Immunohistochemical Study of the Epithelial-Mesenchymal Transition Phenotype in Cancer of Unknown Primary: Incidence, Correlations and Prognostic Utility

AIKATERINI STOYIANNI[1,6], ANNA GOUSSIA[3], GEORGE PENTHEROUDAKIS[1,2], VASSILIKI SIOZOPOULOU[3], ELLI IOACHIM[7], DIMITRIOS KRIKELIS[1,8], VASSILIOS GOLFINOPOULOS[1], ANDRES CERVANTES[5], MATTHEOS BOBOS[4], THEODOROS FOTSIS[6], SOFIA BELLOU[6], GEORGIOS FOUNTZILAS[2,8], VASSILIKI MALAMOU-MITSI[3] and NICHOLAS PAVLIDIS[1,2]

[1]*Department of Medical Oncology, Medical School, University of Ioannina, Greece;*
[2]*Hellenic Cooperative Oncology Group, Athens, Greece;*
[3]*Department of Pathology, Medical School, University of Ioannina, Greece;*
[4]*Department of Pathology, Medical School, Aristotle University of Thessaloniki, Greece;*
[5]*Department of Medical Oncology, Institute of Health Research Hospital Clinic of Valencia (INCLIVA), University of Valencia, Valencia, Spain;*
[6]*Institute of Biomedical Research, FORTH-BRI, Ioannina, Greece;*
[7]*Department of Pathology, Xatzikosta General Hospital, Ioannina, Greece;*
[8]*Department of Medical Oncology, Medical School, Aristotle University of Thessaloniki, Papageorgiou Hospital, Thessaloniki, Greece*

Abstract. *Background: The epithelial to mesenchymal transition (EMT) has been associated with metastatic dissemination and poor outcome in several solid tumour types. Our aim was to study its incidence and its prognostic significance in cancer of unknown primary (CUP). Patients and Methods: One hundred tumour samples of CUP were loaded in tissue microarrays and were studied for immunohistochemical (IHC) expression of E-cadherin, N-cadherin, vimentin, the EMT transcription factor (SNAIL) and the stem cell marker octamer-binding transcription marker 4(OCT4). An EMT phenotype was defined as low expression of E-cadherin, expression of N-cadherin with/without vimentin with concomitant expression of SNAIL, as assessed by percentage of tumour cell staining. Results: Among 100 CUP cases, the histological diagnosis was adenocarcinoma in 55, squamous carcinoma in 20 and undifferentiated carcinoma in 15, with a high grade seen in 46. Therapy consisted of palliative chemotherapy, mostly platinum based. The median progression-free survival and overall survival (OS) were 7 and 12 months respectively. Distributional studies resulted in selection of IHC cut-offs for E-cadherin (negative when expressed in <60% of tumour cells), N-cadherin, vimentin (positive when expressed in ≥40% of tumour cells), SNAIL (positive when stained in ≥80% of tumour cells). An EMT phenotype was observed in 8 cases (8.1%) and was strongly associated with poor OS (median OS EMT^{-}=13 months vs. median OS EMT^{+}=8 months, p=0.023). When we used staining intensity (H-Score), an EMT phenotype was observed in 16 patients and carried borderline adverse prognostic utility for outcome (median OS 9 vs. 14 months, p=0.07). The presence of the EMT phenotype correlated significantly with male gender, high grade and presence of visceral metastases (χ^2 p<0.05), while EMT mediator expression was correlated to high NOTCH 2/3 expression. Other factors, prognostic for poor survival, were male gender, PS≥2, non-platinum therapy (χ^2 p<0.05). Conclusion: EMT is infrequently seen in tumours of CUP. However, an adverse prognostic significance for patient outcome has been identified and may warrant studies of therapeutic targeting.*

Correspondence to: Nicholas Pavlidis, Department of Medical Oncology, Ioannina University Hospital, Niarchou Avenue 45500 Ioannina Greece. Tel/Fax: +30 2651099394, e-mail: npavlid@uoi.gr

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Metastatic cancer of unknown primary site (CUP) accounts for approximately 3% of all malignant neoplasms and is one of the ten most frequent cancers diagnosed in men. CUP manifests as an heterogeneous group, mostly of epithelial carcinomas which share a unique clinical behaviour and

presumably a unique biology (1, 2). The natural history of patients with cancer of unknown primary is extremely heterogeneous, with a reported median survival from 4 to 11 months. However, several patient subgroups, identified by means of unique clinical and pathological characteristics, have been described to favourably respond to treatment and enjoy long-term disease control. However, the vast majority of patients (80%) harbour systemic visceral metastases of adenocarcinoma or poorly differentiated carcinoma and belong to the poor-risk CUP subset, which has as hallmark features systemic spread, resistance to therapy and dismal outcome.

Late-stage cancer is invariably accompanied by metastasis, which constitutes the most common cause of death of cancer patients. The metastatic cascade comprises of several steps, ultimately leading to the emergence of secondary tumours at distant sites (3). One process contributing to the first phase of metastasis is the epithelial-mesenchymal transition (EMT). EMT refers to a complex molecular and cellular programme by which epithelial cells lose their differentiated characteristics, *i.e.* cell–cell adhesion, planar and apical-basal polarity, and lack of motility, and acquire instead mesenchymal features, including motility, invasiveness and heightened resistance to apoptosis. The dysregulation of this programme can have pathogenic consequences (4). EMT is characterized by down-regulation of epithelial markers, particularly E-cadherin, expression of its repressor, the transcription factor SNAIL, and up-regulation of mesenchymal markers, such as N-cadherin and vimentin (5). In the context of epithelial cells, EMT provides a mechanism by means of which tumour cells leave the primary site and invade into the local tissue and blood vessels, setting the stage for metastatic spread. Furthermore, metastasizing cancer cells seem to acquire self-renewal capability, similar to the one exhibited by stem cells, in order to spawn macroscopic metastases (6). This raises the possibility that the EMT process is the mechanism that imparts a self-renewal capability to disseminating cancer cells (7). Therefore, since EMT is hypothesized to contribute to tumour progression and to be related to stemness features, we sought to study its incidence in a retrospective series of 100 patients diagnosed with CUP and to investigate its associations with stem-like cellular phenotype, NOTCH protein expression and several clinicopathological characteristics. Moreover, our aim was to screen for the potential predictive utility of EMT phenotype for response to therapy and outcome.

Patients and Methods

Patients. The patient pool consisted of 150 patients diagnosed with CUP treated in the Hellenic Cooperative Oncology Group (HeCOG)-affiliated centres as well as at centres in Valencia University Hospital, Spain, from whom a tumour block was obtained in 100 cases. All referred patients had undergone a standardised diagnostic work-up consisting of medical history; thorough physical examination; computerized tomography of chest, abdomen- pelvis; mammography in women; complete hematological and biochemical serum profile; serum testing of prostate-specific antigen and α-fetoprotein; and sign/symptom-directed endoscopic studies of respiratory and alimentary tracts. All patients had provided written informed consent for use of their data and bioptic material.

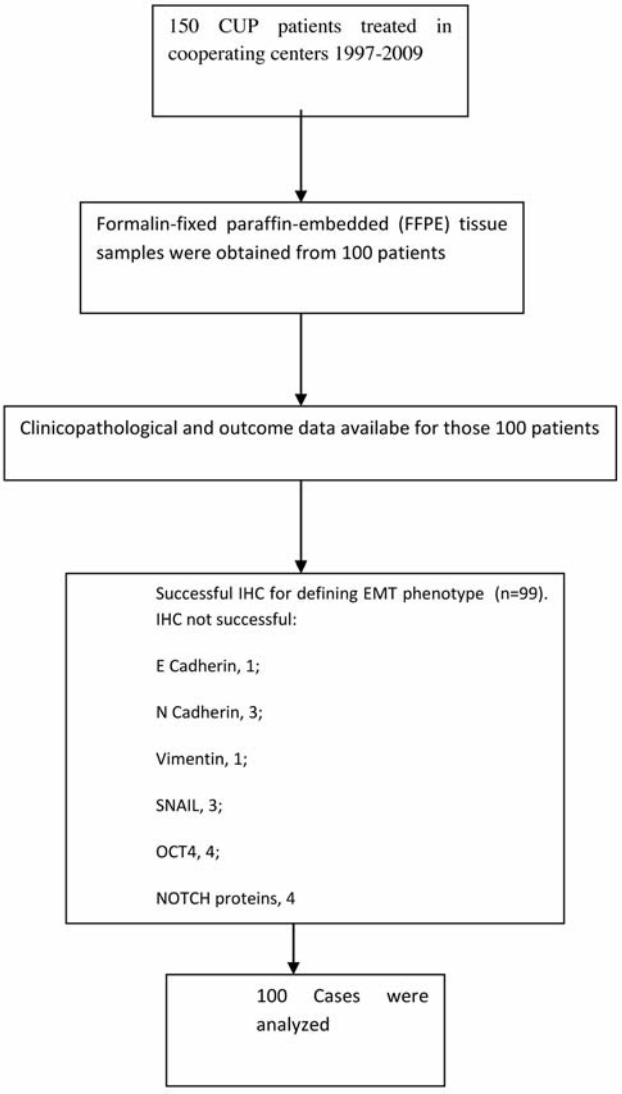

Figure 1. *REMARK flow chart*.

Tissue Microarrays. One hundred formalin-fixed paraffin-embedded (FFPE) tissue blocks from CUP were retrospectively identified and retrieved. Each case was centrally reviewed histologically by experienced pathologists (A.G., V.S.) in order to confirm the presence of tumour, the pathological diagnosis of an epithelial carcinoma and to rule out diagnoses of lymphoma, melanoma, germ cell and mesenchymal tumours. Metastatic deposits that were most accessible to biopsy were those sampled (liver, 16; lung, 9; lymph-nodes, 44; peritoneal surfaces, 20). Tissue cores as small as 1.5 mm were inserted in a recipient paraffin block in a precisely spaced, array pattern for tissue microarrays in the Pathological Department

Table I. *Patient and tumour characteristics*.

Parameter	No of cases
Gender	
Male/female	47/53
Age, years	
Median (range)	61 (35-85)
PS category	
PS 0-1 *vs*. 2	69/23
Missing data	8
Histology	
Adenocarcinoma	55
Squamous cell	20
Poorly differentiated/undifferentiated carcinoma	15
Undefined neoplasm	9
Grade	
Well-differentiated	7
Moderately differentiated	38
Poorly differentiated	46
Missing data	9
Number of metastatic organ sites	
1	51
2 or more sites	49
Subgroup	
Visceral	27
Axillary nodal	8
PC	20
Squamous cervical or inguinal	11
Nodal	25
Missing data	9
EMT positivity	
By % cells/By H-score	8/16
Chemotherapy administration	
Palliative chemotherapy	68
Platinum based	49
Response to chemotherapy	
CR or PR	40

PS: performance status, PC: peritoneal carcinomatosis, CR: complete response, PR: partial response.

of the Aristotle University of Thessaloniki. Two spots from each tumour with a thickness of 5 μm were mounted on each of the five master array slides, one for each biomarker under study. These five master arrays were sent to the Department of Pathology, Ioannina University Hospital, for immunohistochemical staining and evaluation. A REMARK flow diagram is provided in Figure 1(8).

Immunohistochemistry (IHC). The tissue slides were studied for IHC expression of the epithelial surface marker E-Cadherin; the mesenchymal surface markers N-cadherin and vimentin; the EMT-inducing transciption factor SNAIL and the stem cell marker OCT4 [OCT-4A, C52G3 rabbit monoclonal antibody; Cell Signaling, Danvers, MA, USA), SNAIL (rabbit polyclonal to SNAIL – aminoterminal end, ab70983; Abcam Cambridge, UK), N-cadherin (monoclonal mouse anti-N-cadherin, Clone 3B9; Invitrogen, NY, USA), E-cadherin (mouse anti-E-cadherin, Clone 4A2C7; Invitrogen, NY, USA) and VMN (mouse anti-vimentin, Clone V9; Invitrogen, NY, USA)]. Moreover, the sections were stained with antibodies

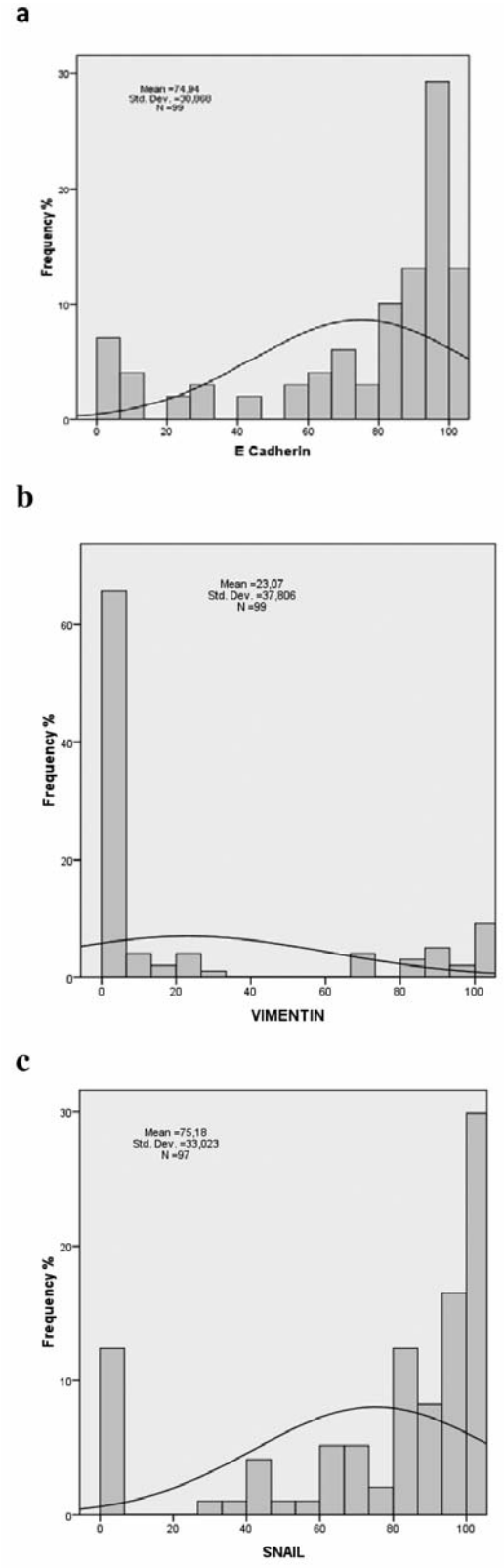

Figure 2. *Frequency histograms showing the distribution of (IHC) expression values (% of staining cells in each case) of E-cadherin (a), vimentin (b) and SNAIL (c).*

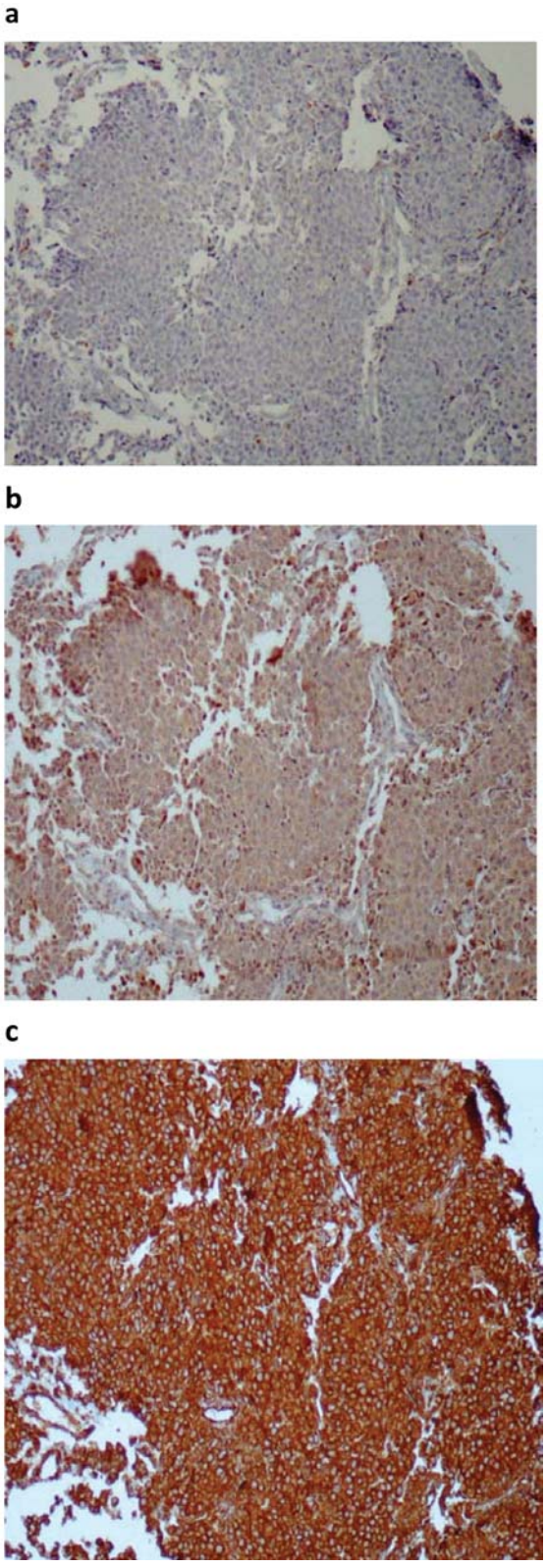

Figure 3. *A case of cancer of unknown primary with epithelial-mesenchymal transition phenotype by immunohistochemistry: a) low E-cadherin expression, b) high SNAIL expression, c) high vimentin expression.*

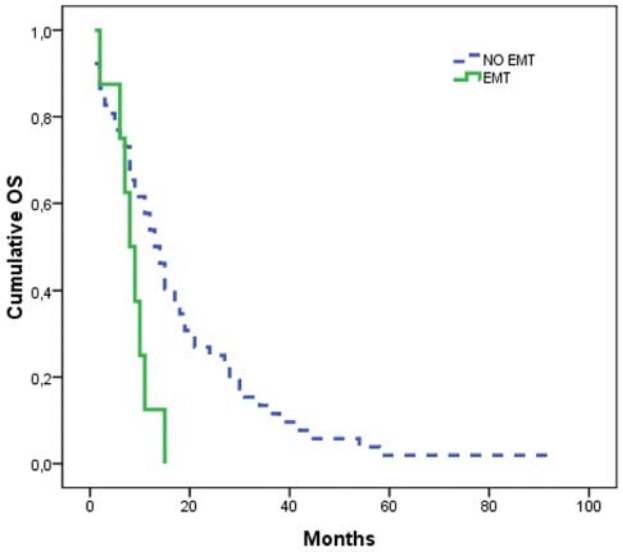

Figure 4. *Overall survival by tumour (EMT) status.*

against: NOTCH1 [anti-activated NOTCH1 (ab8925) at 1:300 dilution; Abcam, Cambridge, UK], NOTCH 2 [anti-activated NOTCH2 (ab72803) at 1:100 dilution; Abcam, Cambridge, UK], NOTCH3 [anti-NOTCH3 (ab23426) at 1:1600, Abcam, Cambridge, UK]. Five micrometers thick tissue sections of each TMA block were used. The automated slide processing system BenchMark XT IHC/ISH Staining Module (Ventana Medical Systems, Inc., Tucson, AE, USA) was used for optimization and performance evaluation of OCT-4A, SNAIL, NCADH, ECADH and VIM antibodies respectively. A protocol was established so that the entire assay procedure consisting of baking, deparaffinization, pretreatment, cell conditioning, titration, stringency wash, amplification, signal detection, counterstaining and post counterstaining was completed as a one-step fully automated assay (XT iVIEW DAB v3 procedure). Specifically, the slide processing system automatically performed the process starting with tissue deparaffinization followed by three cycles of cell conditioning (short 8 min conditioning, mild 30 min conditioning, standard 60 min conditioning). Titration took place by placing the primary antibody (OCT-4A dilution 1:50; SNAIL dilution 1:10; N-cadherin dilution 1:30; E-cadherin dilution 1:30; and vimentin dilution 1:30) and then incubation. Antibody amplification in several stages followed, and finally, counterstaining with 10% haematoxylin (4 min), application of bluing reagent, incubation for 4 min and coverslipping were performed.

Positive control samples included breast, colon, renal and thyroid carcinoma; normal placenta; and tonsil lymphoma; while for negative controls the primary antibody was omitted. The EMT phenotype was defined as partial loss of E-cadherin expression with simultaneous expression of N-cadherin/vimentin along with concomitant expression of SNAIL, as assessed by the percentage of staining tumour cells(9). Two experienced pathologists (A.G. and V.S.) examined the slides simultaneously using a double-headed light microscope with no prior knowledge of the clinical and pathological data. Positively stained inflammatory cells or areas of necrosis were excluded from counting. Complete clinicopathological and management data were electronically recorded for analysis.

Table II. *(IHC) expression data and epithelial-mesenchymal transition (EMT) phenotype.*

	E-Cadherin	N-Cadherin	Vimentin	SNAIL	OCT4	EMT positive
N	99	97	99	97	98	99
IHC						
Cut-off (% of stained cells)	≤60%	≥40%	≥40%	≥85%	≥1%	
Cases (%)	21 (21.2)	13 (13.4)	23 (23.2)	60 (61.9)	0	8 (8.1)
Median	90	0	0	90	0	
Interquartile range	65-95	0-10.38	0-25	65-100	0	
H-Score						
H Score cut-off	<140	>20	>20	>100		
Cases (%)	37 (37.4)	23 (23.5)	31 (31.3)	47 (48.5)	0	16 (16)
Median	180	0	0	100	0	
Interquartile range	67-278	0-18.5	0-55	66-200	0	

H Score (0-300): % of stained cells × intensity.

IHC Cut-offs. Since there is no validated scoring system for interpreting IHC staining for the biomolecules under study, and given the fact that there is no previous experience with these in CUP, IHC cut-off values were defined prospectively, based on distributional analyses in frequency histograms. In particular, IHC staining evaluation was based on the following cut-offs: partial loss of E-cadherin expression when staining was observed in <60% of tumour cells; positive cases for N-cadherin nd vimentin expression were those tumours in which staining was seen in ≥40% of tumour cells; positive by SNAIL expression was established when staining was seen in >80% of tumour cells; while positive cases for OCT4 were those with staining in at least 1% of tumour cells. For NOTCH protein expression, IHC staining was considered to be positive based on the following cut-offs: NOTCH1 staining in ≥5% of tumour cells, NOTCH2 >20%, NOTCH3 >80%. Subsequently, in order to avoid missing cut-off values with prognostic utility, we ran receiver-operating curve (ROC) analysis of IHC staining scores with death as the indicator parameter and failed to find any cut-off with significant sensitivity and specificity.

Statistics. Correlations among all markers under study were assessed using the Spearman correlation coefficient (Rho). The Kruskal-Wollis test was utilized as a non-parametric analog of (ANOVA). The univariate Cox proportional hazard regression model was applied to study prognostic significance of variables for survival and the χ^2 exact test for the predictive significance of variables for response to therapy. Progression-free survival (PFS) was calculated from diagnosis to disease progression or death; overall survival (OS) was calculated from diagnosis to date of death or last follow-up with the Kaplan-Meier product limit method and compared by means of the Log-rank test. All calculated *p*-values were two-sided and findings considered significant when $p<0.05$. Analyses were performed with the use of the SPSS 16.0 statistical software package (Chicago, Illinois, USA).

Results

Patient and tumour characteristics. The study population consisted of 100 patients diagnosed with CUP (47 males and 53 females), with a median age of 61 years and mostly fit performance status (PS 0-1 in 75%). Histological diagnosis was adenocarcinoma in the majority of the cases, while high histological grade was seen in 46%. Sixty-eight patients were managed with palliative chemotherapy, mostly platinum-based combination regimens (49). Detailed patient, tumour and management data are shown in Table I.

Table III. *Correlations of IHC expression (% of stained tumour cells) between various biomolecules in cancer of unknown primary. IHC staining intensity was considered a continuous variable and the non-parametrical Spearman's rho test was utilized in all cases.*

Spearmann Rho test	N-Cadherin	Vimentin	Snail
E-Cadherin	–0.246	–0.324	
	$p=0.015$	$p=0.001$	
Notch2			0.240
			$p=0.018$
Notch3		0.215	
		$p=0.036$	

IHC expression of biomolecules. Biomolecules with the most frequent IHC expression according to the cut-off values used were E-cadherin (78.8% of cases) and SNAIL (61.9% of cases). On the contrary, N-cadherin, vimentin and OCT4 were less frequently expressed. Of note, vimentin displayed a dichotomous IHC expression pattern, with most of the cases being either negative or fully positive. Subcellular localisation of staining was membrane and cytoplasmic for E-cadherin, N-cadherin and vimentin; cytoplasmic and nuclear for SNAIL; and nuclear for OCT4. The distribution frequency histograms of E-cadherin, vimentin and SNAIL IHC expression (% of staining tumour cells in each case) in all CUP cases are shown in Figure 2. Among patients studied, 21 (21.2%) had low E-cadherin expression, whereas 13 (13.8%) had positive expression of N-cadherin, 23 (23.2%) of vimentin and 60 (61.9%) of SNAIL. No cases positively stained for OCT4. When we looked at the number

Table IV. *Correlation with clinicopathological factors of epithelial to mesenchymal transition phenotype.*

	EMT		
	Present	Absent	*p*-Value
Characteristic n(90)			
Gender			
Male	8 (17.4)	38 (82.6)	0.002
Female	0 (0)	52 (100)	
Grade			
High	6 (13.6)	38 (86.4)	0.05
Low	2 (4.5)	42 (95.5)	
Metastasis			
Visceral	2 (13.3)	13 (86.7)	0.05
Non-visceral	3 (5.1)	55 (94.9)	
Responder/non responder (%)	52.9%/47.1%	50%/50%	0.893
PFS (months)			
Median	5	8	0.112
95% CI	3.000-7.000	5.311-10.689	
Survival (months)			
Median	8	13	0.023
95% CI	5.226-10.772	9.859-16.141	
1-year (%)	0	75	
By H-SCORE			
PFS			
Median (months)	5	8	0.541
95% CI	5.658-6.342	5.415-10.585	
Survival			
Median	9	14	0.074
95% CI	7.281-10.719	10.648-17.352	
1-Year (%)	7.7	72.3	

PFS: Progression-free survival, CI: confidence interval.

Table V. *Clinicopathological prognostic factors.*

	1-Year OS (%)	Median OS (months)	95% CI	*p*-Value
Chemotherapy				
Non-platinum based	45.8	7	4.13-9.87	0.029
Platinum-based	66.7	15	12.1-17.9	
Gender				
Male	43.3	9	6.3-11.68	0.0005
Female	64.5	17	8.27-25.73	
PS				
PS 0-1	70	14	10.9-17.1	0.001
PS≥2	42.9	5	0.51-9.5	

PS: Performance status.

of CUP cases that fulfilled the IHC definition of EMT as partial loss of E-cadherin with expression of N-cadherin, vimentin or both and expression of the SNAIL transcription factor, only 8 patients (8.1%) fulfilled this criterion. A representative CUP tumour with the EMT IHC phenotype is shown in Figure 3.

In view of the lack of data on the optimal way to assess IHC expression of EMT markers, we sought to explore a semi-quantitative method of IHC biomolecule staining, used previously in other studies (10). In addition to counting the percentage of staining cells, we also evaluated the staining intensity and derived the H-score.

We applied distributional analyses based on frequency histograms in order to select natural cut offs for H Score values for each biomolecule, resulting in slightly different data on the incidence of EMT IHC Phenotype. Thirty seven patients (37.4%) had down-regulation of E-cadherin, whereas 23 (23.5%), 31 (31.3%), 47 (48.5%) and 0 had positive expression of N-cadherin, vimentin, SNAIL and OCT4, respectively. Accordingly, the EMT phenotype based on the IHC H-Score was noted in 16 patients (16%). When we compared the EMT-positive cases based on the percentage of staining cells *versus* the H-Score evaluation, we observed that the additional eight cases with tumour EMT phenotype by H-score, consisted of carcinomas with loss of E-cadherin expression with intense (2+/3+) staining of N-cadherin and vimentin staining in less than 40% of tumour cells per case.

Correlation of IHC EMT phenotype with other clinicopathological characteristics. The correlations of the EMT phenotype with standard clinicopathological factors are shown in Table II. In general, the EMT phenotype was significantly associated with tumour characteristics with adverse prognostic impact such as male gender, high histological grade and presence of visceral metastases. Specifically, 17.4% of male patients had EMT-positive tumours whereas none of the women had tumours with EMT characteristics (χ^2 p=0.002). Furthermore, only 4.5% of well- to moderately-differentiated tumours were of EMT phenotype (χ^2 p=0.05) compared with 13.6% of grade 3 lesions. EMT was also correlated with localization of metastases in visceral sites. A total of a 13.3% of cases with visceral metastases were EMT-positive *versus* only 0-7% of those with non-visceral deposits (χ^2 p=0.05).

We investigated whether the expression of mesenchymal markers, the loss of the epithelial marker E-cadherin and the expression of the orchestrator of EMT, the transcription factor SNAIL, were related to each other as continuous variables. The inverse correlation of E-cadherin with N-cadherin (Spearman's Rho correlation coefficient=–0.246, p=0.015) as well as with vimentin (Spearman's Rho correlation coefficient=–0.324, p=0.001) were statistically significant. Moreover, they confirmed the biological plausibility of our observations, as the appearance of N-cadherin and vimentin in the cell surface and cytoplasm of tumour cells went hand-in-hand with the down-regulation of E-cadherin epithelial marker, as reported in EMT.

In the context of a broader research project, in profiling the activity of several signalling pathways in CUP, we observed a statistically significant association of NOTCH receptor IHC expression with that of specific EMT biomarkers. However, this was an unplanned, post hoc analysis and results should be interpreted cautiously. In regards to high SNAIL expression cases, 33% were with low NOTCH2 expressing tumours, while 67% were seen with high NOTCH2 expressing tumours (χ^2 p=0.012, low NOTCH2 expression defined as staining in <20% of tumour cells). Moreover, vimentin expression status was significantly associated with NOTCH3 expression (cut-off of staining in 80% or more of tumour cells). In fact, on cases with high vimentin expression, only 9.5% were seen in low NOTCH3-expressing tumours while 90.5% were seen in those with high NOTCH3 expression (χ^2 p=0.05). The induction of SNAIL by NOTCH2 and the induction of vimentin by NOTCH3 were held as a statistically significant correlation, even when the biomolecules were analysed as continuous variables (p<0.05, as shown in Table III).

Prognostic and predictive utility. At the time of the analysis (June 2011), the median follow-up time was 40 months. During this time, 47 patients (47%) had developed a relapse and 61 patients (61%) had died. The median PFS was 7 months (95% confidence intervals CI=4.761-9.239) while the median OS was 12 months (95% CI=8.723-15.276).

Malignant relapse occurred in 6 patients and death in all 8 patients with an EMT-positive phenotype *versus* 51 relapses and 54 deaths in the 92 patients harbouring non-EMT tumours, respectively. The Kaplan-Meier survival curves representing the probability of survival as a function of EMT phenotype are presented in Figure 4. Patients affected by EMT-positive tumours showed a trend towards an earlier relapse as they had a median PFS of 5 (95% CI=3.0-7.0) months *versus* a median PFS of 8 (95%CI=5.31-10.69) months for those with EMT-negative tumours (log-rank p=0.112). The difference in OS reached statistical significance as EMT-positive tumour patients reached a median OS of 8 (95% CI=5.226-10.772) months with none of them surviving for 1 year, while patients with EMT-negative tumours had a median OS of 13 (95%CI=9.859-16.141, log-rank p=0.023) months and a 1-year survival rate of 75%. The EMT phenotype did not seem to possess any predictive utility for response to chemotherapy, as the ratio of responders to non-responders was similar (approximately 50%/50%) both in the EMT and the non-EMT groups (χ^2 p=0.893). Prognostic analyses are summarized in Table IV.

As an exploratory, hypothesis-generating analysis, we investigated whether the tumoural EMT phenotype carried prognostic significance specifically in clinicopathological subgroups of CUP. Indeed, the CUP subgroup of patients with midline nodal metastases with EMT-negative tumours had a median OS of 14 (95% CI=12.3-15.6) months *versus* only 10 (95% CI=8.4-11.6) months for those with EMT-positive tumours (log-rank p=0.039). Moreover, patients in the subgroup of peritoneal carcinomatosis had numerically higher median OS (21 months) in the absence of EMT *versus* a median OS of only 6 months in the presence of EMT, although the difference was not statistically significant (log-rank p=0.23). In view of the small sample size of these subgroups and the multiple analyses run, it is not certain whether the adverse prognostic utility of EMT in CUP subgroups of nodal and peritoneal carcinomatosis reflects a genuine biological phenomenon or is a random event.

We sought to investigate whether the use of the H-Score for the definition of EMT improves its prognostic utility. This hypothesis seemed plausible, as the EMT H-Score incorporates staining intensity of the examined biomolecules and results in an increase of the number of EMT-positive cases. Survival analysis depending on the EMT by H-Score showed comparable results with prior analyses but not improved prognostic power. Patients with EMT-positive tumours by H-Score reached a median OS of 9 (95% CI=7.281-10.719) months *versus* 14 (95% CI=10.648-17.352) months for those with EMT-negative tumours (p=0.074, Table IV).

Univariate Cox regression analysis showed a non-significant trend for increased risk of death in patients affected by tumours with adverse clinicopathological features such as advanced age, of grade 3 and with multiple metastases upon presentation, specifically visceral (data not shown) (Table V).

Multivariate analysis. Multivariate Cox regression analysis including age, gender, performance status category (PS 0-1 *versus* PS 2 or more), CUP subgroup (visceral *versus* peritoneal *versus* midline nodal metastases), number of metastatic sites (1-2 *versus* 3 or more), platinum-based treatment and presence or absence of the EMT phenotype revealed that only female gender, PS category and platinum-based therapy remained significant independent prognosticators of OS. More specifically, the female gender (male sex hazard ratio HR=2.90, 95% CI=1.520-5.541, p=0.001), PS 0-1 (HR=0.455, 95% CI=0.252-0.821, p=0.009) and treatment with platinum salts (HR=01.749, 95% CI=1.005-3.046, p=0.048), were assosiated with decreased risk of death. However, the relatively small sample size warrants attention in the interpretation of multivariate regression output that can be due to random statistical effects.

Discussion

CUP is a model disease for early dissemination of the primary tumour to distant sites. The metastatic spread occurs early during the disease course before the primary reaches a detectable size, produces a high tumour burden and is related to resistance to antineoplastic therapies. Consequently, we considered it biologically relevant and appropriate to

investigate the incidence, correlations and prognostic utility in CUP of one of the pivotal phenomena implicated in metastatic dissemination, the EMT. EMT occurs during embryonic morphogenesis in multicellular organisms, in which embryonic mesenchymal cells are formed and become motile following the loss of epithelial cell polarity. In recent years, EMT has also been recognised as a potential mechanism for cancer progression (9). During progression to metastatic competence, carcinoma cells acquire altered adhesive properties, activate proteolysis and motility programmes, metastasize and establish deposits at distant sites (10, 11).

A central event in EMT is the down-regulation of E-cadherin, which leads to the loss of cell–cell contact and the consecutive progression of cells towards a mesenchymal-like phenotype with up-regulation of N-cadherin and vimentin. The transcription factor SNAIL is a major suppressor of E-cadherin and a strong inducer of EMT. SNAIL has been shown to down-regulate E-cadherin in cell cultures, transfection and knock-out experiments in transgenic mice and in different types of human tumours, *e.g.*, hepatocellular carcinomas (12), carcinoma from the esophagus, cardia and stomach (13), and colorectal carcinomas (14, 15). Expression of SNAIL in epithelial tumours increases their aggressiveness, as seen in experimentally induced breast tumours. Moreover, high SNAIL expression correlated with an increased risk of tumour relapse and poor survival rates in human breast and gastric cancer(16). Recently, EMT has been linked to the acquisition of a stem cell-like phenotype as seminal *in vitro* experiments showed that epithelial cells induced to undergo EMT become spindle shaped, evade apoptosis and anoikis-related death, express a battery of stemness markers and have the ability to create colonies and mammospheres that re-acquire the cellular heterogeneity of the initial tumour from which they were harvested (17).

Our retrospective analysis of 100 patients with CUP incorporated IHC staining of TMAs, examining two cores for each patient for specific EMT biomarkers in order to minimize the impact of random sampling from tumour areas with unique characteristics. The combination of E-cadherin, N-cadherin, vimentin and SNAIL was selected in order to define a more complex but also more secure definition of the EMT phenotype, whereas OCT4 was selected in order to explore its relation to stem-like features. We prospectively selected one cut-off value only, for each biomarker, based on distributional and ROC analyses and taking into account the relevant literature rather than performing multiple, exploratory analyses using multiple cut-offs. Moreover, when possible, we analysed correlations of biomolecules by examining them as continuous variables rather than as categorical ones. Despite these precautions, it is uknown if the IHC cut-offs selected represent those with genuine biologic relevance, and their validation in independence, larger cohorts of patients with CUP is warranted.

Despite methodological limitations, common in such novel research projects, we showed that EMT markers have an inverse relation to epithelial markers in cells from CUP, that EMT is correlated to adverse clinicopathological characteristics (male gender, anaplasia, visceral metastases) and that it carries a strong adverse prognostic signifance for patient survival. Moreover, we demonstrated a possibly causal role of NOTCH2 and 3 activation towards induction of the EMT cellular phenotype, as already reported by others (18, 19). Consequently, the EMT phenotype demonstrated here could be proven to be a prognostic marker for patient risk classification and a therapeutic target for modulation with smart drugs.

Our findings concerning the adverse prognostic impact of tumoural EMT in patients with CUP seem to provide further evidence to the already established knowledge of the dismal prognosis conferred by EMT in known primary tumours. Overexpression of the EMT-inducing transcription factors SNAIL, SLUG, TWIST and ZEB1 and 2 in combination with suppression of E-cadherin significantly correlated with poor prognosis in patients with breast, ovarian and uterine cancer through promoting aggressive tumour characteristics, nodal metastasis and invasion (20-23). However, despite these robust differences in survival according to EMT status, it is indisputable that the EMT phenotype is quite rare in our group of patients, with an incidence of only 8% based on staining cell percentage and 16% based on the H-Score. Amongst hypotheses that could interpret this finding, we could cite protein degradation in the tumour block by inadequate fixation, problematic antigen retrieval or even protein staining underestimation in old archival material derived from biopsied metastases. Another factor limiting the EMT rate may be that TMAs were obtained from the centre of the tumour in contrast to recent studies suggesting that EMT processes take place at the tumour periphery, the invasive front (24, 25). Finally, the complexity of the signalling networks that regulate the induction of EMT and the reversibility of the acquired mesenchymal phenotype also pose significant challenges. If systemic dissemination and EMT occur at early stages of tumour development, as suggested by recent studies, then at the time of diagnosis, it may already be too late to identify such an unstable phenomenon (26).

To conclude, our work is the first to examine EMT in a model disease for metastatic dissemination such as CUP and the first that established EMT as having an adverse prognostic significance for patient outcome. Studies of larger and more homogeneous populations of patients with CUP are warranted in order to accumulate more data on the molecular mechanisms underlying EMT, solve methodological issues and confirm the importance of this process in cancer. Our group is currently studying the gene expression of biomarkers of EMT in tumour subpopulations by selectively extracting mRNA from the centre and the invasive front of tumours. Such studies may pave the way for promising therapeutic strategies for the prevention of metastasis, the fatal consequence of tumorigenesis.

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Conflict of Interest

None declared.

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