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Epithelial–mesenchymal transition in malignant mesothelioma

Ambrogio Fassina[1], Rocco Cappellesso[1], Vincenza Guzzardo[1], Lisa Dalla Via[2], Stefano Piccolo[3], Laura Ventura[4] and Matteo Fassan[1]

[1]*Department of Diagnostic Medical Sciences and Special Therapies, Surgical Pathology and Cytopathology Unit, University of Padova, Padova, Italy;* [2]*Department of Pharmaceutical Sciences, University of Padova, Padova, Italy;* [3]*Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, Padova, Italy and* [4]*Department of Statistics, University of Padova, Padova, Italy*

Epithelial–mesenchymal transition is a physiopathological process by which epithelial cells acquire mesenchymal shape and properties. Malignant mesothelioma is histologically characterized by the concomitant presence of epithelioid and sarcomatoid features, the latter being associated to worse prognosis, thus suggesting a role of epithelial–mesenchymal transition in this dual phenotype. We studied 109 malignant mesotheliomas (58 epithelioid, 26 sarcomatoid, and 25 biphasic) by immunohistochemistry and qRT–PCR analysis, and demonstrated a substantial switch from epithelial markers (E-cadherin, β-catenin, and cytokeratins 5/6) to mesenchymal markers (N-cadherin, vimentin, α-smooth muscle actin, Snail, Slug, Twist, ZEB1, ZEB2, S100A4, MMP2, and MMP9) through epithelioid to biphasic and sarcomatoid histotypes. In agreement with these findings, the ectopic expression of *miR-205* (a repressor of *ZEB1* and *ZEB2* expression) in MeT-5A (mesothelial cell line), H2452 (an epithelioid malignant mesothelioma cell line) and MSTO-211H (a biphasic malignant mesothelioma cell line) not only induced a significant reduction of *ZEB1* and *ZEB2* and a consequent up-regulation of E-cadherin gene expression, but also inhibited migration and invasion. Moreover, *miR-205* was significantly down-regulated in biphasic and sarcomatoid histotypes (qRT–PCR and *in situ* hybridization analyses). Collectively, our findings indicate that epithelial–mesenchymal transition has a significant part in the morphological features of malignant mesothelioma. In particular, *miR-205* down-regulation correlated significantly with both a mesenchymal phenotype and a more aggressive behavior.
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Epithelial–mesenchymal transition is a cellular process by which epithelial cells lose some of their typical characteristics, such as polarization and immotility, and acquire mesenchymal shape and properties.[1,2] Epithelial–mesenchymal transition has a central role in both physiological (ie growth and wound healing) and pathological processes (ie fibrosis and cancer).[3,4]

In cancer, epithelial–mesenchymal transition is involved in many mechanisms, especially invasion and motility, but also in resistance to apoptosis, senescence, immunotolerance, immunosuppression, drug resistance, and the acquisition of stem-cell-like properties.[5,6]

Epithelial–mesenchymal transition involves a loss of intercellular adhesion and polarity markers, cytoskeletal reorganization, and degradation of the basement membrane.[2] Commonly used molecular markers of epithelial–mesenchymal transition include a reduced expression of E-cadherin, cytokeratins (CK), and β-catenin (in the membrane), and an increased expression of Snail, Slug, Twist, ZEB1, ZEB2, N-cadherin, vimentin, α-smooth muscle actin (αSMA), S100A4, and matrix metalloproteinases (MMP).[5,7]

In particular, activation of the transcriptional regulators Snail, Slug, Twist, ZEB1, and ZEB2 enables the changes in gene expression patterns underlying epithelial–mesenchymal transition to be regulated.[8–12] These molecular markers repress the

Correspondence: Professor A Fassina, MD, Department of Medical Diagnostic Sciences and Special Therapies, Surgical Pathology and Cytopathology Unit, University of Padova, Via Aristide Gabelli, 61, 35121 Padova, Italy.
E-mail: ambrogio.fassina@unipd.it

gene expression of E-cadherin, a marker of the epithelial phenotype.[7–9,13–16]

MicroRNAs (or miRNAs) are a class of short non-coding RNAs that regulate gene expression in several physiological and pathological cellular processes.[17,18] Recently, *miR-205* has been identified as a key regulator of epithelial–mesenchymal transition enforcing the epithelial phenotype.[19–22] This miRNA participates directly with ZEB1 and ZEB2 in a signaling network that is postulated to facilitate the maintenance of stable epithelial or mesenchymal states, but that also allows for reversible switching between these states in response to epithelial–mesenchymal transition effectors.[19–23]

Malignant mesothelioma is the tumor arising from the cells lining serosal cavities.[24] According to the WHO classification, malignant mesothelioma is subclassified as epithelioid (mostly composed of epithelial-shaped cells), sarcomatoid (mostly composed of spindle-shaped cells), or biphasic (composed of both types of cell).[24] The morphological patterns of malignant mesothelioma are therefore likely to be the outcome of different steps in an epithelial–mesenchymal transition process.[25] Several authors reported the association between malignant mesothelioma subtype and patient prognosis.[25–29] Indeed, a purely epithelioid histology involved the longest survival, a purely sarcomatoid histology the worst, and a biphasic pattern an intermediate survival.[26–28]

This study was conducted to investigate the role of epithelial–mesenchymal transition in malignant mesothelioma histological subtyping. Judging from the results obtained, malignant mesothelioma could be considered an original *in vivo* model for studying the epithelial–mesenchymal transition process.

Table 1 Sites and histotypes of malignant mesothelioma

	Epithelioid	*Biphasic*	*Sarcomatoid*
Pleural	35	21	18
Peritoneal	23	5	7

Materials and methods

Patients and Tissue Specimens

One hundred and nine malignant mesotheliomas (58 epithelioid, 26 biphasic, and 25 sarcomatoid) reported between 2002 and 2009 were retrieved from the archives of the Surgical Pathology and Cytopathology Unit at Padova University. Overall, the male/female ratio was 76/33 and the patients mean age was 68.9 ± 9.9 years (median = 69.0; range = 47–83). Of these 109 cases, 74 malignant mesotheliomas were of pleural origin and 35 were peritoneal (Table 1). All diagnoses were based on the WHO criteria and confirmed in all instances by two pathologists (AF and MF) on clinical, histological, and immunohistochemical data.[24] Written informed consent to the study from the patients was obtained.

Immunohistochemistry and Immunocytochemistry

Immunohistochemical reactions were obtained on 4–5 μm-thick formalin-fixed and paraffin-embedded sections from each tumor sample. All immunohistochemical stains were performed automatically (Bond™—maX, Menarini, Florence, Italy) with the panel of primary antibodies listed in Table 2, according to the manufacturer's instructions. Sections were then lightly counterstained with hematoxylin. For immunocytochemistry, cells were seeded (10^4 cells/well) on fibronectin-coated cover slides on a 24-well plate, and stained as described elsewhere.[30] Appropriate positive and negative controls were run concurrently. E-cadherin and N-cadherin immunoreactions were defined as immunoreactions detectable in the membrane, while for β-catenin, MMP2, and MMP9, the membrane and the cytoplasm were considered, for CK 5/6, vimentin, αSMA, and S100A4 only the cytoplasm, and for ZEB1 and ZEB2 the nucleus. Immunohistochemical reactions were semiquantitatively scored in a four-tier scale: negative = 0–5% positive cancer cells, score 1 = 6–33%, score 2 = 34–66%, score 3 = 67–100%. Slides were scored

Table 2 Antibodies used in the immunohistochemical study

Antigen	Clone	Source	Vendor	Dilution
E-cadherin	Clone NCH38	Mouse	DakoCytomation, Glostrup, Denmark	1:200
N-cadherin	Clone 6G11	Mouse	DakoCytomation	1:100
β-Catenin	Clone 17C2	Mouse	Novocastra, Newcastle-upon-Tyne, UK	1:100
CK 5/6	Clone D5/16B4	Mouse	DakoCytomation	1:50
Vimentin	Clone SRL33	Mouse	Leica Microsystems, Newcastle, UK	1:300
αSMA	Clone 1A4	Mouse	DakoCytomation	1:400
S100A4	Polyclonal	Rabbit	DakoCytomation	1:100
MMP2	Clone 17B11	Mouse	Leica Microsystems	1:40
MMP9	Clone 15W2	Mouse	Leica Microsystems	1:40
ZEB1	Polyclonal	Rabbit	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:100
ZEB2	Polyclonal	Rabbit	Santa Cruz Biotechnology	1:100

independently by three pathologists (AF, RC, MF) and a consensus score was reached.

In Situ Hybridization

Reactions were obtained on formalin-fixed and paraffin-embedded sections 4–5 μm thick using the GenPointTM Catalyzed Signal Amplification System (DakoCytomation, Glostrup, Denmark) and applying the biotin-labeled miRCURY LNA detection probe (Exiqon, Vedbæk, Denmark) for *miR-205* (probe sequence: 5′-cagactccggtggaatgaagga-3′) or the scrambled negative control probe (U6, Exiqon) at 200 nM final concentration, as explained elsewhere.[31]

Reverse Transcription and Quantitative Real-Time PCR

In all, 2 mm cores of tissue were obtained from the microdissected paraffin block of each tumor sample and deparaffinized with xylene at 50 °C for 3 min. Total RNA was extracted using the RecoverAll kit (Ambion, Austin, TX, USA), as reported elsewhere.[32,33] For cell lines, total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was done using 100 ng of total RNA, M-MLV Reverse Transcriptase (Invitrogen) and 250 μM random primers (Invitrogen). Quantitative real-time PCR analyses for *CDH1* (E-cadherin), *CDH2* (N-cadherin), *SNAI1* (Snail), *SNAI2* (Slug), *TWIST1* (Twist), *ZEB1*, *ZEB2*, and *B2M* (β-2-microglobulin) were performed with the LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). The primer sequences (shown in Table 3) and the respective probes were designed using the Universal ProbeLibrary (Roche) and the ProbeFinder software (http://www.roche-applied-science.com/). Experiments were performed according to a standard protocol using the LightCycler 480 Probes Master (Roche). *B2M* was included as housekeeping gene control to adjust for unequal RNA amounts. To detect and quantify mature *miR-205* (primer sequence in Table 3), the NCodeTM miRNA qRT–PCR method (Invitrogen) was used on the LightCycler 480 Real-Time PCR System (Roche), according to the manufacturer's instructions, normalizing the results with the small nuclear RNA U6B (*RNU6B*; Invitrogen). All the reactions were run in triplicate, including no-template controls.

Cell Cultures

The immortalized by SV-40 transfection mesothelial cell line MeT-5A and the H2452 (epithelioid) and MSTO-211H (biphasic) malignant mesothelioma-derived cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained as recommended.

Cell Proliferation Assay

Cells were seeded in triplicate (10^4 cells/well) into a 24-well plate and cultured in their specific medium, replacing the culture medium every 24 h. Cell proliferation was assessed after 24, 48, 72, and 96 h, by manually counting the cells in the Burker chamber under the microscope using the Trypan Blue exclusion method to count only viable cells.

Migration Assay

Cells were starved of serum for 24 h, then seeded in triplicate (10^3 in 200 μl) in Boyden chambers (upper chamber) (BD Biosciences, Bedford, MA, USA). Lower chambers contained 500 μl of serum-free medium (SFM) or 500 μl of medium supplemented with 10% fetal bovine serum. After 24 h, the cells in the upper chamber were removed, whereas the cells that migrated to the lower chamber were counted under the microscope after fixing and staining in Coomassie blue solution, as described.[34,35]

Table 3 Primers used for qRT–PCR analysis

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product base pairs
Gene of interest			
CDH1	CCCGGGACAACGTTTATTAC	GCTGGCTCAAGTCAAAGTCC	72
CDH2	GGTGGAGGAGAAGAAGACCAG	GGCATCAGGCTCCACAGT	72
SNAI1	GCTGCAGGACTCTAATCCAGA	ATCTCCGGAGGTGGGATG	84
SNAI2	TGGTTGCTTCAAGGACACAT	GTTGCAGTGAGGGCAAGAA	66
TWIST1	CGGCCAGGTACATCGACT	CATCTTGGAGTCCAGCTCGT	62
ZEB1	GCCAACAGACCAGACAGTGTT	TCTTGCCCTTCCTTTCCTG	96
ZEB2	CAAGAGGCGCAAACAAGC	AACCTGTGTCCACTACATTGTCA	71
hsa-miR-205	CTTCATTCCACCGGAGTCTG	—	42
Loading control			
B2M	CCTTGAGGCTATCCAGCGTA	TCAGGAAATTTGACTTTCCATTC	80
RNU6B	ACGCAAATTCGTGAAGCGTT	—	30

Invasion Assay

Cell invasion through a three-dimensional extracellular matrix was assessed using a Matrigel invasion assay with BD Matrigel Invasion Chambers (BD Biosciences), as described.[34,35] Briefly, cells were starved of serum for 24 h, then seeded in triplicate (10^3 in 200 μl) in Boyden chambers (upper chamber) (BD Biosciences). Lower chambers contained 500 μl of SFM or 500 μl of medium supplemented with 10% fetal bovine serum. After 24 h, the cells on the upper surface of the filters were removed with cotton swabs, whereas cells that had invaded to the lower surface of the filter were counted under the microscope after fixing and staining in Coomassie blue solution for 5 min.

Cell Transfection

Cells were seeded (10^4 cells/well) into a 24-well plate and cultured in their specific medium. After 24 h of incubation, the medium was removed and replaced with 500 μl of transfection medium composed of 2.5 μl DharmaFECT 1 reagent (Dharmacon, Thermo Fisher Scientific, Waltham, MA, USA), 2.5 μl of 5 μM miR-205 miRIDIAN miRNA mimic (double-stranded, chemically modified RNA oligonucleotides; Dharmacon), and 95 μl antibiotic and SFM, with a final concentration of 25 nM miRNA mimic. Transfection of miRNA mimic was confirmed by qRT–PCR for *miR-205*. Negative miRIDIAN mimics were transfected as matched controls. After 24 h, cells were processed and analyzed for migration and invasion as described above.

Statistical Analysis

All statistical analyses were performed using the R software (R Development Core Team, version 2.9; R Foundation for Statistical Computing, Vienna, Austria). Survival was calculated as the time of diagnosis to the date of death or last contact (after a follow-up of at least 18 months). Survivor curves with censored data were estimated from the univariate Cox model; comparisons between groups were performed using log-rank test. Statistical significance was determined for immunohistochemical and qRT–PCR results using Student's *t*-test and the Kruskal–Wallis test. A *P*-value <0.05 was considered statistically significant.

Results

Malignant Mesothelioma Histotypes Are Associated with Different Survival Time

As expected, the median survival time for epithelioid malignant mesothelioma was 13 months (95% CI = 13–21 months), for biphasic malignant mesothelioma was 14 months (95% CI = 11–27 months),

and for sarcomatoid malignant mesothelioma was 6 months (95% CI = 6–13 months). Survivor curves for the epithelioid, biphasic, and sarcomatoid histotypes with censored times are showed in Figure 1. The log-rank test revealed differences in survival time for the three histotypes (LR = 17.3, *P* < 0.001). The Cox model showed that patients with sarcomatoid malignant mesothelioma had a significantly worse prognosis than those with epithelioid and biphasic malignant mesothelioma (*P* < 0.001), while there was no difference for those with these last subtypes (*P* = 0.146).

Malignant Mesothelioma Phenotypic Subclassification Corresponds to Specific Expression Profiles of Epithelial–Mesenchymal Transition Markers

Immunohistochemical analyses were performed on our series of 109 formalin-fixed paraffin-embedded malignant mesothelioma samples. Immunostaining showed that epithelial hallmarks such as E-cadherin and CK 5/6 were positive and reliable markers of epithelioid commitment, while their expression was weak in biphasic and absent in sarcomatoid malignant mesothelioma samples (Figures 2 and 3; *P* < 0.001 for all markers). The *β*-catenin levels were likewise higher in epithelioid and biphasic histotypes than in sarcomatoid malignant mesotheliomas (Figures 2 and 3). On the other hand, the typical mesenchymal markers vimentin, S100A4, and ZEB1 displayed a strong reaction in all sarcomatoid samples, a moderate reaction in biphasic malignant mesotheliomas, and a weak and focal reaction in epithelioid malignant mesotheliomas (Figures 2, 4, and 5). MMP9, N-cadherin, αSMA, ZEB2, and MMP2 did not differ significantly between biphasic and sarcomatoid malignant mesotheliomas, while they were lower in the epithelioid histotype (Figures 2, 4, and 5). The log-rank test showed that the survival time for the epithelioid malignant
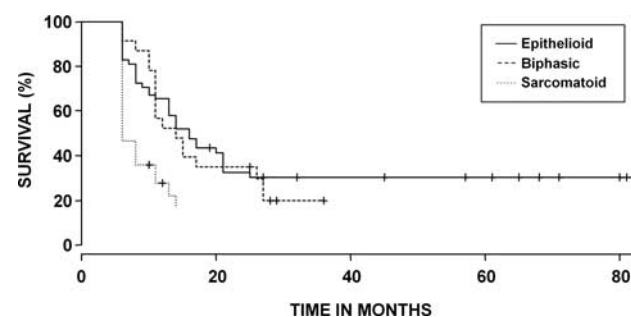

Figure 1 Kaplan–Meier survival curves with censored times (crosses) in malignant mesothelioma patients according to the histological subtype. Curves were compared by univariate (log-rank) test, which revealed differences in survival time for the three histotypes (LR = 17.3, *P* < 0.001) and Cox model displayed that the survival time of patients with sarcomatoid malignant mesothelioma was significantly shorter than epithelioid and biphasic malignant mesotheliomas (*P* < 0.001), while there was no difference between these last subtypes (*P* = 0.146).
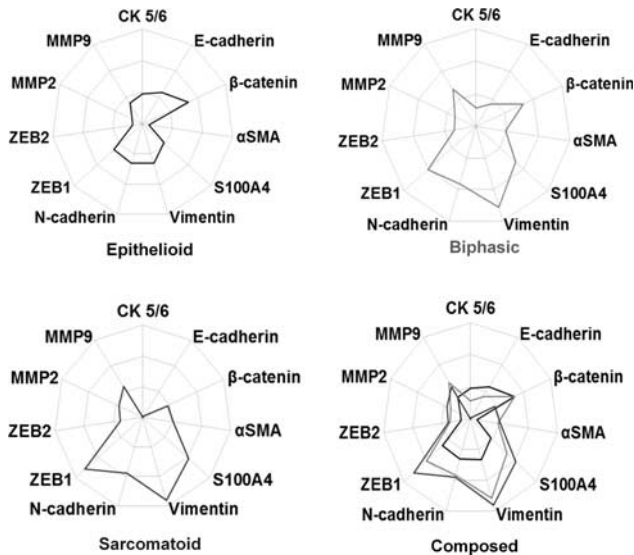
Figure 2 Epithelial–mesenchymal transition marker expression in malignant mesotheliomas. Radar charts show the mean immunohistochemical scores observed in epithelioid, biphasic, and sarcomatoid malignant mesotheliomas; center of the chart correspond to negative immunohistochemical reaction while the edges to a score of 3. Note that the epithelial markers CK 5/6, E-cadherin, and *β*-catenin levels are consistently higher in epithelioid than in sarcomatoid malignant mesotheliomas ($P < 0.001$ for all markers), and in biphasic than in sarcomatoid malignant mesotheliomas ($P < 0.001$ for all markers); and CK 5/6 and E-cadherin are significantly more over-expressed in epithelioid than in biphasic samples ($P = 0.005$ and $P = 0.028$, respectively). Typical mesenchymal markers, such as *α*SMA, S100A4, vimentin, N-cadherin, ZEB1, ZEB2, MMP2 and MMP9, are significantly more over-expressed in sarcomatoid and biphasic than in epithelioid malignant mesotheliomas ($P < 0.01$ for all markers).

mesothelioma was associated with the immunohistochemical level of the epithelial marker E-cadherin ($P = 0.042$). No differences were observed relating to the anatomical site of involvement (pleura or peritoneum). These findings were further supported by qRT–PCR analysis, which partially confirmed the shift from the epithelial to the mixed and mesenchymal phenotypes. Indeed, qRT–PCR revealed the cadherin switch, showing that *CDH1* mRNA levels were much higher in epithelioid malignant mesotheliomas than in biphasic and sarcomatoid malignant mesotheliomas, and that *CDH2* mRNA levels were markedly increased in the sarcomatoid histotype by comparison with the biphasic or epithelioid histotypes (Figure 6). Analysis of the transcriptional repressors regulating epithelial–mesenchymal transition showed that *SNAI2*, *TWIST1*, and *ZEB2* mRNA levels were higher in sarcomatoid than in epithelioid samples, and *TWIST1* and *ZEB2* mRNA levels were higher in sarcomatoid than in biphasic malignant mesotheliomas (Figure 6). Notwithstanding *SNAI1* and *ZEB1* amounts were not statistically different among the three histotypes, ZEB1 mRNA levels showed an upward trend from epithelioid to biphasic and sarcomatoid malignant mesotheliomas (Figure 6).

Epithelial–Mesenchymal Transition Dysregulation Is a Key Determinant of the Phenotypic and Aggressive Features of Mesothelial/Mesothelioma-Derived Cell Lines

To establish the effect of epithelial–mesenchymal transition on malignant mesothelioma phenotype and behavior, we performed immunocytochemistry, proliferation, migration, and invasion assays in the mesothelial cell line MeT-5A and in the established H2452 (epithelioid) and MSTO-211H (biphasic) cell lines. The immunocytochemical results indicated that the epithelial markers E-cadherin and CK 5/6 were weakly expressed only in the mesothelial cell line MeT-5A, while *β*-catenin expression was strong in MeT-5A, weak in H2452, and absent in MSTO-211H (Figure 7). Conversely, all mesenchymal markers, *α*SMA, S100A4, vimentin, N-cadherin, ZEB1, ZEB2, MMP2, and MMP9 occurred in higher levels in the biphasic than in the epithelioid or non-tumorigenic cell lines (Figures 7 and 8). Immunostaining also showed that *α*SMA, S100A4, vimentin, ZEB2, and MMP2 were expressed more in H2452 than in MeT-5A (Figure 7). Proliferation assays showed that both the malignant mesothelioma cell lines had a considerably greater increase in cell number than MeT-5A, and that the biphasic cells grew faster than all the others (Figure 9). The more aggressive behavior of H2452 and MSTO-211H, compared with MeT-5A, was also confirmed by migration and invasion assays, as shown in Figure 10.

MiR-205 Expression Is Dysregulated in the Different Mesothelioma Subtypes and Inhibits Migratory and Invasive Phenotypes

MiR-205 is known to be a negative regulator of *ZEB1* and *ZEB2* mRNA thus maintaining the epithelial phenotype.[19–22] A qRT–PCR analysis, performed in solid malignant mesotheliomas and in cell lines, showed that *miR-205* levels were higher in epithelioid than in biphasic or sarcomatoid samples, and in MeT-5A than in malignant mesothelioma cell lines (Figure 11; $P < 0.01$ for both). To support this observation, a *miR-205*-specific *in situ* hybridization was performed in 15 malignant mesotheliomas corresponding to the three different phenotypic forms (five cases of each subtype), randomly selected from the immunohistochemical series. *In situ* hybridization displayed a strong and diffuse reaction in the epithelioid samples, a moderate reaction in the biphasic samples, and a weak and focal reaction in the sarcomatoid samples (Figure 12). To assess the effect of miR-205 dysregulation on cellular behavior, mesothelial/malignant mesothelioma-derived cell lines were transfected with a *miR-205* mimic. In treated and untreated cells, in all the cell lines, qRT–PCR analysis revealed a consistent down-regulation of *ZEB1* and *ZEB2* and a slight up-regulation of *CDH1* coinciding
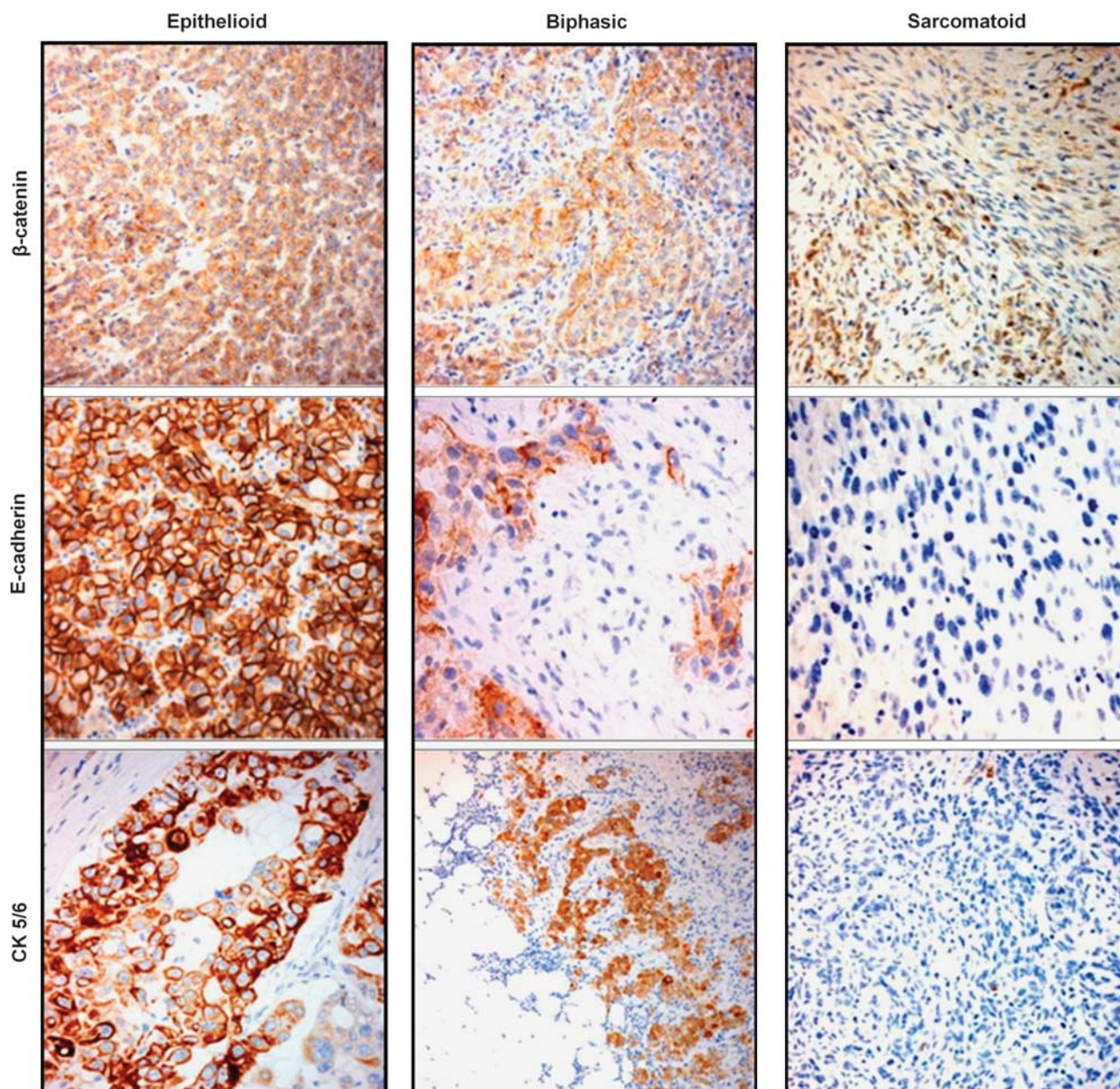
Figure 3 Epithelial–mesenchymal transition marker expression in malignant mesotheliomas. Representative immunostains for epithelial (CK 5/6, E-cadherin, and β-catenin) markers in the different malignant mesothelioma histotypes. (Original magnifications $\times 20$ and $\times 40$).

with the increase of *miR-205* levels (Figure 13). To investigate the role of *miR-205* in cell migratory and invasive phenotypes, we conducted *in vitro* migration and invasion assays using the biphasic MSTO-211H cell line: *miR-205* over-expression inhibited the cells' capacity of migration and invasion, indicating that miR-205 negatively regulates the metastatic phenotype of mesothelioma cancer cells (Figure 14). We repeated these assays in the other two cell lines and obtained similar results, albeit without reaching statistical significance (data not shown).

Discussion

The current histological classification recognizes three major subtypes of malignant mesothelioma: epithelioid malignant mesothelioma is composed of epithelial-shaped cells, sarcomatoid malignant mesothelioma consists of cells with a mesenchymal appearance, and biphasic malignant mesothelioma is a combination of the two.[24] This phenotypic triad reflects the whole spectrum of cell commitments occurring during the epithelial–mesenchymal transition process, and this was the working hypothesis
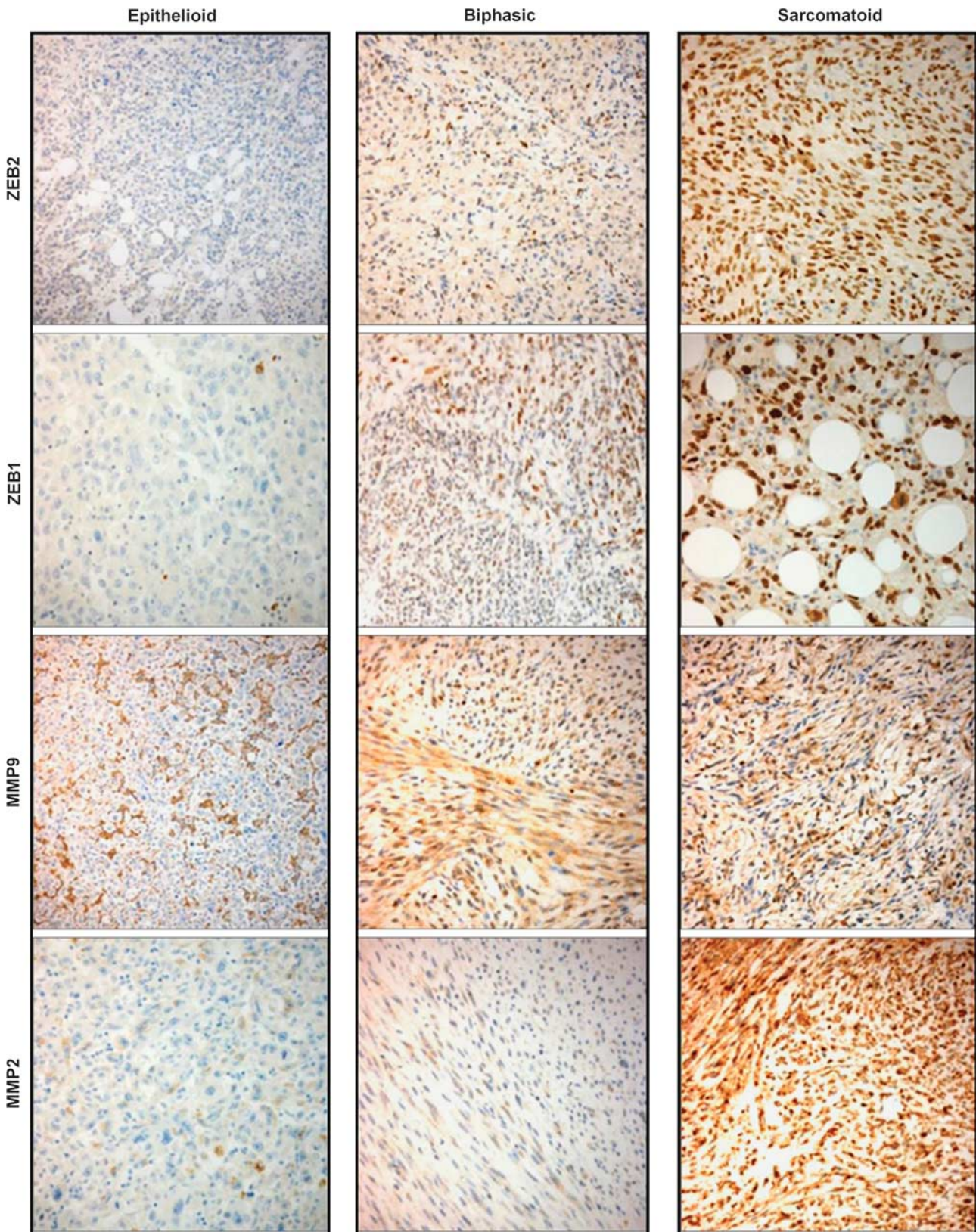
Figure 4 Epithelial–mesenchymal transition marker expression in malignant mesotheliomas. Representative immunostains for sarcomatoid (ZEB1, ZEB2, MMP2, and MMP9) markers in the different malignant mesothelioma histotypes. (Original magnifications ×20 and ×40).
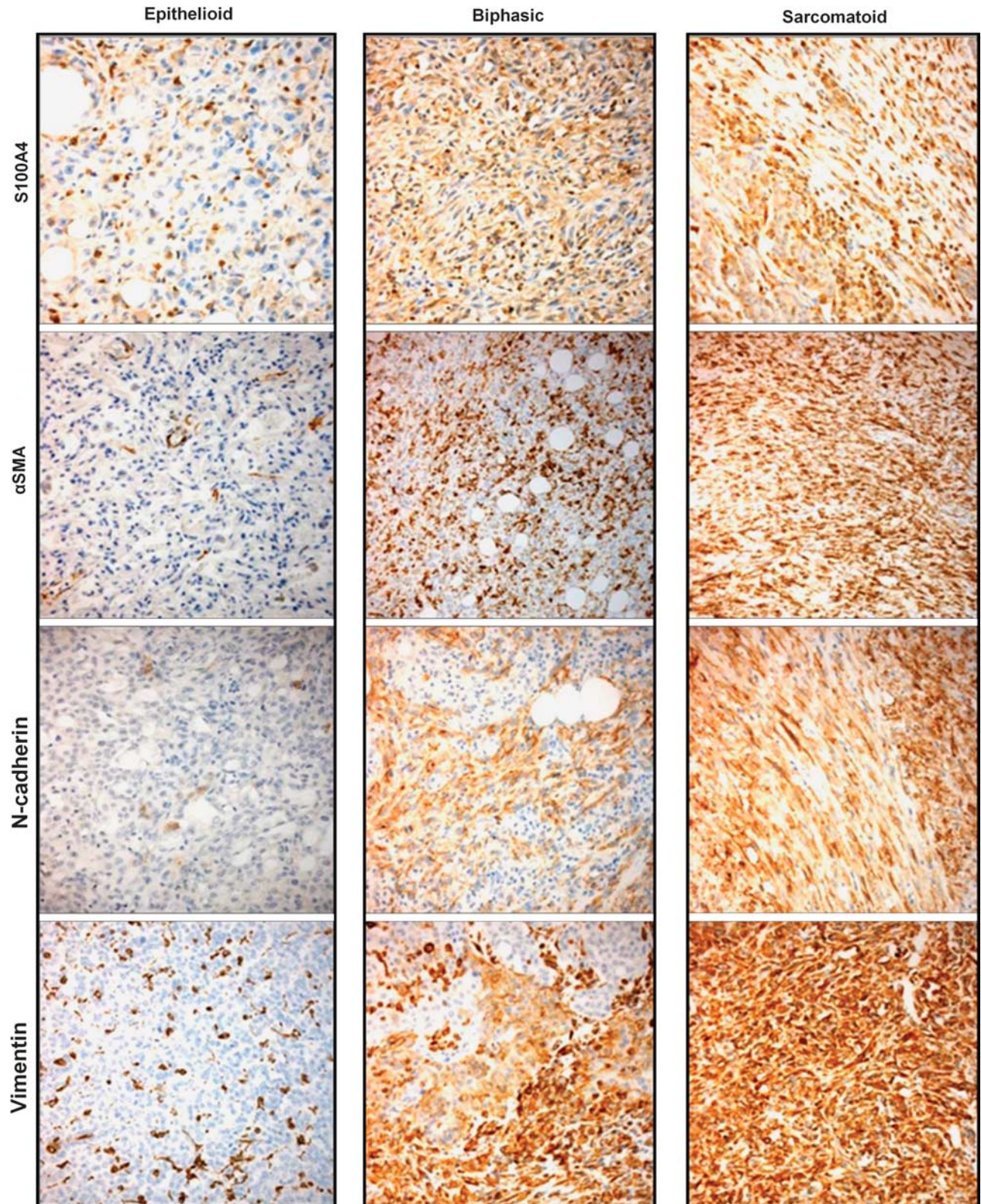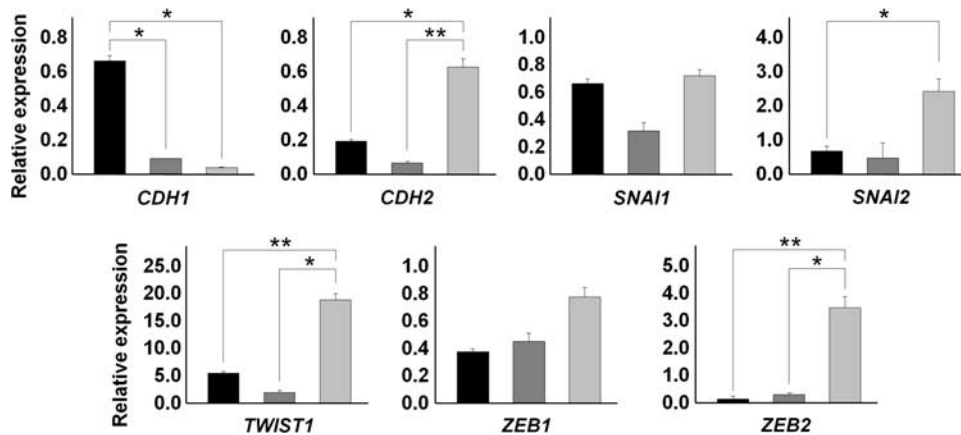
Figure 5 Epithelial–mesenchymal transition marker expression in malignant mesotheliomas. Representative immunostains for sarcomatoid (N-cadherin, vimentin, αSMA, and S100A4) markers in the different malignant mesothelioma histotypes. (Original magnifications ×20 and ×40).

Figure 6 Relative fold of *CDH1*, *CDH2*, *SNAI1*, *SNAI2*, *TWIST1*, *ZEB1*, and *ZEB2* mRNA levels in 58 epithelioid (black), 26 biphasic (dark gray), and 25 sarcomatoid (bright gray) malignant mesotheliomas. qRT–PCR highlighted the differences in cadherin levels: *CDH1* was significantly higher in epithelioid than in biphasic or sarcomatoid samples ($P = 0.049$ and $P = 0.039$, respectively), while *CDH2* was more over-expressed in sarcomatoid than in biphasic or epithelioid malignant mesotheliomas ($P = 0.009$ and $P = 0.028$, respectively). Mesenchymal transcriptional regulators *SNAI2*, *TWIST1*, and *ZEB2* were consistently more over-expressed in the sarcomatoid than in the epithelioid histotype ($P = 0.029$, $P = 0.009$, and $P = 0.006$, respectively); *TWIST1* and *ZEB2* mRNA levels were also higher in biphasic than in epithelioid malignant mesotheliomas ($P = 0.016$ and $P = 0.049$, respectively). *SNAI1* and *ZEB1* amounts were not statistically different among the three histotypes; however, *ZEB1* showed an upward trend from epithelioid to biphasic and sarcomatoid malignant mesotheliomas. Columns, representative images of reactions run in triplicate; bars, s.d. *$P < 0.05$; **$P < 0.01$.
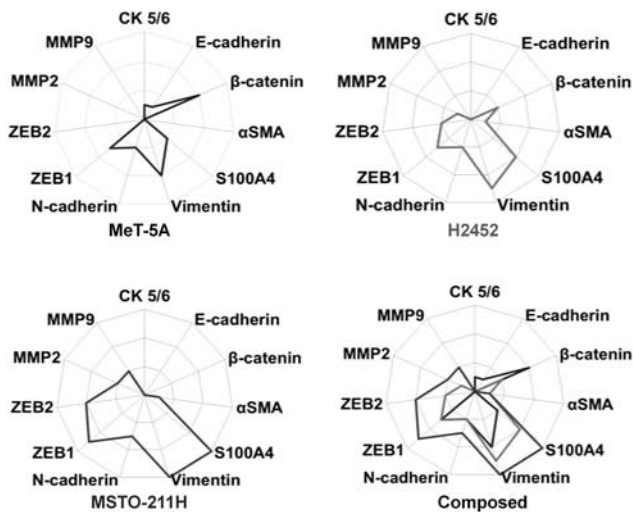

Figure 7 Mesothelial-derived cellular commitment corresponds to specific epithelial–mesenchymal transition marker profiles. Radar charts show the mean immunocytochemical scores in the mesothelial cell line MeT-5A and the established H2452 (epithelioid) and MSTO-211H (biphasic) malignant mesothelioma cell lines. The figures are the mean of three different immunoreactions obtained from three different cell passages; center of the chart correspond to negative immunohistochemical reaction while the edges to a score of 3. Overall, marker expression increased from MeT-5A to H2452 and MSTO-211H.

behind the present study, in which we investigated the role of epithelial–mesenchymal transition in malignant mesothelioma histological subtyping.

In a large series of malignant mesotheliomas, we observed a consistent epithelial-to-mesenchymal trend in marker representation coinciding with the epithelioid to biphasic, to sarcomatoid histotypes by immunohistochemistry and qRT–PCR analysis.

In the sarcomatoid samples, E-cadherin virtually disappeared, as reported in other solid tumors undergoing epithelial–mesenchymal transition.[36–38] This crucial passage coincided with an increased expression of Slug, Twist, ZEB1, and ZEB2, a set of well-known nuclear factors that inhibit E-cadherin gene transcription by binding to its promoter.[5,7,19,21,22]

E-cadherin has recently been found to be regulated at protein level by mechanisms implicating miRNAs, a class of small non-coding RNAs that modulate gene expression post-transcriptionally.[19–22] Indeed, it has been demonstrated *in vitro* that *miR-205* controls ZEB1 and ZEB2 levels by targeting their messenger RNA, thus enhancing E-cadherin expression and maintaining the epithelial phenotype.[19]

Based on these data, we found that changes in E-cadherin expression varied according to the *miR-205* levels in the different histotypes. Moreover, when *miR-205* was over-expressed, a significant *ZEB1/ZEB2* down-regulation and a minor *CDH1* up-regulation were consistently seen in the mesothelial/mesothelioma-derived cell lines.

The other proteins involved in epithelial–mesenchymal transition mechanisms (implicated in cell–cell and cell–matrix interactions, cell structure, and motility) also showed a different expression profile for the three different malignant mesothelioma subtypes. In addition to E-cadherin, epithelioid malignant mesotheliomas expressed *β*-catenin and CK 5/6, which are needed to maintain cell contact and epithelial shape.[39,40] On the other hand, biphasic and sarcomatoid malignant mesothelioma variants lost these proteins and gained a mesenchymal phenotype by (i) increasing the amount of N-cadherin on the cell surface, which results in weaker cell adhesion; (ii) expressing
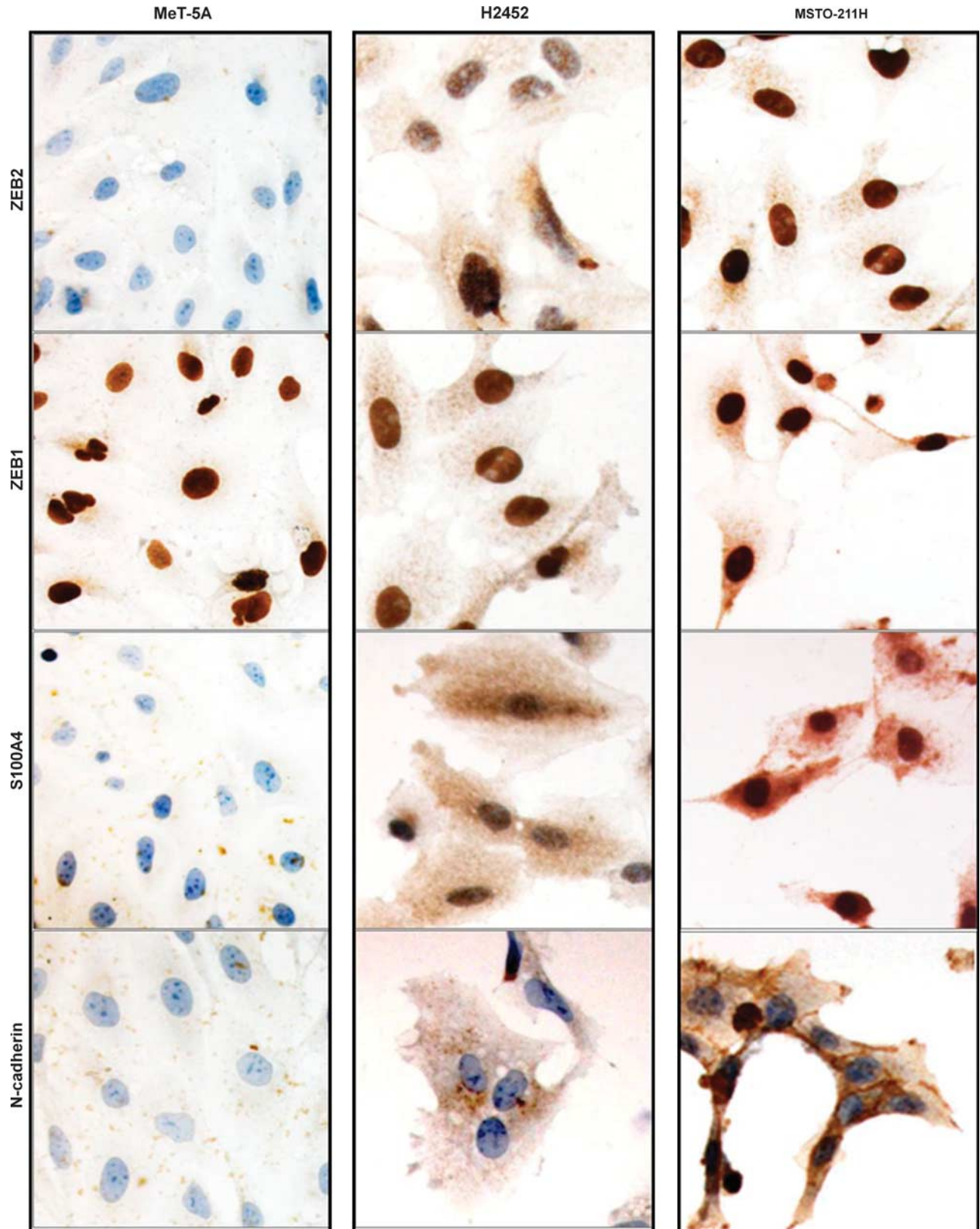
Figure 8 Mesothelial-derived cellular commitment corresponds to specific epithelial–mesenchymal transition marker profiles. Representative images of immunostaining for the mesenchymal markers N-cadherin, S100A4, ZEB1, and ZEB2 in the three cell lines studied (original magnifications ×40). Overall, marker expression increased from MeT-5A to H2452 and MSTO-211H.
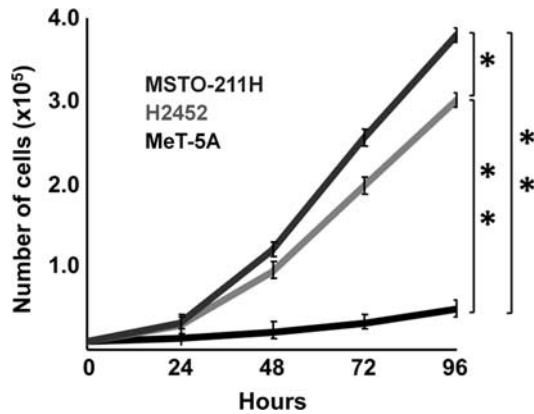
Figure 9 Growth curves show a significant drop in normal (MeT-5A) and epithelioid (H2452) cells compared with the biphasic cell line (MSTO-211H). The figures are the mean of three independent experiments. *$P<0.05$, **$P<0.01$.
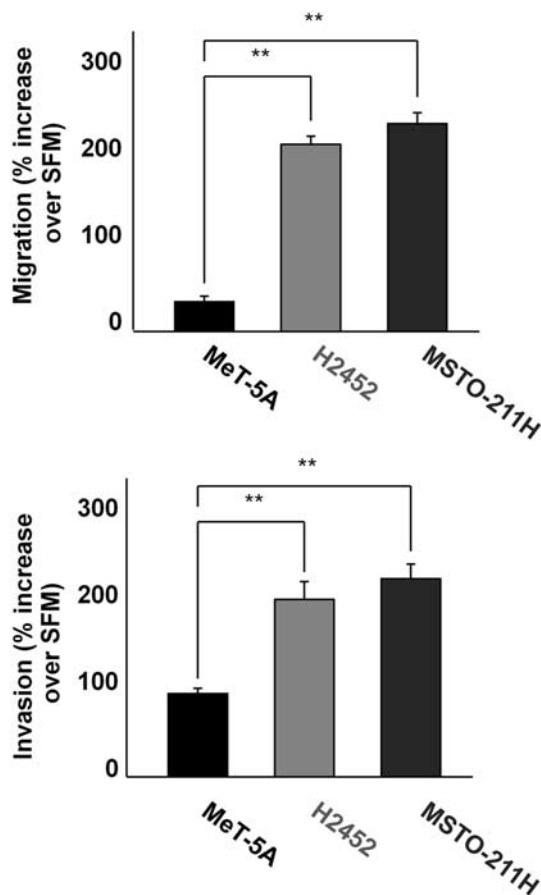

Figure 11 *MiR-205* is key factor of epithelial–mesenchymal transition in malignant mesothelioma. The relative expression of *miR-205* was analyzed in both solid malignant mesotheliomas and mesothelial cell line MeT-5A, established H2452 (epithelioid) and MSTO-211H (biphasic) malignant mesothelioma cell lines. qRT–PCR analysis showed that *miR-205* levels were significantly higher in epithelioid than in biphasic or sarcomatoid samples ($P<0.01$ for both), and in non-tumorigenic and epithelioid than in biphasic cell lines ($P<0.01$ for both). Columns, representative images of reactions run in triplicate; bars, s.d. **$P<0.01$.

Figure 10 MeT-5A (normal mesothelial cell line) showed a less aggressive behavior than H2452 of MSTO-211H (epithelioid and biphasic malignant mesothelioma cell lines), in both the migration and the invasion assays. Migration and invasion experiments on the three cell lines were done using SFM or medium supplemented with 10% fetal bovine serum. Percent increase over control (SFM). Columns, representative images of triplicates from three independent experiments; bars, s.e.m. *$P<0.05$; **$P<0.01$.
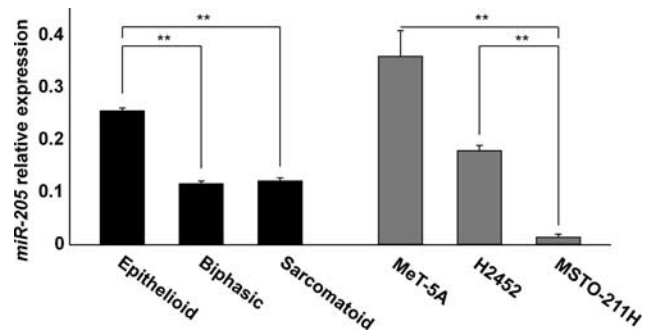
vimentin, αSMA, and S100A4 in the cytoskeleton, achieving a resistance to deformation and motility; and (iii) producing MMP2 and MMP9 to degrade the basement membrane.[1–3,5,7] In this study, however, we evaluated only the loss or acquisition of marker physiological expression and localization, while other mechanisms involved in epithelial–mesenchymal transition, such as the nuclear localization of β-catenin and S100A4, were not considered.

Epithelial–mesenchymal transition is reported to be implicated in many tumoral mechanisms, mainly invasion and motility, which lead to a more aggressive behavior of tumors and, consequently, to a worse prognosis for patients.[5,6] As reported by several authors, our data confirmed the association between sarcomatoid malignant mesothelioma and worst outcome, once again supporting the hypothesis of an epithelial–mesenchymal transition involvement.[25–28] Moreover, our findings highlighted the link between the epithelial marker E-cadherin and the longer survival time of epithelioid malignant mesothelioma than the other subtypes. Further larger studies should investigate the possible correlations of the epithelial–mesenchymal transition markers with survival among the histological variants of epithelioid malignant mesothelioma (and the pleomorphic subtype, in particular) and also with the presence/absence of lymph node metastasis.[29]

Overall, the demonstration of epithelial–mesenchymal transition in malignant mesothelioma is a suitable model for studying this process *in vivo*. One of the most important arguments against the existence of epithelial–mesenchymal transition in human solid tumors is that it has not been identified in most cases.[40,41] This could be due to the clonal heterogeneity of epithelial–mesenchymal transition in primary tumors, that is, cells that have undergone
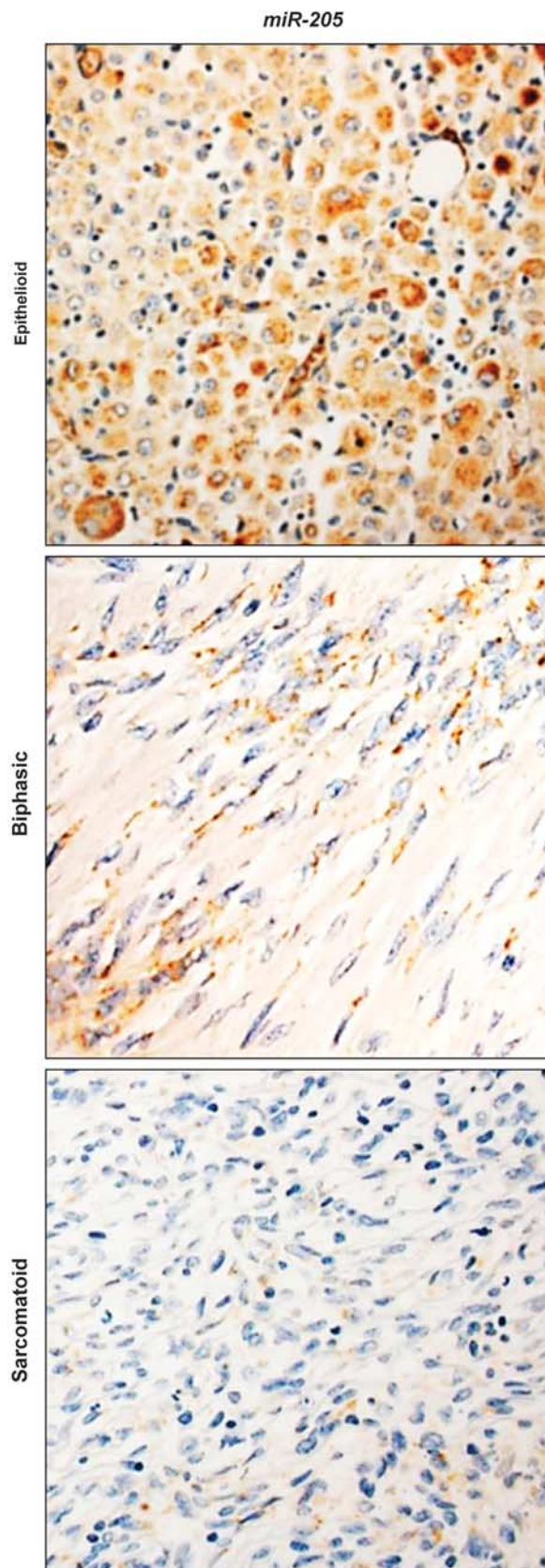
Figure 12 *MiR-205*-specific *in situ* hybridization analysis clearly showed a significant drop in *miR-205* expression from epithelioid to biphasic, to sarcomatoid malignant mesotheliomas (original magnifications ×20 and ×40).
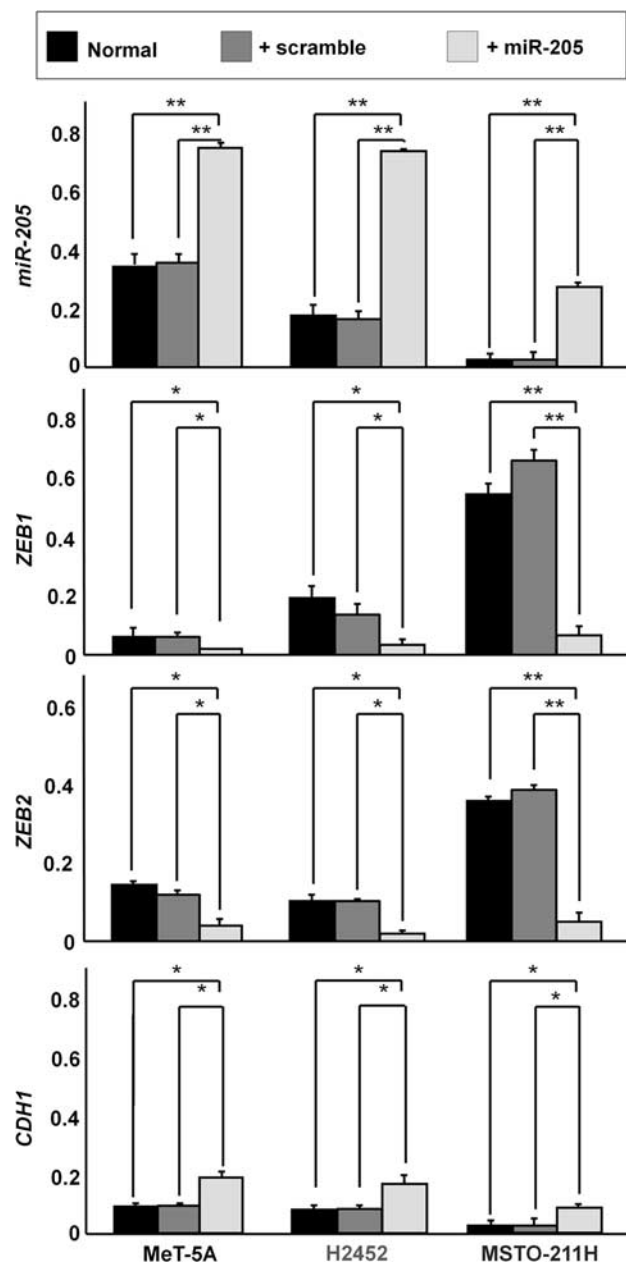

Figure 13 *MiR-205* regulated *ZEB1* and *ZEB2* and consequently *CDH1* expression in MeT-5A, H2452, and MSTO-211H cell lines. Cells were transfected with miR-205 mimic or a negative control (scramble) and analyzed by qRT–PCR. Columns, representative images of reactions run in triplicate; bars, s.d. *$P<0.05$; **$P<0.01$.

epithelial–mesenchymal transition are just a sub-population located in certain neoplastic areas, and the process could be difficult to recognize using routine histopathological investigations.[42] Another reported reason for the difficulty encountered in identifying this process *in vivo* is the reverse mesenchymal–epithelial transition process occurring in metastatic tumors.[43] In contrast, malignant mesothelioma histotype subtyping is relatively straightforward using a suitable immunohistochemical panel, and malignant mesothelioma is therefore a reasonably easy model to study.
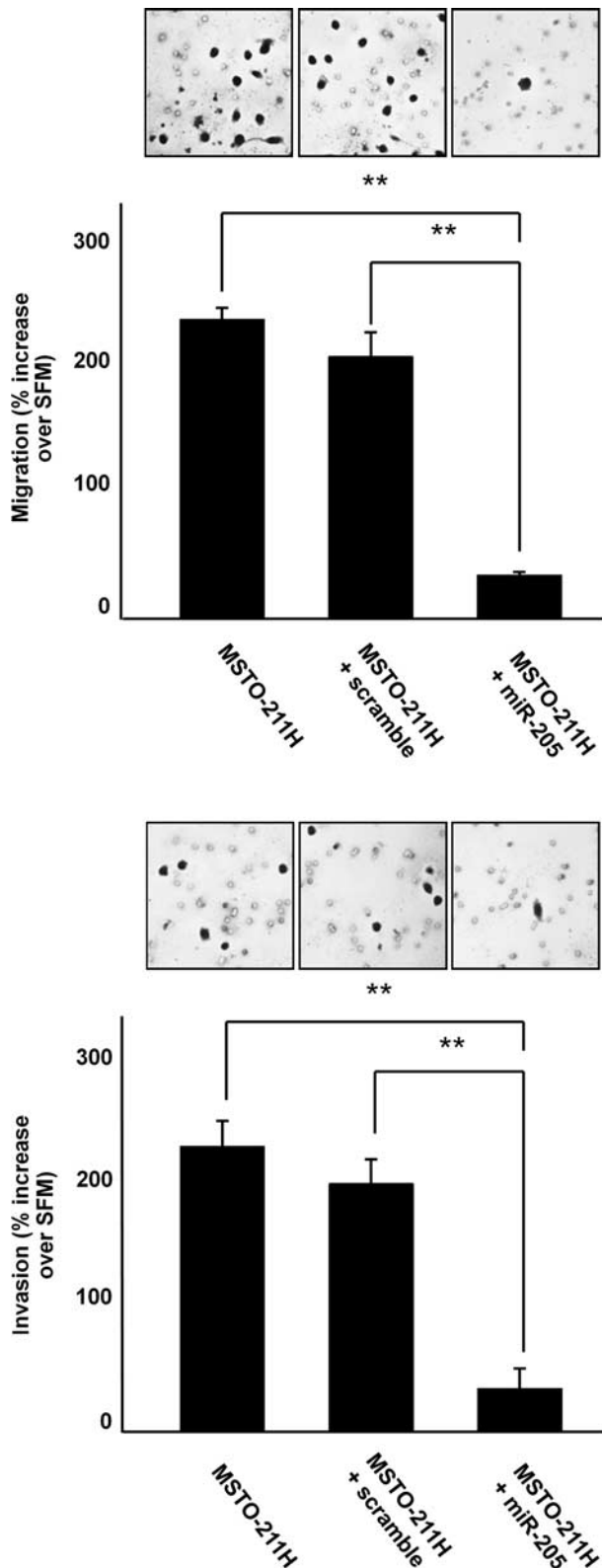
Figure 14 Transfection with *miR-205* mimic inhibited the migratory and invasive properties of MSTO-211H cancer cells. Migration and invasion experiments on MSTO-211H cancer cells were done using SFM or medium supplemented with 10% fetal bovine serum. Percent increase over SFM. Columns, representative images of triplicates from three independent experiments; bars, s.e.m. **P < 0.01.

Our findings on the migratory and invasive capacity of malignant mesothelioma cell lines confirm the link between cellular phenotype and behavior, as reported by many other authors in other neoplastic diseases.[1–3,5,7] As expected, the biphasic malignant mesothelioma cell line showed a more aggressive behavior, in terms of both cell growth and migration/invasion, than MeT-5A and epithelioid malignant mesothelioma cell lines. The ectopic expression of *miR-205* also contributed to the mesenchymal–epithelial transition by (i) reducing *ZEB1* and *ZEB2* expression and consequently increasing *CDH1* expression and (ii) interfering with cell motility and invasiveness.[19]

Based on the present study, we conclude that the different morphological patterns of malignant mesothelioma are part of a continuous spectrum determined by epithelial–mesenchymal transition, initiated by ZEB1 and ZEB2 activation and correlated with a worse outcome. In particular, we found that loss of *miR-205* expression correlated significantly with both a mesenchymal phenotype and a more aggressive behavior. Malignant mesothelioma may be a powerful model for studying epithelial–mesenchymal transition *in vivo*. Regulating epithelial–mesenchymal transition marker expression might be a novel strategy for the chemoprevention of metastatic/aggressive human cancers.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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