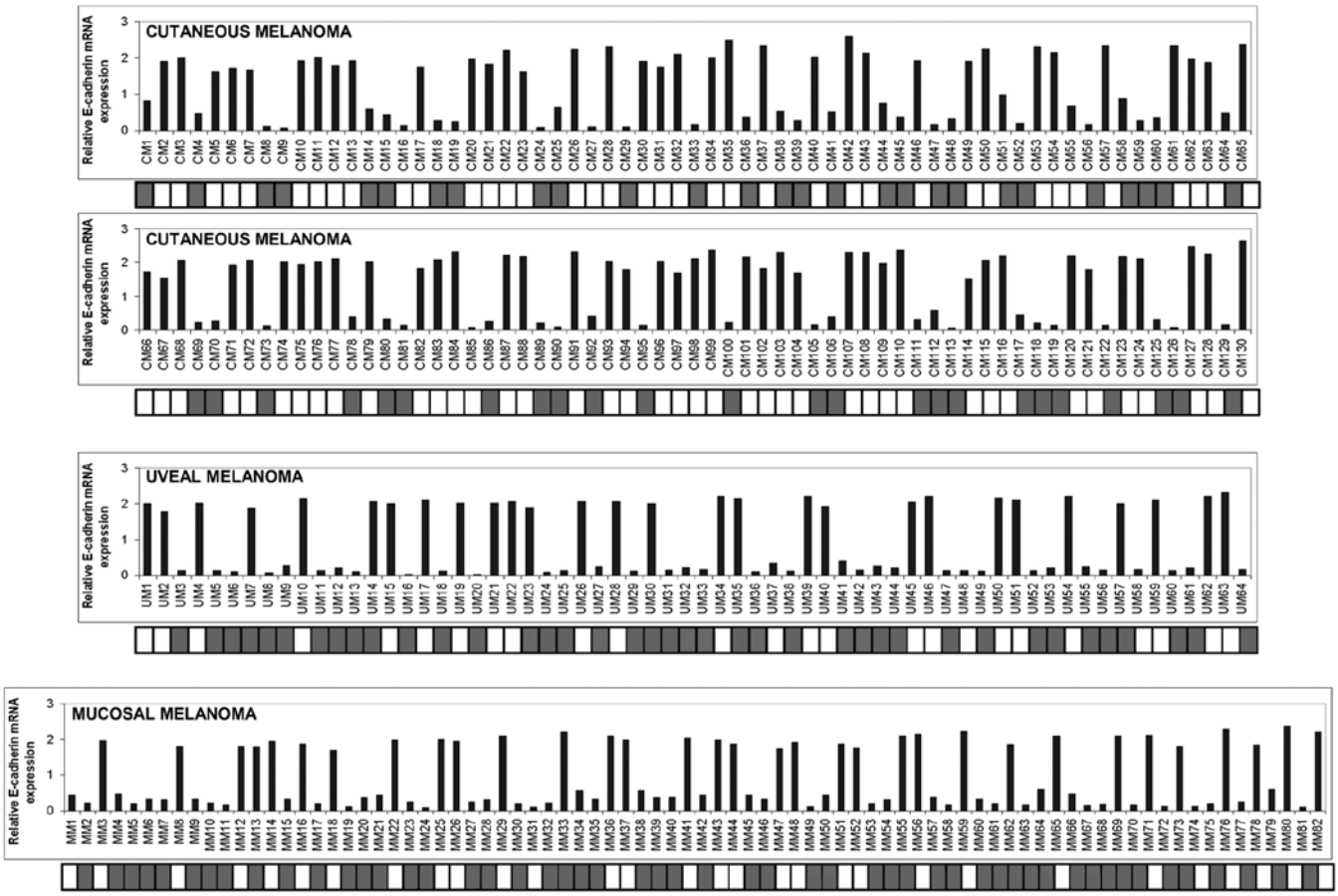

Figure 1. Expression levels and the methylation status of *E-cadherin* in cutaneous, uveal and mucosal melanoma specimens. (A) Total RNA was extracted from 130 cutaneous melanomas (CM), 64 uveal melanomas and (UM) 82 mucosal melanomas (MM), reverse-transcribed, and analyzed by qPCR. mRNA levels of E-cadherin were normalized using the housekeeping gene β-actin as the inner control. Data are depicted as the mean ± SD of three independent experiments. The detailed methylation pattern of E-cadherin as determined by methylation specific-PCR (MSP) was placed and aligned just beneath the expression graphs. White square, unmethylated CpG regions; grey square, methylated CpG regions.

Quantification of methylation levels. A single cytosine signal at the corresponding CpG site was considered 100% methylation, a single thymine signal was considered no methylation, and overlapping cytosine plus thymine signals were considered partial methylation. In the latter condition, the percentage of methylation was expressed as the ratio of the peak values of the cytosine to cytosine plus thymine signals.

Transient transfections. For transient knockdown of *DNMT1*, *DNMT3a* and *DNMT3b*, cells were transfected with specific targeting small interfering RNA (siRNA) or non-targeting control siRNA (Invitrogen, Milan, Italy) at a final concentration of 100 nM 24 h after plating using siPORT *Lipid* Transfection Agent (Ambion, Milan, Italy).

Invasion assay. The anti-invasive activity of 5-aza-dC was assessed using the Cultrex® BME Cell Invasion assay (Trevigen, Gaithersburg, MD, USA).

Statistical analysis. The Pearson's correlation test was used to assess the association of E-cadherin expression with promoter methylation. Differences in E-cadherin expression levels and clinical characteristics were evaluated by χ^2 test between patient subgroups. Kaplan-Meier method was used in the evaluation of the overall and disease-free survival time. The log-rank test was used in comparing the differences between the periods of survival among the examined patients. A p-value of <0.05 was considered to indicate a statistically significant result.

Results

E-cadherin expression correlates with promoter methylation in cutaneous, uveal and mucosal melanoma. E-cadherin mRNA values were firstly measured in 65 normal skin samples from healthy donors and in NHEM cells in order to determine the cut-off point for abnormal E-cadherin expression. E-cadherin levels (as defined by the ratio between the values measured in skin samples over those of NHEM cells) ranged between 1.52 and 2.1 (mean, 1.84±0.133). Values equal or below 1.44 (determined as the mean minus 3 SD) were considered to represent under-expression of E-cadherin. As shown in Fig. 1, a strong reduction or loss of expression of E-cadherin was detected in 55/130 cutaneous (42.3%), 36/64 uveal (56.2%) and 49/82 mucosal (59.7%) melanoma samples. In order to investigate whether promoter hypermethylation correlated with *E-cadherin* expression, we performed MSP analysis in the series of melanoma specimens examined. Of the 55 cutaneous melanomas down-expressing E-cadherin, 51 samples were

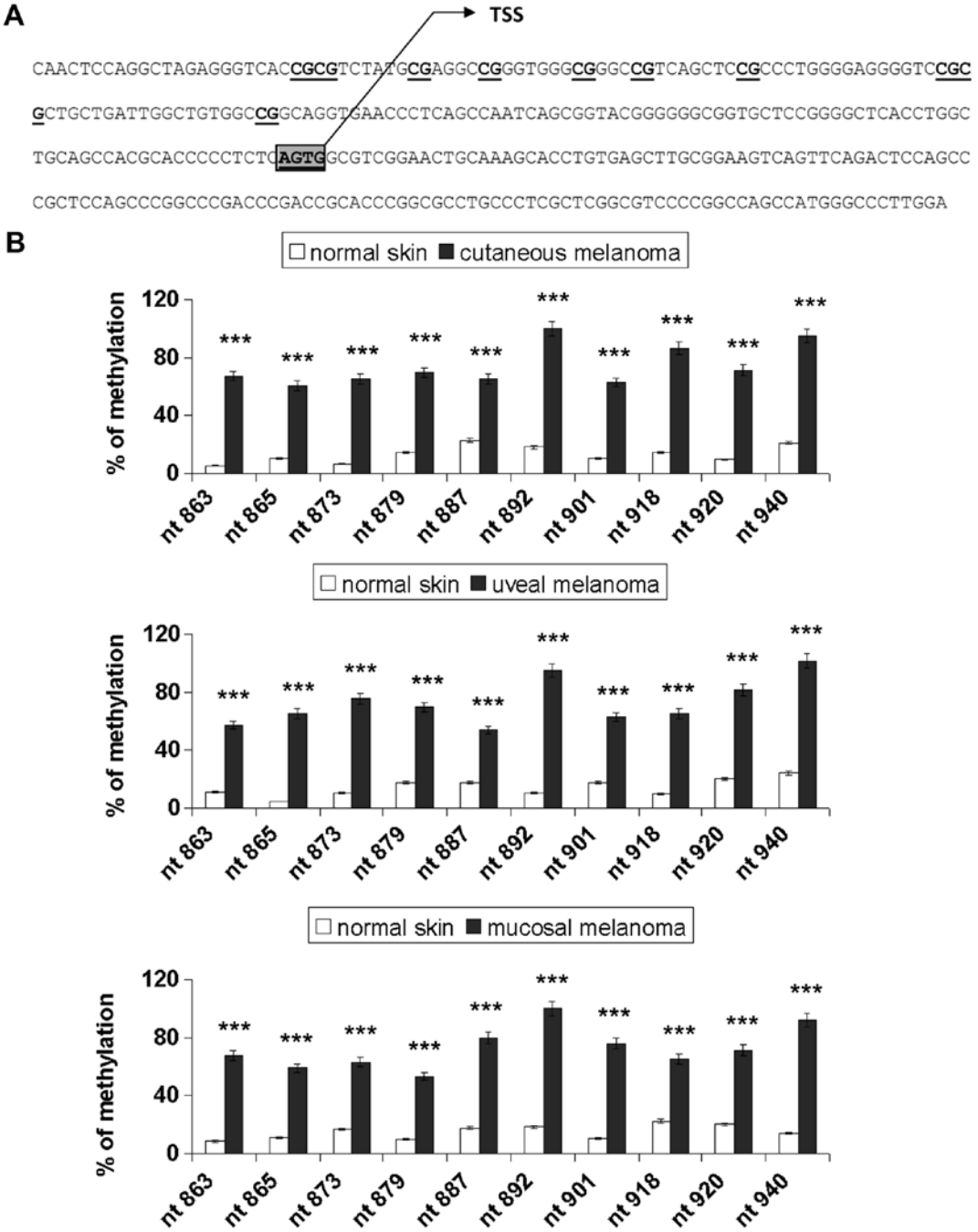

Figure 2. Methylation status of individual CpG sites in the melanoma samples. (A) Schematic representation of the E-cadherin promoter region analyzed. Individual CpG sites are underlined. (B) Direct bisulfite sequencing analysis of E-cadherin promoter methylation in melanoma. PCR products from normal skin and cutaneous, uveal and mucosal melanoma samples were sequenced after amplification with primers S1/S2, as described in the Materials and methods section. Data are depicted as the mean ± SD of three independent experiments. Significant $^{***}p<0.001$, melanoma samples vs. normal skin.

hypermethylated (92.7%) and 4 samples were unmethylated (7.3%). All of the 75 cutaneous melanoma sections which expressed amounts of E-cadherin mRNA above the cut-off point were unmethylated. Of the 36 uveal melanoma samples exhibiting levels of mRNA under the cut-off point, 33 (91.7%) were methylated and 3 (8.3%) were unmethylated, whereas only 6 out of 28 (21.4%) specimens exhibiting consistent amounts of E-cadherin expression were methylated. Among the 49 mucosal melanomas that expressed lower mRNA levels of E-cadherin, 46 specimens (93.9%) exhibited promoter methylation and 3 specimens (6.1%) were unmethylated. Six out of 33 (18.2%) mucosal melanoma samples with high levels of E-cadherin were found to be methylated while the rest were unmethylated. Pearson correlation analysis revealed that E-cadherin mRNA levels were inversely correlated with promoter methylation (for cutaneous melanoma, correlation coefficient, -0.899, $p<0.001$; for uveal melanoma, correlation coefficient, -0.729, $p<0.001$; for mucosal melanoma, correlation coefficient, -0.754, $p<0.001$).

Sodium bisulfite DNA sequencing was carried out to assess the promoter methylation pattern for 10 specific CpG sites (genomic positions nt 863, 865, 873, 879, 887, 892, 901,

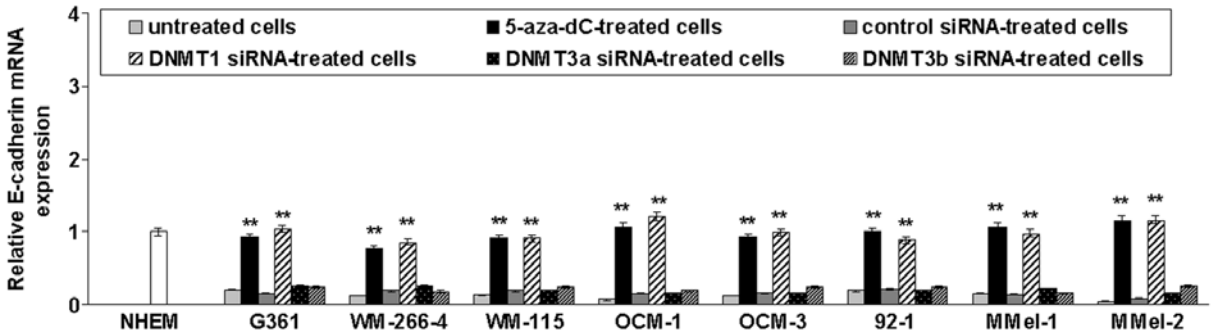

Figure 3. Restoration of E-cadherin gene expression in melanoma cell lines after demethylation. Three cutaneous (G361, WM-266-4 and WM-155), three uveal (OCM-1, OCM-3a and 92.1), and two mucosal (MMel-1 and MMel-2) melanoma cell lines were plated in 6-well plates and exposed to 5-aza-dC (10 μM). Where indicated, cells were transfected with a control siRNA or siRNAs targeting *DNMT1*, *DNMT3a* and *DNMT3b*. Total RNA was isolated, reverse-transcribed and analyzed by qRT-PCR. mRNA levels of E-cadherin were normalized using the housekeeping gene β-actin as the inner control. Data are depicted as the mean ± SD of three independent experiments. Significant $^{**}p<0.01$, 5-aza-dC-treated or DNMT1-silenced cells vs. untreated control cells.

918, 920 and 940; GeneBank accession no. L34545), as represented in Fig. 2A. Fig. 2B shows the percentage of methylation of E-cadherin in the normal skin and melanoma samples. Cancer samples showed significantly higher methylation levels compared with the normal tissues ($p<0.001$). Notably, the CpG sites nt 892 and 940 were 90-100% methylated in all the melanoma types examined and the others were partially methylated (range, 53-86%). The methylation rate of the E-cadherin gene was low in the normal tissues (range, 5-24%).

To test whether promoter methylation was responsible for repression of *E-cadherin* transcription, cutaneous (G361, WM-266-4 and WM-155) and uveal (OCM-1, OCM-3 and 92.1) melanoma cell lines previously found to be E-cadherin-methylated (11) as well as primary mucosal melanoma cells (M-Mel-1 and M-Mel-2) that are methylated for this gene (data not shown), were treated with the demethylating drug 5-aza-dC or transfected with siRNAs targeting *DNMT1*, *DNMT3a* or *DNMT3b*. As shown in Fig. 3, E-cadherin mRNA levels were significantly increased following 5-aza-dC treatment or *DNMT1* silencing. In contrast, *DNMT3a* or *DNMT3b* knockdown did not restore E-cadherin expression.

Correlation between E-cadherin expression and clinical parameters in cutaneous, mucosal and uveal melanoma. We next examined the correlation between E-cadherin expression and different clinicopathological features. Low E-cadherin expression was observed in 78.5% of the head and neck cutaneous melanomas as compared to 12.8% of melanomas occurring in other regions ($p<0.0001$; χ^2 test). Ulcerated lesions exhibited reduced levels of E-cadherin in 95.7% of the cases relative to 12.1% observed in the non-ulcerated tumors ($p<0.0001$; χ^2 test). A high percentage of melanomas with elevated mitotic index (84.2% for 11-15 mitoses/10 HPFs; 92.3% for 15-20 mitoses/10 HPFs) or with metastasis (68.75%) showed decreased amounts of E-cadherin as compared with melanomas with a low number of mitoses (3.5% for 1-5 mitoses/10 HPFs; 4.7% for 6-10 mitoses/10 HPFs) ($p<0.0001$; χ^2 test), or no metastatic melanomas (12.1%; $p<0.0001$; χ^2 test), respectively. No statistical differences were found between E-cadherin expression and clinical stage or Breslow thickness (Fig. 4A). Mucosal melanoma presented a similar pattern, since a very significant correlation was found between decreased E-cadherin expression and head/neck localization ($p<0.0001$; χ^2 test), ulceration ($p<0.0001$; χ^2 test), mitotic index ($p<0.0001$; χ^2 test) and metastasis ($p<0.0001$; χ^2 test) (Fig. 4B). Data relative to uveal melanoma showed that a high frequency of reduced E-cadherin levels occurred in melanomas of the choroid ($p<0.0001$; χ^2 test) as well as in those with elevated mitotic index ($p=0.002$; χ^2 test) or metastasis ($p<0.0001$; χ^2 test) (Fig. 4C).

E-cadherin expression discriminates site-specific melanoma metastasis. Analysis of the expression of *E-cadherin* in metastatic tumor according to the metastasis site revealed that the reduction in *E-cadherin* expression was significantly associated with lymph node metastatic localization (Fig. 5).

Restoration of E-cadherin negatively correlates with the invasive potential of melanoma cells. Since a relationship between *E-cadherin* silencing and melanoma metastasis was noted (Fig. 4), we aimed at evaluating the effect of 5-aza-dC-induced *E-cadherin* reactivation on melanoma cell invasiveness. As shown in Fig. 6, demethylation markedly inhibited the invasion of all the examined melanoma cell lines.

Correlation between E-cadherin levels and patient survival. The overall and disease-free survival of melanoma patients were also explored and the association with E-cadherin expression was analyzed. As shown in Fig. 7, a shorter survival time for patients with reduced levels of E-cadherin was documented ($p<0.0001$). Compared to the overall survival time for patients with preserved levels of E-cadherin (from 2 to 60 months, average 32.3 months, median 36 months in cutaneous melanoma; from 2 months to 60 months, average 33.2 months, median 36 months in mucosal melanoma; from 2 to 60 months, average 25.5 months, median 24 months in uveal melanoma), a shorter overall survival time in patients with reduced expression of E-cadherin was observed (from 2 to 52 months, average 19.4 months, median 20 months in cutaneous melanoma; from 2 to 44 months, average 19.2 months, median 24 months in mucosal melanoma; from 2 to 28 months, average 15.2 months, median 14 months in uveal melanoma). Disease-free survival time was also shorter in patients with loss of expression of E-cadherin, compared with the other patients. In the presence

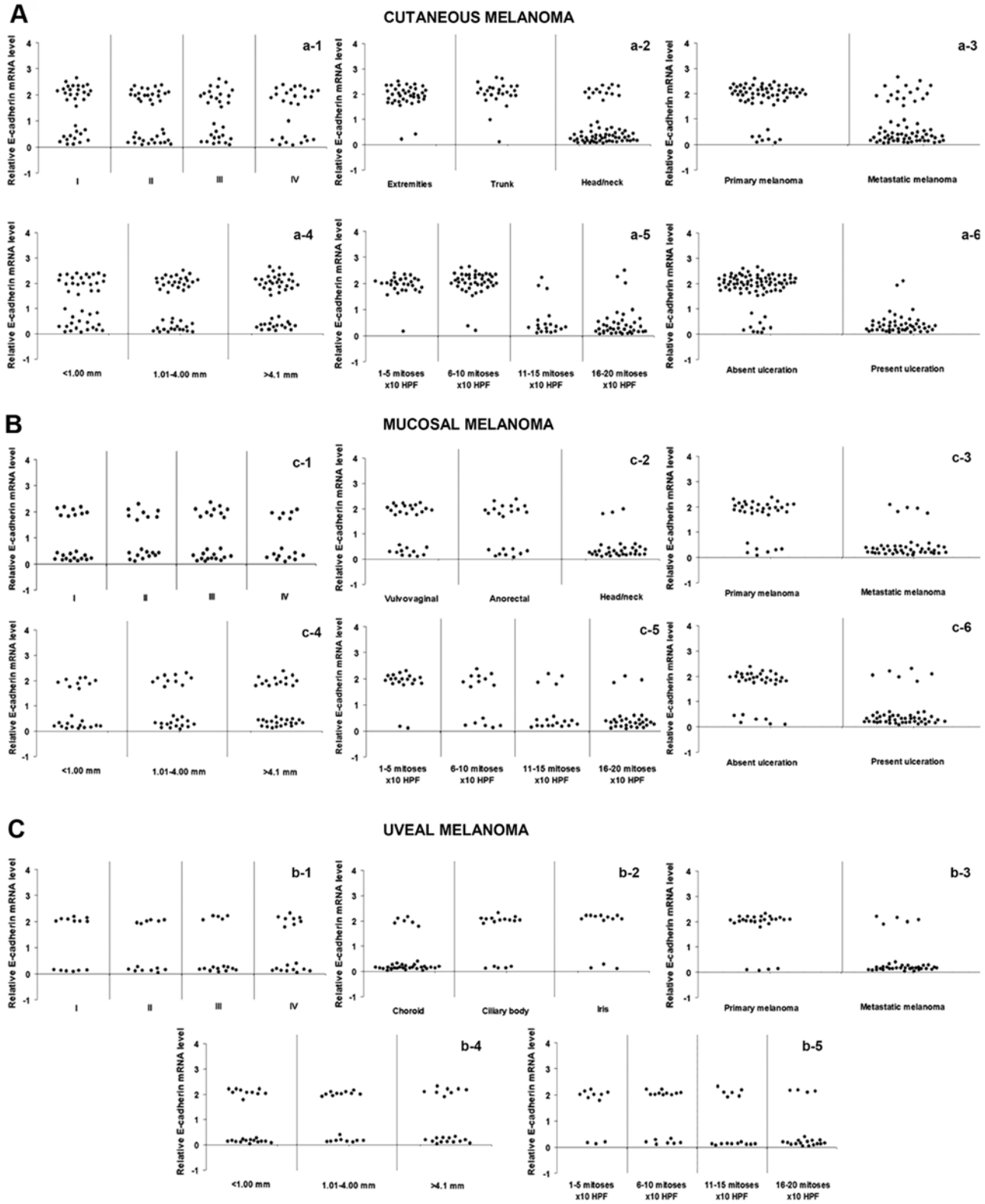

Figure 4. Correlations between E-cadherin expression and clinicopathological characteristics in cutaneous, mucosal and uveal melanoma. In (A) cutaneous and (B) mucosal melanomas, decreased E-cadherin expression was correlated with head/neck localization ($p<0.0001$; χ^2 test) (a-2 and b-2), metastasis ($p<0.0001$; χ^2 test) (a-3 and b-3), mitotic count ($p<0.0001$; χ^2 test) (a-5 and b-5) and ulceration ($p<0.0001$; χ^2 test) (a-6 and b-6). No association was found between E-cadherin expression and AJCC stage (cutaneous melanoma, $p=0.336$; mucosal melanoma, $p=0.358$; χ^2 test) (a-1 and b-1) and Breslow thickness (cutaneous melanoma, $p=0.161$; mucosal melanoma, $p=0.207$, χ^2 test) (a-4 and b-4). In (C) uveal melanoma, decreased E-cadherin expression was correlated with choroidal localization ($p<0.0001$; χ^2 test) (c-2), metastasis ($p<0.0001$; χ^2 test) (c-3) and mitotic index ($p=0.002$; χ^2 test) (c-5). No association was found between E-cadherin expression and AJCC stage ($p=0.424$; χ^2 test) (c-1) and Breslow thickness ($p=0.385$; χ^2 test) (c-4).

of E-cadherin expression it was: 2 months, 60 months, average 32.7 months, median 28 months in patients with cutaneous melanoma; 2 months, 60 months, average 31.6 months, median 28 months in patients with mucosal melanoma; 2 months

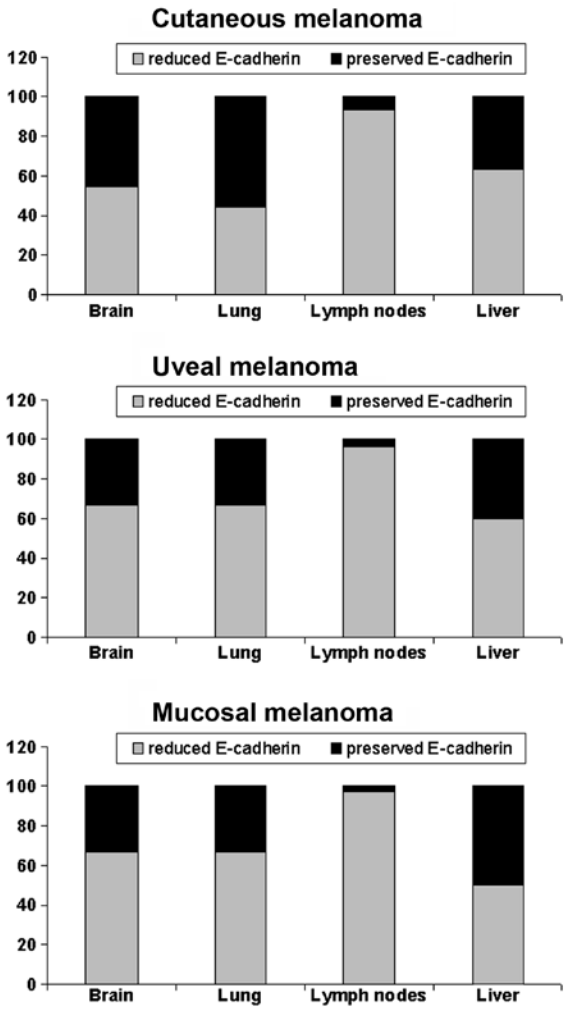

Figure 5. Correlation between reduced E-cadherin expression and organ-specific metastasis. Graph shows the organ distribution of metastases. The plot indicates the percentage of cases with reduced vs. preserved E-cadherin mRNA expression levels.

60 months, average 31.9 months, median 32 months in patients with uveal melanoma. In the rest of the patients it was: 2 months, 40 months, average 21.4 months, median 20 months in patients with cutaneous melanoma; 2 months, 56 months, average 21.5 months, median 20 months in patients with mucosal melanoma; 2 months, 36 months, average 16.9 months, median 16 months in patients with uveal melanoma.

Discussion

The role of E-cadherin as a 'suppressor of invasion' (15) has been well established in the context of melanoma, since its re-expression was accompanied by suppression of tumor invasiveness and metastasis (16). Moreover, the significant role played by E-cadherin in melanoma was very recently evidenced by observations that the resistance to BRAF inhibitors displayed by melanomas harboring mutations in *BRAF* was strictly associated with a significant decrease in E-cadherin expression (17). However, although reduced *E-cadherin* expression has been well documented in malignant melanoma and is associated with specific features of the tumor (17-19), to the best of our knowledge, the underlying mechanisms have not been extensively determined. In fact, in only two cellular melanoma models the relationship between *E-cadherin* silencing and promoter methylation was evidenced (19,20). In the present study, we showed that in cutaneous, uveal, and mucosal melanomas the methylation at specific CpG sites in the *E-cadherin* promoter was responsible for reduced gene expression (Figs. 1 and 2) and several clinicopathological features. Indeed, E-cadherin downregulation was assumed to be an important step in the progression from a melanoma *in situ* to an invasive and metastasizing lesion and a determinant of tumor depth (8,21). Thus, the demonstration that *E-cadherin* expression is epigenetically regulated through promoter methylation and that it can be reverted by demethylating tools, such as 5-aza-dC treatment or *DNMT1* silencing it may be of clinical significance (Fig. 3). This latter data accounts for the prominent role of *DNMT1* in repressing E-cadherin transcription, as already shown in another cancer model (22), and is in agreement with studies reporting the near selective activity of 5-aza-dC in inhibiting *DNMT1* and neglecting *DNMT3a* and *DNMT3b* enzymes (23,24). The identification of a likely mechanism for E-cadherin downregulation in melanoma may pave the way to alternative therapeutic strategies. This possibility is further suggested by additional results of the present study, which indicate that methylation and reduced expression of E-cadherin are closely associated with clinicopathological markers, either specific or common

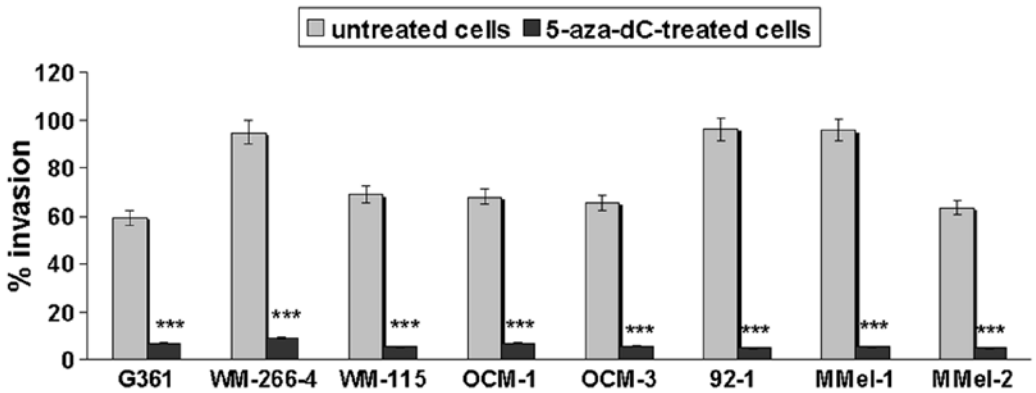

Figure 6. Reduced melanoma invasiveness after 5-aza-dC treatment. Invasive activity was evaluated by the Cultrex® BME cell invasion assay. Experiments were run in triplicate and the data are expressed as the mean ± SD. Significant ***p<0.001, 5-aza-dC-treated vs. untreated melanoma cells.

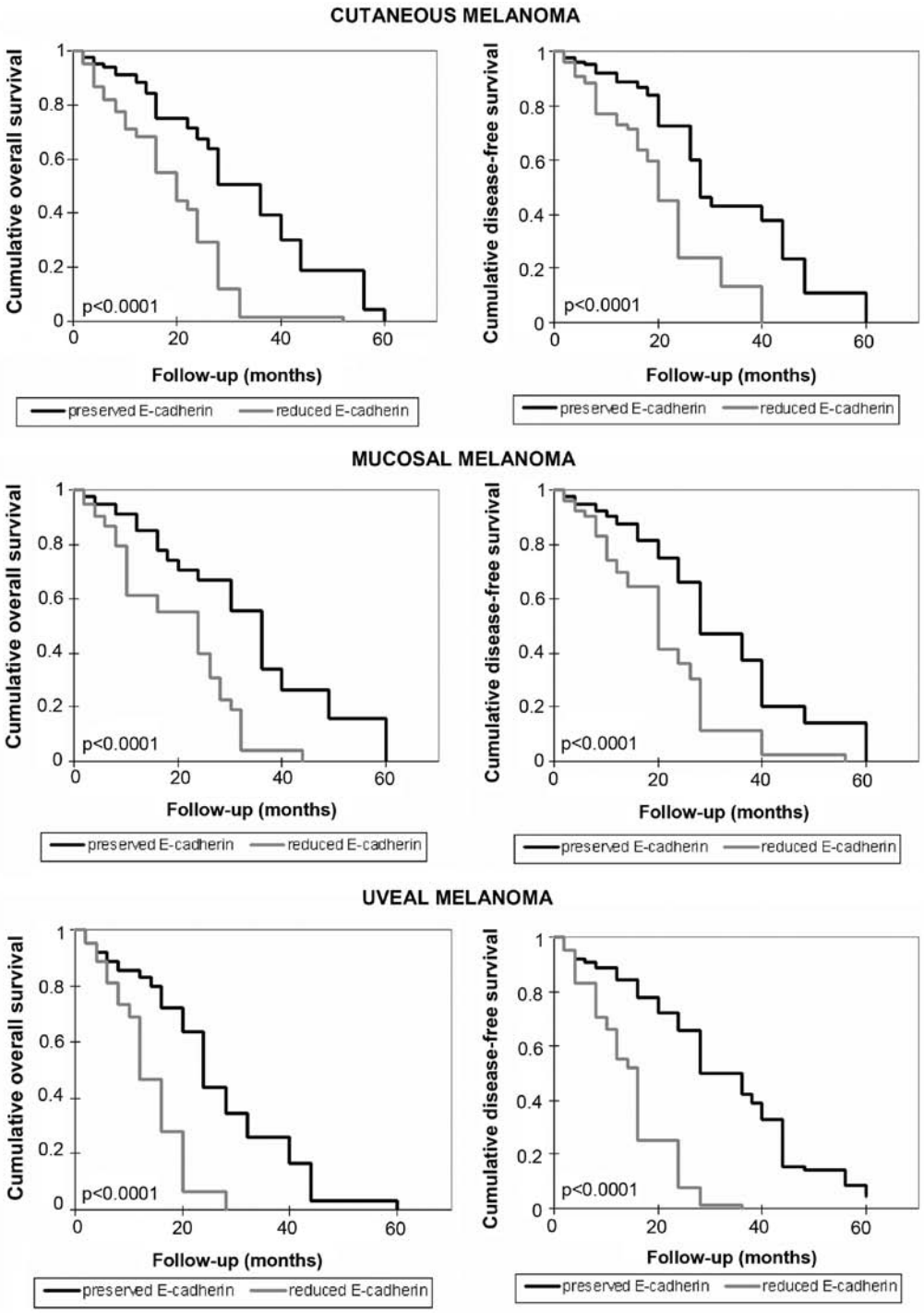

Figure 7. Relationship between E-cadherin content and cumulative overall and disease-free survival in melanoma patients. Kaplan-Meier survival curves relative to patients with high and low E-cadherin expression are shown.

to each type of melanoma. This may be of some interest, since up-to-date conflicting data emerge from the literature. Initially, E-cadherin expression was shown to be rare in both nevocellular nevi and early-stage melanoma, but preserved in advanced-stage melanoma and melanoma metastases (25). More recently, however, E-cadherin expression was found to be altered in different stages of melanoma progression (19). Moreover, information with regard to the actual meaning of *E-cadherin* epigenetic silencing in melanoma was lacking. In the present study, we showed that in cutaneous melanoma reduced E-cadherin expression via promoter aberrant methylation was strongly associated with several poor prognostic factors, namely ulceration, head/neck localization, mitotic count and metastasis as well as with reduced overall/disease-free survival (Figs. 4 and 7). No statistical association was found with clinical stage or Breslow thickness. Our findings allow us to hypothesize that reduced E-cadherin expression through promoter hypermethylation may have a major impact on the proliferation rate of melanoma. The inverse association between E-cadherin expression and the mitotic index found by us is in line with various studies, which reveal that downregulation of E-cadherin prevents the control

of melanocyte proliferation by keratinocytes and is involved in melanocyte transformation (16,26). Moreover, other studies have reported that expression of E-cadherin results in inhibition of cell motility and local invasion in melanoma cells (16,27), as well as in other cell systems (28,29). The higher frequency of *E-cadherin* methylation and silencing in cutaneous melanoma occurring in head and neck regions, as detected in the present study, deserves to be considered separately. The incidence of melanoma of the head and neck has increased in proportion to sun exposure, and it is often linked to poor prognosis and reduced survival (30). Intense vascularization and abundance of lymphatic vessels in the region may account for these malignancy characteristics, but although it is generally believed that molecular mechanisms different from those involved in melanoma occurring in sun-protected areas are engaged (31,32), little information about distinctive molecular markers of head/neck melanoma are available. The positive correlation between the methylation of *E-cadherin* and the location of melanoma in the head and neck region highlighted here may explain the apparent discrepancies with other studies reporting a low frequency of methylation of the gene in this tumor. Indeed, in case studies by Liu *et al*, melanomas of the head and neck were not included (33). The silencing of *E-cadherin* through aberrant promoter methylation may contribute to add new insights into the mechanisms accounting for the higher malignancy of head/neck melanoma. A similar hypothesis may be advanced for head/neck mucosal melanomas, which, although rare (34), represent half of all mucosal melanomas and exhibit a more aggressive behavior as compared to mucosal melanoma of other regions (35). Indeed, it is quite intriguing that, among the various types of mucosal melanoma, methylation-mediated downregulation of E-cadherin is almost entirely restricted to the head and neck area (Fig. 4). Likewise, we showed that the presence of ulcerated lesions, high mitotic index, metastasis and reduced overall/disease-free survival are conditions frequently associated with epigenetic downregulation of E-cadherin in mucosal melanomas (Figs. 4 and 7). These data allow us to hypothesize that cutaneous and mucosal melanomas not only use the same mechanism for regulating E-cadherin expression, but also that both adopt it in the same circumstances of transformation. Regarding uveal melanoma, it is worth mentioning that the expression of E-cadherin, along with other biomarkers, is considered to be the most accurate predictor of metastasis (36). Therefore, our findings that the downregulation of E-cadherin correlates inversely with the mitotic index and directly with the choroidal site, metastasis and reduced overall/disease-free survival (Figs. 4 and 7) are not surprising, albeit indicative from a clinicopathological point of view. In fact, it is well recognized that high expression of E-cadherin is strictly associated to the epithelial phenotype, while loss of E-cadherin is indicative of mesenchymal transition and acquirement of invasiveness characteristics (37,38). In addition, it is even more interesting that all the melanoma types share the association of methylation-induced E-cadherin reduction with the metastatic phenotype (Fig. 4), the lymph nodal metastasis localization (Fig. 5), the invasive character (Fig. 6), and the reduced overall/disease-free survival (Fig. 7). Such data, streaming from the well known role exerted by E-cadherin as cell-cell adhesion molecule and central hub of inhibitory signals of cell motility (39,40), contribute to clarify why melanoma preferentially metastasizes to distant sites and lymph nodes. Taken together, the results reported in the present study highlight the crucial role of E-cadherin in the events affecting melanoma progression and support the proposal that E-cadherin itself may be considered as a prognostic factor for melanoma.

In conclusion, in the light of the above considerations, the findings reported in the present study, indicate that promoter hypermethylation plays a significant role in switching off *E-cadherin* gene expression in malignant melanoma and suggest that loss of *E-cadherin* may contribute to increased cell proliferation, invasiveness, ulcerative process, metastasis, prevalently at lymph nodes, head and neck or choroidal localization, and reduced disease-free/overall survival. Taken together, the findings reported in the present study suggest that E-cadherin downregulation may itself be considered a poor prognostic factor in melanoma and raise the possibility that reactivation of *E-cadherin* through promoter demethylation may represent a promising therapeutic strategy for the treatment of melanoma.

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