Subcellular Localization of Activated Leukocyte Cell Adhesion Molecule Is a Molecular Predictor of Survival in Ovarian Carcinoma Patients

Delia Mezzanzanica,[1] Marina Fabbi,[6] Marina Bagnoli,[1] Samantha Staurengo,[2] Marco Losa,[2] Emanuela Balladore,[1] Paola Alberti,[1] Lara Lusa,[1,4] Antonino Ditto,[3] Silvano Ferrini,[6] Marco A. Pierotti,[4,5] Mattia Barbareschi,[7] Silvana Pilotti,[2] and Silvana Canevari[1]

Abstract

Purpose: Currently available clinicopathologic prognostic factors are imperfect predictors of clinical course in advanced-stage epithelial ovarian cancer patients. New molecular predictors are needed to identify patients with higher risk of relapse or death from disease. In a retrospective study, we investigated the prognostic impact of activated leukocyte cell adhesion molecule (ALCAM) expression in epithelial ovarian cancer.

Experimental Design: We analyzed the effect of cell-anchorage loss on ALCAM cellular localization *in vitro* and assessed ALCAM expression by immunohistochemistry in a series of 109 well-characterized epithelial ovarian cancer patient samples. Chi-square test, Kaplan-Meier method, and Cox proportional hazard analyses were used to relate ALCAM cellular localization to clinical-pathologic parameters and to overall survival (OS) rate.

Results: Loss of epithelial ovarian cancer cell anchorage was associated both *in vitro* and *in vivo* with decreased ALCAM membrane expression. *In vivo*, ALCAM was localized to cell membrane in normal surface ovarian epithelium, whereas in 67% of the epithelial ovarian cancer samples, membrane localization was decreased or even lost, and the molecule was mainly expressed in cytoplasm. Median OS in this group of patients was 58 months, whereas a median OS was not yet reached in patients with ALCAM membrane localization ($P = 0.036$, hazard ratio [HR] = 2.0, 95% confidence interval [CI] 1.1 to 3.5). In a multivariate Cox regression model including all the available clinicopathologic variables, loss of ALCAM membrane expression was an independent factor of unfavorable prognosis ($P = 0.042$, HR = 2.15, 95% CI: 1.0 to 4.5).

Conclusions: Decreased/lost ALCAM membrane expression is a marker of poorer outcome in epithelial ovarian cancer patients and might help to identify patients who could benefit from more frequent follow-up or alternative therapeutic modalities.

Epithelial ovarian cancer is the fifth leading cause of cancer-related death in women in Western countries and is the leading cause of gynecologic cancer death (1). Epithelial ovarian cancer is characterized by only a few early symptoms, presentation at an advanced stage, and poor survival. The high mortality rate reflects the difficulty in the early detection of epithelial ovarian cancer, the frequency of tumor relapses, and the development of resistance to chemotherapy despite high initial response rates. About 80% of patients are diagnosed with advanced stage disease (2). The clinical course of advanced disease is difficult to predict because currently available clinical-pathologic prognostic factors are imperfect predictors and do not provide insights into biologic mechanisms underlying the clinical behavior (3). The heterogeneity of clinical outcomes in patients with advanced epithelial ovarian cancer points to the need for accurate prognostic factors that can identify patients who, despite initial complete response, are likely to relapse and die of disease and thus might be appropriate candidates for new therapeutic approaches.

Recent efforts to develop accurate predictors of clinical outcome have focused on techniques such as cDNA microarrays to assess global gene expression. This technology has provided a wealth of data on differential gene expression in a number of tumors, including ovarian cancer (4, 5) and has identified large sets of dysregulated genes and profiles associated with early relapse (6) or patient survival (7, 8). Using cDNA microarrays,

Authors' Affiliations: Departments of [1]Experimental Oncology and Laboratories, [2]Pathology, and [3]Surgery, Fondazione IRCCS Istituto Nazionale dei Tumori, [4]Molecular Genetics of Cancer, Fondazione Istituto FIRC di Oncologia Molecolare (IFOM), Milan, Italy, [5]Fondazione IRCCS Istituto Nazionale dei Tumori, [6]Department of Translational Oncology, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy, and [7]Department of Pathology, S. Chiara Hospital, Trento, Italy

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Note: D. Mezzanzanica and M. Fabbi contributed equally to this work.

Requests for reprints: Silvana Canevari, Unit of Molecular Therapies, Department of Experimental Oncology and Laboratories, Fondazione IRCCS Istituto Nazionale dei Tumori, Via Venezian 1, 20133 Milan, Italy. Phone: 39-02-23902567; Fax: 39-02-23903073; E-mail: silvana.canevari@istitutotumori.mi.it.

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we recently identified (9) a molecular signature on a subset of epithelial ovarian cancer characterized by numerous genes related to the extracellular matrix and its remodeling and to elements of the fibroblast growth factor 2 signaling pathway. These observations suggested a complex modulation network at the level of fibroblast growth factor receptor signaling functions, in which adhesion molecules such as members of the immunoglobulin-like cell adhesion molecule (Ig-CAM) family might play a relevant role. Ig-CAMs have been implicated in tissue morphogenesis and in progression of tumors other than epithelial ovarian cancer (10).

Activated leukocyte cell adhesion molecule (ALCAM or CD166) is a member of the Ig superfamily, with five extracellular Ig-like domains that promote heterophilic (ALCAM-CD6) and homophilic (ALCAM-ALCAM) cell-cell interactions. Its pattern of expression in human tissues and cells is broad, including epithelia, neurons, lymphoid and myeloid cells, as well as hematopoietic and mesenchymal stem cells. Developmental biology studies suggest the involvement of ALCAM-dependent cell adhesion in cell migration and guided outgrowth in neurogenesis, in hematopoiesis, and in immune responses (11, 12). Altered ALCAM expression has also been associated with differentiation state and progression in melanoma (13, 14), prostate (15, 16), colorectal (17), and breast (18) cancers.

We have previously shown that ALCAM is expressed at high levels on the membrane of human ovary carcinoma cell lines and that it can be endocytosed and recycled back to the cell surface (19). Furthermore, the ligand-triggered internalization of the ALCAM molecule was maximal at the cleavage furrow during cytokinesis (19), suggesting a role for this molecule in rearrangement of cell-cell contacts. We also recently showed that ALCAM ectodomain can be cleaved from epithelial ovarian cancer cell surface and that this process is involved in epithelial ovarian cancer cell motility (20). Here we provide evidence that loss of epithelial ovarian cancer cell anchorage is accompanied with a loss of ALCAM expression at membrane level in both cell line and primary ovarian tumor cells derived from ascitic fluid of advanced-stage epithelial ovarian cancer patients. We therefore evaluated by immunohistochemistry, the prognostic value of ALCAM expression on tumor samples from a series of epithelial ovarian cancer patients with known clinical history, by correlating the reduction/loss of membrane expression with clinicopathologic variables and patient survival.

Materials and Methods

Study subjects. We performed the study on archival formalin-fixed, paraffin-embedded material collected at surgery, from 109 patients with primary epithelial ovarian cancer who underwent surgical resection at the Fondazione IRCCS Istituto Nazionale dei Tumori (INT) between 1990 and 2001 and at the S. Chiara Hospital in Trento between 1992 and 1999. All histologic sections and paraffin blocks were obtained from the Departments of Pathology of both institutes. Pathologists (SP and MB) with specialized expertise in gynecological pathology reviewed all pathologic data. All clinical data and follow-up information were available from the Units of Gynecologic Oncology of both institutes.

Clinicopathologic characteristics of patients are summarized in Table 1. Patients' ages ranged from 25 to 84 years, with an average age of 55.7 years. Tumor staging was in accordance with FIGO criteria; 36 patients with disease at clinical stage I to II and 73 patients with disease at clinical stage III to IV were included in this study. Residual tumor size ranged from 0 to 10 cm; according to the extent of residual disease after primary surgery, the patient population was divided into two groups: 0 to 1 cm (optimal debulking) and >1 cm (suboptimal debulking) (ref. 3). Seven patients with a residual tumor >1 cm but <2 cm were included in the suboptimally debulked group. Presence of malignant tumor cells in peritoneal fluids (ascites or washing) was recorded for 72 stage III to IV patients. After surgery, 101 patients received a front-line treatment, with platinum-based therapeutic schedules according to the time of accrual and institutional involvement in international trials (43 patients were treated with platinum alone, 43 with the standard platinum-paclitaxel combination, and 15, selected for suboptimal debulking, were treated with platinum-paclitaxel and topotecan). Follow-up time was based on patient date of death or the last information provided in the medical records. The median of follow-up period for all patients was 48 months (range, 7-120 months). Overall survival (OS) was defined as the time interval between the date of surgery and the date of death; 52 patients (48%) had died and all but one observed death was cancer related. The Institutional Review Boards approved the use of tissue blocks and patient records.

Isolation of tumor cells from ascitic fluid. Data are available only for stage III to IV patients. We collected ascitic fluids during surgical procedures from patients undergoing debulking surgery at INT at the first diagnosis and not previously treated with chemotherapeutic regimens. All clinical specimens used for this study were obtained with Institutional Review Board approval from patients who gave informed consent to use leftover biological material for investigative purposes. Tumor cells were isolated as described (21). After separation from nontumor adherent cells, cell population growing in suspension (tumor cell clumps) was recovered, formalin fixed, and paraffin embedded. Slides were processed and stained for ALCAM detection as described below.

In vitro *epithelial ovarian cancer cell detachment from substrate.* Human serous ovarian carcinoma cell line A2774 (J. Bénard, Institute Gustave Roussy, Villejuif, France), which expresses ALCAM at the membrane level (19), was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine and streptomycin (100 μg/mL), in a humidified atmosphere of 5% CO_2 at 37°C and routinely tested for mycoplasma infection using the mycoplasma PCR ELISA kit (Roche, Basel, Switzerland). For cell detachment studies, A2774 cells were seeded (10^5 per well) on cover glasses in 24-well tissue culture plates. Confluent cells were treated with pervanadate, which mimics hyperactivation of tyrosine kinases by inhibiting tyrosine phosphatase activity. Pervanadate (200 μM) was freshly prepared by mixing stock solution of sodium vanadate (1 M) with H_2O_2 (1 M) and used within 20 minutes of preparation (22). Sodium vanadate or H_2O_2 alone was used as controls. After 50 minutes of treatment, monolayers were washed once with phosphate buffer saline (PBS), fixed for 5 minutes on ice with 4% paraformaldehyde in PBS, pH 7.4, and permeabilized with 0.5% Triton X-100 in PBS. For analysis of cells in suspension, A2774 cells were harvested after treatment with 0.5 mM EDTA in PBS, and cell pellets were formalin fixed and paraffin embedded according to standard techniques. Sections (5-μm thick) were deparaffinized, rehydrated, and treated as described below for tissue samples.

Immunohistochemistry. ALCAM localization was examined by immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded sections from epithelial ovarian cancer using the UltraVision LP detection system HRP polymer (Lab Vision Corporation, Fremont, CA) according to manufacturer's instructions. Briefly, after xylene deparaffinization and alcohol rehydration, sections were subjected to antigen retrieval in 10 mM, pH 6.0, citrate buffer at 95°C for 6 minutes in autoclave. Endogenous peroxidase was quenched by incubating the slide with 3% H_2O_2 for 10 minutes. After washing, slides were incubated in blocking solution (Ultra V Block, Lab Vision Corporation) for 10 minutes, followed by 1-hour incubation at room temperature with primary antibody mouse monoclonal anti-ALCAM (NovoCastra Laboratories) at a 1:80 dilution. After washing, slides were incubated

Table 1. Clinical and pathologic characteristics of epithelial ovarian cancer patients and their association to ALCAM expression as assessed by immunohistochemistry

	No. of cases	ALCAM-cytoplasm	ALCAM-membrane*	P †
Total	109	73	36	
Age, years ‡				0.18
<mean	52	31	21	
≥mean	57	42	15	
Stage				0.91
I	21	13	8	
II	15	10	5	
III	58	39	19	
IV	15	11	4	
Histotype				0.25
Serous	62	41	21	
Undifferentiated	12	11	1	
Endometrioid	18	12	6	
Clear cells	10	6	4	
Others	7	3	4	
Grade				0.08
1	8	3	5	
2	38	24	14	
3	50	34	16	
Undifferentiated	12	11	1	
Not available	1	1	0	
Surgical debulking				0.54
Optimal	51	32	19	
Suboptimal	57	40	17	
Not available	1	1	0	
Malignant tumor cells in peritoneal fluid§				0.60
Present	50	35	15	
Absent or rare	22	14	8	
Not available	1	1	0	

*Membrane expression was defined in samples with a homogeneous membrane staining in at least 80% of the cells.
† *P* values are determined using Chi-square or Fisher's exact test.
‡ Median age of all cases was 56 years; mean age was 55.7 years (range, 25-84 years).
§ Malignant tumor cells: Malignant tumor cells evaluated only for stage III to IV patients.

for 30 minutes at room temperature with Primary Antibody Enhancer (Lab Vision Corporation) and then washed and incubated with HRP Polymer (Lab Vision Corporation) for 30 minutes. The peroxidase reaction was developed with 3,3-diaminobenzidine (Dako S.p.A, Milan, Italy), and sections were counterstained with hematoxylin. Slides incubated with Primary Antibody Enhancer alone provided negative controls.

Staining was recorded by a subjective grading system, considering both staining localization (membranous versus cytoplasmic) and the proportion of cells showing a membrane-positive reaction, because the physiological ALCAM localization is at the cell membrane. In our case material, membrane staining was always associated with cytoplasmic staining. Staining was defined as membranous when at least 80% of the cells maintained homogeneous membrane staining; in all other cases (heterogeneous membrane staining associated with cytoplasmic staining or cytoplasmic staining alone), ALCAM was defined as cytoplasmic. Two independent observers blinded to patient characteristics and outcome evaluated the slides. All cases with discrepant evaluations were discussed during observation with a double-headed microscope, and a consensus was reached.

Statistical methods and data analysis. For statistical analyses, patients were grouped based on similar clinicopathologic parameters (Tables 1-3). Age was used as a categorical variable, and patients were categorized in two classes, below or above the observed mean age value. Chi-square test was used to assess the association of ALCAM localization to the other clinical and pathologic variables.

The Kaplan-Meier method was used to estimate OS curves, and differences between survival curves were assessed with the log-rank test for univariate analysis. For univariate and multivariable analyses, histotype was categorized as serous versus all other tumors. Association of ALCAM with OS was evaluated also with a multivariable Cox proportional hazards regression model in which we included, in addition to ALCAM, all the available clinicopathologic variables and the type of treatment. Covariates that were not statistically significantly associated with OS were not removed from the models (complete model). The *P* values of all statistical tests were two-sided. For all analyses, differences were considered significant at *P* values ≤ 0.05. All analyses were carried out using R statistical language[8] (ref. 23).

Results

Loss of cell anchorage induces ALCAM subcellular relocalization. We previously showed that ALCAM is expressed at the cell surface of epithelial ovarian cancer cell lines and that ALCAM can be internalized upon ligand binding (19). Furthermore, we have evidence that ALCAM is released from epithelial ovarian cancer cells by a metalloprotease-dependent mechanism and that a perturbation of ALCAM-ligand interaction is relevant for epithelial ovarian cancer motility (20). To assess whether a subcellular distribution of ALCAM might be linked to cell detachment from substrate, A2774 epithelial ovarian cancer confluent cell monolayers were treated with

[8] http://www.R-project.org

pervanadate, known to induce the rounding of cells previously attached to culture dishes (24), or with sodium vanadate or H_2O_2 as controls. As shown in Fig. 1, a 50-minute treatment of A2774 cell monolayer with sodium vanadate did not affect cell shape or ALCAM membrane staining, as assessed by IHC (Fig. 1A). On the contrary, pervanadate treatment, which mimics tyrosine kinase activation, resulted in a progressive detachment from the culture dish and rounding of cells, accompanied by a dramatic redistribution of ALCAM localization from the membrane to the cytoplasm, with membrane staining limited to cell-to-cell contact areas (Fig. 1B). Moreover, in A2774 cells completely detached by using a PBS/EDTA solution, which fully preserves cell viability, ALCAM staining was predominantly cytoplasmic (Fig. 1C). In cells that "naturally" lost the substrate anchorage *in vivo*, such as cells recovered from three different epithelial ovarian cancer patients' ascitic fluids, rich in floating cell clumps, the same distribution of ALCAM was observed; ALCAM was localized mainly at the cytoplasmic level, with membrane staining localized only at cell-cell contacts (Fig. 1D).

In epithelial ovarian cancer, ALCAM is mainly localized to cytoplasm. Because the loss of cell anchorage to substrate was associated with a relocalization of the ALCAM molecule from cell membrane to cytoplasm, ALCAM localization was evaluated by IHC on specimens containing normal ovarian surface epithelial (OSE) cells and on archival material from 109 epithelial ovarian cancer patients with known clinical history. IHC showed ALCAM clearly localized at the membrane level in the single layer of OSE (a representative example in Fig. 2A), whereas the majority of tumor samples revealed a cytoplasmic localization of the molecule with a loss of membrane staining. In particular, in 73 samples (67%), the staining was mainly cytoplasmic with a heterogeneous membrane localization (Fig. 2B: cytoplasmic localization), and in 36 samples, the cytoplasmic staining was also associated with a homogeneous membrane staining in at least 80% of the cells (Fig. 2C: membrane localization).

ALCAM expression with respect to associated clinical parameters. Table 1 lists the clinical and pathologic characteristics of the patients and the ALCAM localization results. The average age of all patients was 55.7 years, and 52% of the patients were older than the mean. The series included 19% stage I, 14% stage II, 53% stage III, and 14% stage IV patients. The tumors included serous papillary (57%), undifferentiated (11%), endometrioid (17%), clear cell (9%), and other (6%) cases. Fifty-seven percent of the cases were high-grade or undifferentiated tumors. Following initial surgical debulking, residual disease by size was defined as absent or less than 1 cm in 47% of the cases (optimal debulking) and greater than 1 cm in 52% (suboptimal debulking). In 29% of stage III to IV patients, peritoneal fluids recovered at surgery contained only rare or no malignant tumor cells. ALCAM cellular localization was not associated with any of the analyzed clinicopathologic variables (Table 1).

ALCAM expression and patient survival. As expected for epithelial ovarian cancer, known clinical prognostic factors such as stage of disease, level of surgical debulking, and age at diagnosis showed a statistically significant association with OS in univariate survival analysis (log-rank test $P < 0.05$, Table 2). A statistically significant difference in OS was also observed in advanced-stage patients for the presence of malignant tumor cells in peritoneal fluids (Table 2). In addition, ALCAM subcellular localization showed a statistically significant difference in OS, with cytoplasmic localization associated with worse prognosis (HR = 2.0, 95% CI = 1.1 to 3.5, $P = 0.036$) (Table 2). Figure 3 shows OS curves estimated by the Kaplan-Meier method, stratified by ALCAM subcellular localization. Median OS for patients showing a cytoplasmic ALCAM localization was 58 months, whereas the median survival time for patients with membrane localization of the molecule could not be estimated because more than half of these patients were alive at the end of the study.

A multivariable Cox regression model was fitted on the whole case series, including all the available clinicopathologic variables, treatment, and ALCAM subcellular localization. The variable relative to the presence of malignant tumor cells in peritoneal fluid was not included in the model because this information was available only for stage III to IV patients. In this model, in addition to the expected clinicopathologic variables (level of surgical debulking and tumor stage) the type of treatment and ALCAM localization were also independent prognostic factors for OS ($P < 0.05$, Table 3). Patients with ALCAM localization in the cytoplasm had a statistically significant higher risk of death than patients with ALCAM localized on the membrane (HR = 2.15, 95% CI = 1.0 to 4.5, $P = 0.042$). This result confirms the prognostic impact of ALCAM subcellular localization even after adjusting for standard clinical and pathologic covariates and even considering only the subgroup of 73 advanced-stage patients, in which we could adjust the analysis also for the presence of

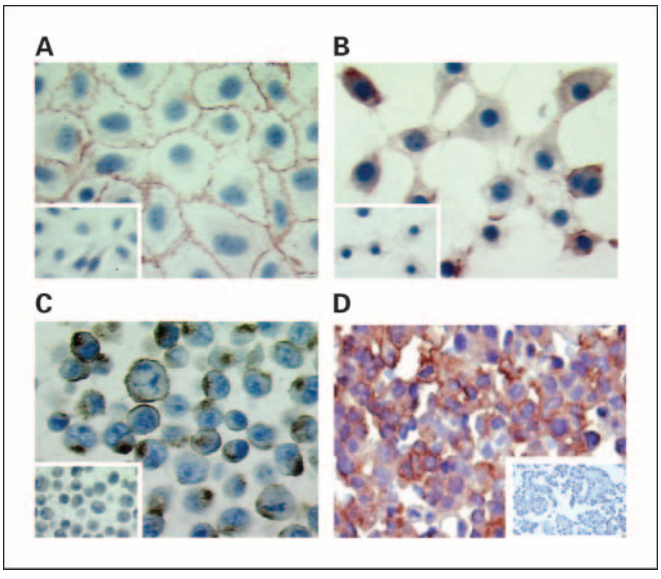

Fig. 1. Anchorage loss in epithelial ovarian cancer cells relocalizes ALCAM from cell membrane to cytoplasm. Localization of ALCAM was evaluated by immunohistochemistry in formalin-fixed, paraffin-embedded sections containing A2774 epithelial ovarian cancer cells and cells from epithelial ovarian cancer ascites using monoclonal anti-ALCAM Ab. A2774 cell monolayer treated for 50 min with sodium vanadate (*A*) shows a marked ALCAM membrane staining, whereas the cell monolayer treated with pervanadate (*B*) progressively detaches from the culture dish and shows membrane signal limited to the cell-to-cell contact region. With the rounding of the cells, cytoplasmic localization of ALCAM is detected. *C*, A2774 cells detached from the culture flask with PBS/EDTA and immunostained for ALCAM localization show cytoplasmic localization of ALCAM. *D*, epithelial ovarian cancer ascites cells show ALCAM localization mainly in the cytoplasm, with some membrane staining at cell-cell contacts. A representative sample of three tested is shown. Insets show negative controls. (Original magnification, ×400).

malignant tumor cells in peritoneal fluids (ALCAM HR = 2.6, 95% CI = 1 to 6.6, $P = 0.05$, complete results from the model not shown).

Discussion

The heterogeneity of clinical outcomes, particularly in advanced-stage epithelial ovarian cancer patients points to the inadequacy of currently available clinical pathologic prognostic factors (3). The identification of diagnostic, prognostic, and predictive biomarkers based on a better knowledge of the underlying biological mechanisms is an urgent need, and many efforts are focused in this direction (25–29).

Using a cDNA-microarray-based approach (9), we recently identified a molecular signature of epithelial ovarian cancer linking the fibroblast growth factor receptor signaling functions

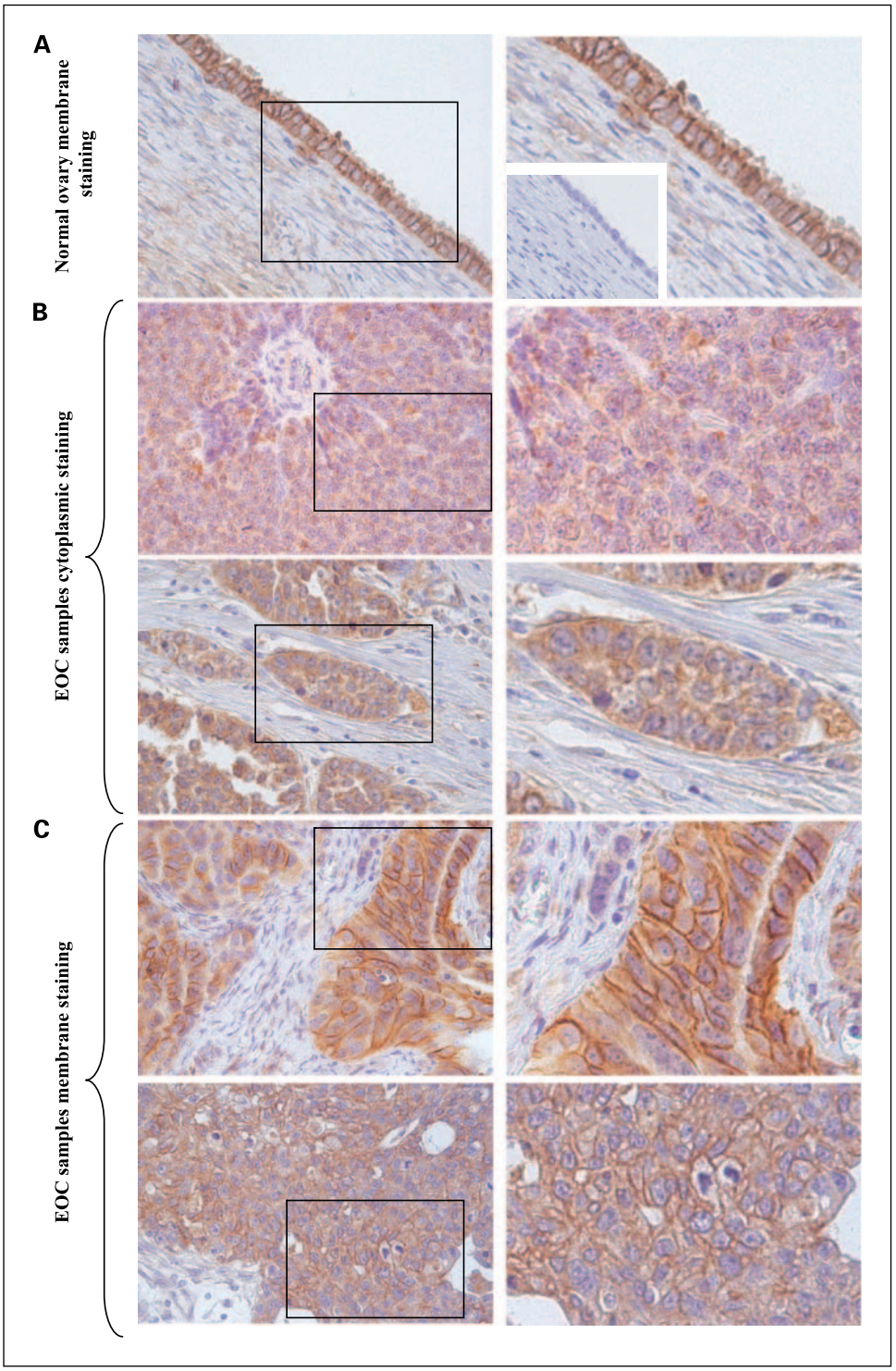

Fig. 2. Cellular localization of ALCAM in epithelial ovarian cancer samples. Immunohistochemical analysis of ALCAM expression in formalin-fixed, paraffin-embedded tissue sections from archival samples stained with monoclonal anti-ALCAM Ab. *A,* Normal ovary with a homogenous membrane staining of OSE cells (negative control stained with secondary antibody alone is shown in inset of right panel). *B,* cytoplasmic ALCAM localization in two representative epithelial ovarian cancer samples. *C,* membrane ALCAM localization in two representative epithelial ovarian cancer samples. (Original magnification ×400 [left panels] and higher magnification (×800) of boxed areas [right panels]).

Table 2. Univariate analysis of overall survival in relation to clinical parameters and ALCAM subcellular localization

	OS					
	No. cases* (*N* = 108)	Survival status		Median OS months	*P* †	HR ‡ (95% CI)
		Alive (*n* = 56)	Dead (*n* = 52)			
Age at diagnosis					0.033	
<mean	51	32	19	NYR		1.8
≥mean	57	24	33	48		(1.0-3.1)
Stage					<0.0001	
I-II	35	28	7	NYR		5.28
III-IV	73	28	45	45		(3.0-9.3)
Histotype					0.134	
Serous	62	29	33	58		1.6
Others	46	27	19	NYR		(0.9-2.7)
Grade					0.17	
1 + 2	44	26	18	NYR		1.5
3 + undifferentiated	62	29	33	58		(0.9-2.6)
Surgical debulking§					<0.0001	
Optimal	50	39	11			5.3
Suboptimal	57	17	40			(3.0-9.5)
ALCAM localization					0.036	
Cytoplasm	73	32	41	58		2.0
Membrane	35	24	11	NYR		(1.1-3.5)
MTC in peritoneal fluids‖					0.0001	
Presence	50	14	36	35		3.8
Absent or rare	22	14	8	NYR		(2.0-7.1)
Front-line chemotherapy					0.78	
Untreated	8	5	3	NYR		
P	43	22	21	87		
PT	42	21	21	53		
PTT	15	8	7	71		

Abbreviations: OS, overall survival; NYR, not yet reached; P, platinum alone; PT, standard platinum-paclitaxel therapy; PTT, platinum-paclitaxel associated with topotecan; MTC, malignant tumor cells; HR, hazard ratio; CI, confidence interval.
*One case was excluded from OS analysis because it was lost during follow-up.
† *P* values determined using log rank test.
‡ Hazard ratios for unfavorable clinical parameter.
§ For one case, the clinical parameter was not available.
‖ Malignant tumor cells in ascitic fluids of III/IV stage patients.

to the modulation of extracellular matrix and adhesion molecules. A remodeling of adhesion molecules might account for tumor cell detachment from the primary tumor and for invasion of the peritoneal cavity. Epithelial ovarian cancer seeds the peritoneal cavity with tumor cell nests that adhere to adjacent organs or float freely and lead to ascites formation in more than 50% of advanced-stage tumors. We recently reported that M-CAM, a member of the Ig-CAM family, is differentially expressed in epithelial ovarian cancer (30). M-CAM expression was associated with advanced-stage tumors, serous and undifferentiated histotypes, and extent of residual disease. Expression of this molecule clearly identified a subgroup of patients initially responsive to front-line therapy but rapidly relapsing and dying of disease. The prognostic impact of Ep-CAM overexpression for reduced survival of epithelial ovarian cancer patients has also been recently described (29).

In the present analysis, we focused on another member of the Ig-CAM family of adhesion molecules, ALCAM/CD166, as its expression has already been correlated to neoplastic processes. Our previous findings indicate that ALCAM can be reduced at cell membrane level by active internalization and that this process may be relevant to the rearrangement of cell-cell contacts (19). Furthermore, we recently found that ALCAM can be released from epithelial ovarian cancer cells by a metalloprotease-dependent mechanism and that inhibition of this process reduced epithelial ovarian cancer cell motility suggesting that perturbation of ALCAM adhesive functions may play a role in epithelial ovarian cancer cell motility and invasiveness (20). Here we show in an epithelial ovarian cancer cell line, that loss of cell anchorage is associated with a relocalization of ALCAM from cell membrane to cytoplasm. Therefore, membrane ALCAM reduction\loss in epithelial ovarian cancer might participate in tumor cell detachment from the primary tumor with consequent peritoneal dissemination of the disease. In fact, ALCAM localization in tumor cell clumps recovered from epithelial ovarian cancer patients' ascitic fluids was mainly cytoplasmic. Immunohistochemical analysis of 109 epithelial ovarian cancer patient specimens revealed ALCAM expression mainly at the cytoplasmic level, in addition to its physiologic expression at the cell membrane. Retrospective analysis showed that patients with cytoplasmic staining had a worse prognosis, as compared with patients who had homogeneous membrane staining. In addition to univariate analyses to evaluate the association of ALCAM with

OS, we also used a multivariable regression model in which we adjusted for all the other known clinicopathologic variables and for the type of treatment and found that subcellular localization of ALCAM is an independent prognostic factor of patients' survival.

The ALCAM molecule is highly expressed in the invasive cells of melanocytic skin lesions, where it correlates with Clark's classification, which reflects local tumor progression (13, 14). In melanoma, ALCAM may function as a cell surface sensor able to regulate cellular signaling and dynamic responses (31). In addition, ALCAM was found upregulated in low-grade prostate cancer and progressively lost in high-grade lesions (15), and cytoplasmic ALCAM expression has been used as a prognostic marker of relapse (16). Although several different functions for ALCAM expression have been reported in different tumor models (17, 18), cytoplasmic overexpression of the protein was associated with disease progression also in breast cancer (32). In this context, our ALCAM IHC data for epithelial ovarian cancer appear consistent with those reported for prostate and breast cancer.

The mechanism underlying the association of cytoplasmic ALCAM expression and poor prognosis in cancer remains unclear. In a prostate cancer model, it has been shown that ALCAM cytoplasmic localization is due to a loss of α-catenin as a function of a loss of E-cadherin activity (33). Furthermore, the metastasis-associated T-lymphoma invasion and metastasis 1 molecule were recently shown to regulate ALCAM localization to the cell membrane, and ALCAM-mediated cell-cell contacts were implicated in the inhibitory regulation of cell migration (34). It is also conceivable that cytoplasmic ALCAM cannot function properly as a cell surface sensor for growth saturation, as recently hypothesized for malignant melanoma (30). In view of our recent observations (19, 20), we can hypothesize that proteolytic release of membrane ALCAM might decrease its membrane expression and that soluble ALCAM may further induce, by means of homophilic interactions, the internalization of those ALCAM molecules still present at the cell surface.

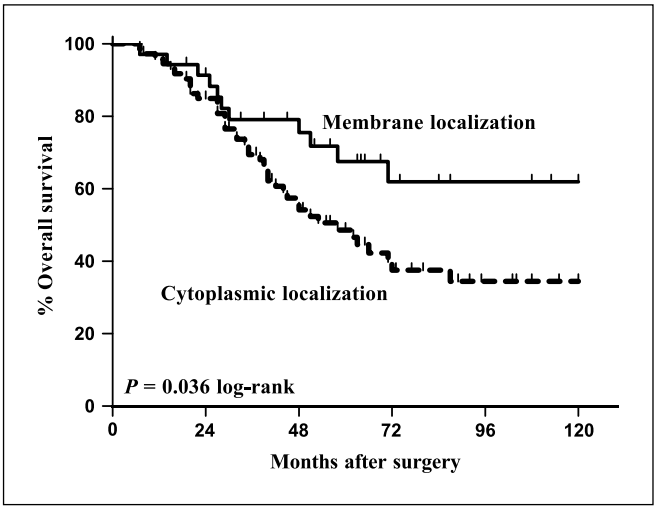

Fig. 3. Association between ALCAM subcellular localization and overall survival (OS) in 108 epithelial ovarian cancer patients. Solid line: membrane localization; dashed line: cytoplasmic localization. Median OS with respect to ALCAM cytoplasmic or membrane localization was 58 and not yet reached, respectively ($P = 0.036$). *P* values were determined using log-rank test.

Table 3. Multivariable analysis (Cox regression) of overall survival for clinical parameters and ALCAM subcellular localization

	OS*	
	P	**HR† (95% CI)**
Age at diagnosis		
≥mean versus <mean	0.24	1.44 (0.8-2.6)
Tumor stage		
III-IV versus I-II	0.005	6.71 (1.8-25.1)
Histotype		
Others versus serous	0.78	1.10 (0.6-2.1)
Grade		
3 + undifferentiated versus 1 + 2	0.81	1.08 (0.6-2.0)
Surgical debulking		
Suboptimal versus optimal	<0.001	4.63 (1.9-11.3)
ALCAM localization		
Cytoplasm versus membrane	0.042	2.15 (1.0-4.5)
Front-line chemotherapy		
No treatment versus P	0.037	5.72 (1.1-29.6)
P versus PT	0.085	1.81 (0.9-3.6)
P versus PTT	0.004	3.86 (1.5-9.8)

Abbreviations: HR, hazard ratio; CI, confidence interval; P, platinum alone; PT, standard platinum-paclitaxel therapy; PTT, platinum-paclitaxel associated with topotecan.
*Evaluated on 106 patients (three observations deleted due to missing data).
†HRs for unfavorable clinical parameter (reported first in the description of the variables).

Thus, ALCAM relocalizing from the cell membrane to cytoplasm might ultimately enhance the migratory properties of malignant cells facilitating peritoneal dissemination of the disease. Considering that ALCAM is a cell surface Ig-CAM superfamily member involved in cell-cell interaction, its expression at membrane level might contribute to avoid cell detachment from the primary tumor and to decrease epithelial ovarian cancer peritoneal dissemination thus improving the patient's outcome. In the subgroup of advanced-stage patients with rare or no malignant cells in peritoneal fluids (22 patients), the maintenance of ALCAM membrane expression identified patients with better prognosis, as seven out of eight patients with ALCAM membrane expression were still alive. These data deserve validation in prospective studies in which the subcellular localization of ALCAM will be simultaneously analyzed in the primary tumor, peritoneal tumor deposits, and free-floating clumps of tumor cells from the same patients and subsequently correlated to tumor recurrence and patient outcome.

Conclusion

Together, our present and previous data (19, 20, 29) suggest a role for altered cell adhesion molecule expression in regulating motility and invasion of ovarian cancer cells. Because it is becoming evident that the measurement of a single biomarker may not provide sufficient prognostic information to be clinically useful, the possibility to measure a panel of biomarkers (e.g., Ig-CAM family members) might produce more informative prognostic indices for epithelial ovarian cancer. In this respect, it might be interesting to examine the combined prognostic value of different members

of the Ig-CAM family, considering that two of them, ALCAM (present data) and M-CAM (30), have been shown to be independent prognostic factors in predicting patients with poorer outcome who may benefit from new therapeutic strategies.

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