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ARTICLE *in* CANCER · APRIL 2008

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Expression of the Nuclear Export Protein Chromosomal Region Maintenance/ Exportin 1/Xpo1 Is a Prognostic Factor in Human Ovarian Cancer

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We thank Martina Eickmann for editorial assistance.

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Received June 29, 2007; revision received September 24, 2007; accepted November 6, 2007.

BACKGROUND. The human nuclear export protein chromosomal region maintenance/exportin 1/Xpo1 (CRM1) mediates the nuclear export of proteins and messenger RNAs and, thus, is an important regulator of subcellular distribution of key molecules. Whereas cell-biologic studies have suggested a fundamental role for CRM1 in the regulation of mitosis, the expression of this protein in human tumor tissue has not been investigated to date.

METHODS. In this study, the expression of CRM1 was analyzed in a cohort of 88 ovarian tumors and 12 ovarian cell lines for the first time to the authors' knowledge.

RESULTS. Immunohistochemistry revealed increased nuclear (52.7%) and cytoplasmic (56.8%) expression of CRM1 in 74 carcinomas compared with the expression revealed in borderline tumors and benign lesions. Similarly, CRM1 expression was increased in ovarian cancer cell lines compared with human ovarian surface epithelial cells. Cytoplasmic CRM1 expression was related significantly to advanced tumor stage ($P = .043$), poorly differentiated carcinomas ($P = .011$), and higher mitotic rate ($P = .008$). Nuclear CRM1 was associated significantly with cyclooxygenase-2 (COX-2) expression ($P = .002$) and poor overall survival ($P = .01$). Because it was demonstrated previously that blocking of CRM1 by leptomycin B (LMB) contributes to the inhibition of nuclear export, the authors used a set of mechanistic assays to study the effects of CRM1 inhibition in cancer cells. Treatment of OVCAR-3 cells with LMB revealed a significant reduction of cell proliferation and increased apoptosis as well as suppressed interleukin-1β-induced COX-2 expression.

CONCLUSIONS. The current results indicated that CRM1 is expressed in a subpopulation of ovarian carcinomas with aggressive behavior and is related to poor patient outcome. A correlation also was demonstrated between CRM1 and COX-2 expression in ovarian cancer tissue. Furthermore, the treatment of ovarian cancer cells with LMB revealed a reduction in COX-2 expression. Therefore, the authors suggest that CRM1 may be an interesting biomarker for the assessment of patient prognosis and a molecular target for anticancer treatment. ***Cancer* 2008;112: 1733–43.** © *2008 American Cancer Society.*

KEYWORDS: chromosomal region maintenance/exportin 1, ovarian cancer, leptomycin B, cyclooxygenase-2.

Ovarian carcinoma is the fifth most common cause of cancer death in women, with an estimated 22,430 new cases and 15,280 deaths in the U.S. expected in 2007, and it has the highest mortality rate among the gynecologic malignancies.[1,2] In particular,

DOI 10.1002/cncr.23354
Published online 27 February 2008 in Wiley InterScience (www.interscience.wiley.com).

60% of patients who have stage III/IV tumors have a 5-year survival rate of only 28%. The main prognostic factors for the planning of therapy are International Federation of Gynecology and Obstetrics (FIGO) stage and residual tumor at surgery. Other factors, such as patient age, tumor grade, and histologic tumor type, have some additional prognostic impact. The identification of further molecularly defined prognostic parameters, particularly for patients with advanced disease, would be very helpful for planning and individualization of treatment. Furthermore, investigation of the molecular mechanisms that contribute to tumor progression will be relevant for the identification of new therapeutic strategies.

The coordinated transport of proteins and nucleic acids across the nuclear envelope between nucleus and cytoplasm is essential for cellular integrity and gene expression. This transport is mediated by soluble transport factors, the karyopherins, which interact with their transport cargos through nuclear localization sequences and nuclear export sequences (NES), which regulate entry into and exit from the nucleus.

Chromosomal region maintenance/exportin 1/Xpo1 (CRM1) originally was identified in the fission yeast *Schizosaccharomyces pombe* in a genetic screen, and investigators determined that it was involved in control of the chromosome structure.[3] Several studies have demonstrated that CRM1 is important in at least 2 areas of cellular functions: nuclear-cytoplasmic transport and control of mitosis. Regarding the transport function, CRM1 is the main nuclear export receptor in humans. This transporter has a comparably broad substrate range and mediates the export of leucine-rich NES-bearing proteins and the transfer of messenger RNAs (mRNAs).[4–8] Mechanistic studies have demonstrated an increasing number of NES-containing signaling molecules that depend on the CRM1 nuclear export pathway, p53,[9] histone deacetylase 5 (HDAC5),[10] protein kinase 1 (Akt1),[11] epidermal growth factor receptor (EGFR),[12] and others.[13,14] Therefore, this shuttling process controls several elements of pathways that have been identified as important in tumor biology.

It also has been demonstrated that CRM1 functions in complex with ras-related nuclear protein-guaosine triphosphatase (RAN-GTPase) to control several processes during cellular mitosis. Thus, CRM1 associates with kinetochores as well as centromeres during mitosis. In particular, this controls chromosome segregation[15] and prevents chromosome reduplication.[16]

CRM1 has been identified in biochemical studies as the specific target molecule of leptomycin B (LMB),[17] an unsaturated, branched-chain fatty acid with a terminal δ-lactone ring that binds covalently to cysteine 529 in the NES binding region of human CRM1.[18] LMB was discovered primarily as an antifungal agent and has demonstrated antiproliferative properties as well as in vivo antitumor activity. Blocking of CRM1 contributes to the inhibition of cargo loading and subsequent nuclear export.[3] It is noteworthy that LMB inhibited the stabilization of COX-2 mRNA in human mammary cancer cells and suppressed interleukin 1β (IL-1β)-induced COX-2 expression in a human colon cancer cell line.[8]

With this cell-biologic background in mind, we have developed the hypothesis that CRM1 plays an important role in the regulation of cellular functions that are involved in the development and progression of human tumors. In this study, for the first time to our knowledge, we analyzed the CRM1 expression in a cohort of primary human ovarian carcinomas and compared expression data with clinicopathologic characteristics, COX-2 expression, and patient survival. We determined the expression of CRM1 in ovarian cancer cell lines and treated cells with LMB, which is a specific CRM1 antagonist.

MATERIALS AND METHODS

Study Population and Tissue Samples

Immunohistochemical examination of CRM1 was performed retrospectively on tissue samples that were taken for routine diagnostic and therapeutic purposes. Normal ovarian tissues and benign and malignant ovarian tumors from 98 patients who were diagnosed at the Institute of Pathology, Charité Hospital Berlin between 1989 and 2003 were included in the study. The tissue specimens consisted of 74 primary invasive ovarian carcinomas, 14 borderline tumors, and 3 cystadenomas as well as 7 normal ovaries. Tissue samples were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Routine hematoxylin and eosin sections were stained for histopathologic evaluation. The stage of tumors was assessed according to the FIGO staging system. For grading of tumors, the Silverberg grading system, which is composed of architectural, nuclear, and mitotic features, was used.[19] The number of mitoses per 10 high-power fields (HPF) was evaluated and separated into 3 groups, <9 of 10 HPF, 10 to 23 of 10 HPF, and >24 of 10 HPF. For statistical evaluation and survival analysis, only the patients with invasive ovarian carcinomas were included. Data on intraoperative residual tumor were available for 50 patients with FIGO stage II through IV disease. Of these, 30 patients (60%) had no residual tumor, 14 patients

(28%) had postoperative residual tumor that measured <20 mm, and 6 patients (12%) postoperative residual tumor that measured >20 mm. Lymphadenectomy was undergone by 81.1% of patients. Most of patients (18.9%) who did not undergo lymphadenectomy had high-stage disease with known peritoneal or distant metastasis. Data regarding postoperative chemotherapy were known for 68 patients; of these, 62 patients (91.2%) received a platinum-based therapy, 2 patients (2.9%) received other nonplatinum regimens, and 4 patients (5.9%) did not receive any chemotherapy. The median follow-up for all patients was 39 months.

Immunohistochemistry

Immunohistochemical examination was performed on tissue microarrays. Therefore, representative areas of the ovarian carcinomas as well as borderline tumors were marked on the routine hematoxylin and eosin-stained histologic sections. Four tissue cores that measured 1.5 mm in greatest dimension from different tumor parts of the donor blocks were punched by using a tissue microarrayer (Beecher Instruments, Woodland, Wis) and positioned in a recipient paraffin array block.

Immunohistochemistry was performed according to standard procedures. Briefly, slides were boiled in citrate buffer (pH 6.0) in a pressure cooker for 5 minutes and incubated with the rabbit polyclonal anti-CRM1 antibody (sc5595; Santa Cruz Biotechnology, Santa Cruz, Calif; 1:100 dilution) overnight at 4 °C. This was followed by incubation with a biotinylated antimouse secondary antibody and the multilink biotin-streptavidin-amplified detection system (Biogenex, San Ramon, Calif). Staining was visualized by using a Fast Red chromogen system (Immunotech, Hamburg, Germany). The intensity of the CRM1 immunostaining in tumor cells was evaluated independently by 2 pathologists (A.N. and C.D.) who were blinded to patient outcomes. In the event of disagreement, the cases were discussed at a multihead microscope. CRM1 expression was evaluated according to the percentage of positive cells and the intensity of staining. We analyzed a complete tumor area of 4 tissue cores (each 1.5 in greatest dimension), approximately 20 HPF. For assessing the percentage of CRM1-expressing cells, the area that contained positive cells was compared with the whole area that was covered by tumor cells. Staining intensity was estimated by comparison with normal ovarian tissue on standard paraffin sections. In addition, a separate scoring for nuclear and cytoplasmic CRM1 expression was performed. The percentage of positive cells was scored as 0 (0%), 1 (<10%), 2 (11%–50%), 3 (51%–80%), or 4 (>80%); and the staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). For the immunoreactive score (IRS), the percentage of positive cells and the staining intensity were multiplied, resulting in a value between 0 and 12. To separate samples with weak or strong expression of CRM1 in tumor tissue, we combined samples that had an IRS from 0 to 6 into 1 group with negative to weak expression (CRM1-negative) and samples that had an IRS from 7 to 12 into another group that was considered CRM1-positive. The minimum requirements for a sample to be scored as positive were either moderate expression in >80% of cells or strong expression in >50% of cells. Normal tissue and benign lesions of the ovary served as negative controls. Expression of COX-2 was determined as described previously[20] by using the monoclonal COX-2 antibody (1:1000 dilution; Cayman Chemical, Ann Arbor, Mich).

Statistical Analysis

The statistical significance of the association between CRM1 and COX-2 expression and clinicopathologic parameters was assessed by using the chi-square test for trends or the Fisher exact test. The probability of overall survival as a function of time was determined by using the Kaplan-Meier method. Differences in survival curves were compared by using the log-rank test. Multivariate survival analysis was performed by using the Cox model of proportional hazards. P values <.05 were considered significant. For statistical evaluations, the SPSS software version 13.0 (SPSS Inc, Chicago, Ill) was used.

Cell Culture and Inhibitors

The human ovarian carcinoma cell lines (OVCAR-3, SKOV-3, CAOV-3, ES-2, A2780, Mdah2744, and OAW42) that were investigated in this study have been described previously.[21,22] EFO21, EFO27, and FU-OV-1 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Heidelberg, Germany); and PA-1 cells, a human ovarian teratocarcinoma cell line, was obtained from the American Type Culture Collection (Rockville, Md). Cells were cultured in Dulbecco Modified Eagle Medium (Bio Whittaker Europe, Verviers, Belgium) supplemented with 10% fetal bovine serum (PAA Laboratories, Linz, Austria). Human ovarian surface epithelial (HOSE) cells,[23] an immortalized human ovarian surface epithelium cell line, were cultivated in a 1:1 mixture of medium 199 (Sigma Chemical Company, St. Louis, Mo) and moisture content, dry basis medium 105 (MCDB 105; Life Technologies,

Inc., Grand Island, NY) supplemented with 10% fetal calf serum and 2 mM glutamine. All cell lines were maintained at 37 °C in a humidified atmosphere of 5% carbon dioxide/95% air. In some experiments COX-2 expression was induced with recombinant human IL-1β (10 ng/mL; R&D Systems, Minneapolis, Minn) for 24 hours.[20]

The following inhibitory agents were used: the inhibitor LMB (L2913; Sigma Chemical Company), which has demonstrated the ability to inhibit CRM1 in other cellular systems at different concentrations (2.5 ng/mL, 5 ng/mL, and 10 ng/mL) for a maximum of 72 hours. Because COX-2 expression may be associated with other tumor signaling pathways, we also used inhibitors of 2 important pathways, the phosphoinositide-3 kinase (PI3K) inhibitor LY294002 (10 mM) and the mitogen-activated protein kinase inhibitor PD98059 (50 mM), to examine any effect of these pathways on COX-2 expression. Both inhibitors were dissolved in dimethyl sulfoxide (Sigma, Deisenhofen, Germany).[24]

Immunoblot Analysis

For protein analysis, cells were lysed in 100 μL of 62.5 mM Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenole blue. One hundred micrograms of protein per sample were separated on a 10% polyacrylamide gel, blotted onto nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany), washed in phosphate-buffered saline (PBS), and blocked in buffer (1 × PBS, 0.1% Tween-20, 5% I-block; Tropix, Bedford, Mass) for 1 hour at room temperature. Membranes were incubated overnight at 4 °C with an anti-CRM1 antibody (Santa Cruz Biotechnology) diluted 1:1000 in blocking buffer, followed by incubation with alkaline phosphatase-conjugated goat-antirabbit secondary antibody (Tropix). Bands were observed by using the CDP-Star RTU luminescence system (Tropix). Expression of COX-2 was analyzed as described previously.[20]

Cell Proliferation Assay

To examine the effect of LMB on cell proliferation, we incubated OVCAR-3 cells (plated in 96-well plates; 3000 cells per well) for 24 hours and 48 hours with LMB (5 ng/mL). Cell numbers were determined by using an XTT (soldium 3′-[1-phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate)-based colorimetric assay (performed in triplicate; Roche, Germany) according to the manufacturer's instructions. Differences in cell proliferation were evaluated statistically by using the 2-sided Student *t* test.

Cell Cycle Analysis

OVCAR-3 cells were plated at 4×10^5 cells per well in 6-well plates. After 24 hours, cells were treated with LMB at concentrations of 2.5 ng/mL, 5 ng/mL, and 10 ng/mL for 48 hours. Next, cells were fixed in 70% ice-cold ethanol at −20 °C for 30 minutes. The percentages of cells in G_0/G_1, S, and G_2/M phase were quantified by using a propidium iodide (PI)-based cellular DNA flow cytometry analysis method. Briefly, cells were rinsed in PBS, and 500 μL diluent buffer (50 μL 0.1% Triton X-100 and 250 mg 0.5% bovine serum albumin in 50 mL PBS) were added. RNAse digestion was performed by adding 4 μL RNAse (10 mg/mL) for 1 hour at 37 °C. Then, cells were centrifuged and diluted in 500 μL diluent buffer containing 20 μL PI and were evaluated by fluorescence-activated cell sorting (FACS) by using a FACScan flow cytometer (BD Biosciences, Heidelberg, Germany). Experiments were performed in 3 separate experiments. Analyses of cell cycle data were done with Cyclchred and WinMDI (Freeware).

Apoptosis Assays

We used different assays to evaluate apoptosis after treatment of ovarian cancer cells with LMB. The cells were incubated with LMB as described above. In the first assay, OVCAR-3 cells were evaluated for evidence of apoptosis by measuring the DNA content in a double-labeling system with PI and annexin V staining. Briefly, cells were seeded at a density of 4×10^5 cells per well in 6-well plates and treated with LMB (2.5 mg/mL, 5 mg/mL, and 10 ng/mL) after 24 hours. After 48 hours, cells and supernatant fluids were collected together and were centrifuged at 2000 revolutions per minute for 5 minutes. The pellet was incubated with 100 μL binding buffer (10 mM N-2-hydroxyethyl piperazine-N′-ethane sulphonate/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$ in 50 mL of H_2O), 2.5 μL annexin V-fluorescein isothiocyanate (FITC) (Roche), and 5 μL PI. After 15 minutes, binding buffer (300 μL) was added. Cells were measured immediately with the FACScan flow cytometer (BD Biosciences). Differences were analyzed by using the 2-tailed Student *t* test.

In an additional set of experiments, apoptosis was examined by caspase-3 activity, a marker for cells undergoing apoptosis, by using the FITC-conjugated monoclonal active caspase-3 antibody apoptosis assay (BD Pharmingen). Briefly, OVCAR-3 cells (2×10^5 per well) were permeabilized, fixed, and stained for active caspase-3 for 30 minutes and analyzed by flow cytometry (after 72 hours) according to the manufacturer's protocol. Campothecin, a potent inhibitor of topoisomerase I, induces apoptosis and

TABLE 1
Characteristics of 74 Patients With Invasive Ovarian Carcinomas

Characteristic	No. of patients (%)
Histologic type	
Serous	45 (60.8)
Nonserous	23 (31.1)
Undifferentiated	6 (8.1)
FIGO stage	
I	15 (20.3)
II	9 (12.2)
III	45 (60.7)
IV	5 (6.8)
Pathologic tumor classification	
pT1	17 (23)
pT2	9 (12.2)
pT3	48 (64.8)
Pathologic lymph node status, n = 60	
pN0	38 (63.3)
pN1	22 (36.7)
Pathologic metastasis status	
pMX	69 (93.2)
pM1	5 (6.8)
Histologic grade	
1	9 (12.2)
2	34 (45.9)
3	31 (41.9)
Age at surgery, y	
≤60	42 (56.8)
>60	32 (43.2)
Intraoperative residual tumor, n = 50	
Residual tumor <2 cm	44 (88)
Residual tumor ≥2 cm	6 (12)
Chemotherapy, n = 68	
Platinum-based	62 (91.2)
Nonplatinum	2 (2.9)
No chemotherapy	4 (5.9)

FIGO indicates International Federation of Gynecology and Obstetrics.

was used as a positive control at a concentration of 5 μM. Finally, the Apo-Direct Kit (BD Pharmingen), which measures the amount of DNA fragmentation, was used according to the manufacturer's instructions followed by flow cytometric analysis.

RESULTS

Clinical and Pathologic Characteristics of Ovarian Tumors

Ovarian carcinoma specimens from 74 patients were investigated for immunoreactivity of CRM1 and COX-2. The mean age of patients at surgery was 57 years (range, 32–84 years). Among the invasive ovarian carcinomas, 45 tumors (60.8%) were serous, 23 tumors (31.1%) were nonserous (4 mucinous, 10 endometroid, 4 clear cell, and 5 transitional cell), and 6 tumors (8.1%) were undifferentiated carcinomas. Of the patients with invasive carcinomas, 15 patients (20.3%) had FIGO stage I disease, 9 patients (12.2%) had stage II disease, 45 patients (60.8%) had stage III disease, and 5 patients (6.8%) had stage IV disease. Lymph nodes from 60 patients were examined; among them, 38 patients (63.3%) had pathologically negative lymph nodes (pN0), and 22 patients (36.7%) had pathologically positive lymph nodes (pN1). Five patients had distant metastases at the time of diagnosis. Clinicopathologic parameters of the patients with invasive carcinoma are summarized in Table 1. Among the 14 borderline tumors, 6 tumors were serous, 7 tumors were mucinous, and 1 tumor was composed of both serous and mucinous parts.

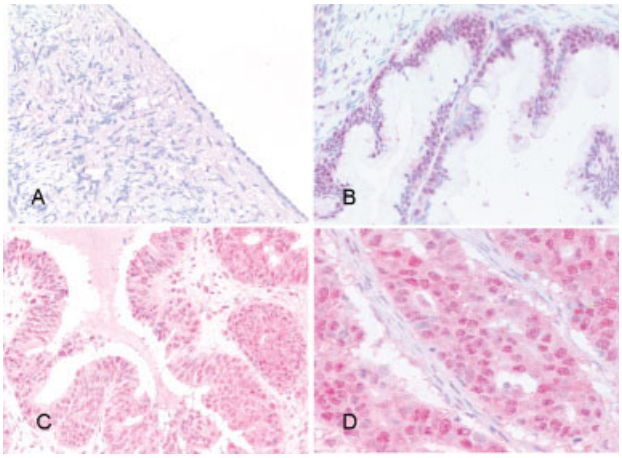

FIGURE 1. Immunohistochemical expression analysis of chromosomal region maintenance/exportin 1/Xpo1 (CRM1) in ovarian carcinomas. (A) No immunoreaction of CRM1 is observed in normal ovarian surface epithelium. (B) In contrast, intense nuclear expression of CRM1 is observed in the epithelium of a mucinous borderline tumor. (C,D) Strong nuclear and moderate cytoplasmic immunostaining of CRM1 are observed in a high-grade serous ovarian cancer.

CRM1 and COX-2 Immunostaining in Ovarian Tumors and Normal Ovaries

An overview of CRM1 immunostaining is shown in Figure 1A-D. We observed both a nuclear expression pattern and a cytoplasmic expression pattern of CRM1 in the ovarian carcinomas; therefore, both patterns were evaluated separately. Nuclear expression of CRM1 was observed in 39 of 74 tumors (52.7%), and cytoplasmic expression of CRM1 was observed in 42 of 74 tumors (56.8%). In the majority of tumors, we observed both expression patterns simultaneously but with different intensities. Only a small group of tumors had solely nuclear (14.9%) or cytoplasmic (18.9%) expression. Ovarian surface epithelium of normal ovaries and benign ovarian tumors showed only weak or no immunostaining of CRM1.

TABLE 2
Increased Expression of Chromosomal Region Maintenance/Exportin 1/Xpo1 in Invasive Carcinomas of the Ovary

	No. of patients (%)			
CRM1 expression	Invasive carcinomas, n = 74	Borderline tumors, n = 14	Benign lesions, n = 10	*P**
Nuclear and cytoplasmic				.001
Negative	22 (29.7)	13 (92.9)	10 (100)	
Positive	52 (70.3)	1 (7.1)	0 (0)	
Nuclear expression				.002
Negative	35 (47.3)	13 (92.9)	10 (100)	
Positive	39 (52.7)	1 (7.1)	0 (0)	
Cytoplasmic expression				.000
Negative	32 (43.2)	14 (100)	10 (100)	
Positive	42 (56.8)	0 (0)	0 (0)	

CRM1 indicates chromosomal region maintenance/exportin 1/Xpo1.
* Chi-square test.

TABLE 3
Correlation Between Nuclear Chromosomal Region Maintenance/Exportin 1/Xpo1 With Cyclooxygenase-2 Expression and Pathologic Factors in Patients With Invasive Ovarian Carcinoma

	No. of patients (%)			
Characteristic	All patients	CRM1 nuclear negative	CRM1 nuclear positive	*P*
All carcinomas	74 (100)	35 (47.3)	39 (52.7)	
COX-2 expression				.002
Negative	55 (100)	32 (58.2)	23 (41.8)	
Positive	19 (100)	3 (15.8)	16 (84.2)	
Pathologic tumor classification				.982
pT1	17 (100)	8 (47)	9 (53)	
pT2/pT3	57 (100)	27 (47.4)	30 (52.6)	
Histologic grade				.084
1/2	43 (100)	24 (55.8)	19 (44.2)	
3	31 (100)	11 (35.5)	20 (64.5)	

CRM1 indicates chromosomal region maintenance/exportin 1/Xpo1; COX-2, cyclooxygenase-2.

Only 1 of 14 borderline tumors exhibited moderate nuclear expression (IRS = 9). According to a univariate correlation analysis, CRM1 expression levels were increased significantly in invasive carcinomas compared with the levels in borderline tumors, benign lesions, and normal ovaries (Table 2). COX-2 expression was observed in 19 of 74 ovarian carcinomas (25.7%).

Correlation Between CRM1, COX-2, and Clinicopathologic Features

In univariate analysis, we investigated correlations among CRM1, COX-2, and clinicopathologic parameters (Tables 3, 4). We observed a significant association between nuclear and cytoplasmic CRM1 expression ($P = .006$). Nuclear CRM1 staining was correlated significantly with increased COX-2 expression ($P = .002$), as illustrated in Figure 2A. Cytoplasmic CRM1 expression was related significantly to advanced tumor stage ($P = .043$) and poorly differentiated carcinomas ($P = .011$). Because the Silverberg grading system is based on 3 morphologic parameters (nuclear polymorphism, mitotic rate, and growth pattern), we evaluated the association between CRM1 expression and each of those parameters separately. There was a significant correlation between cytoplasmic CRM1 expression and higher mitotic rate ($P = .008$), but no correlation was observed between CRM1 and nuclear polymorphism or tumor growth pattern.

Association of CRM1 With Patient Survival

In univariate Kaplan-Meier analysis, nuclear CRM1 expression was associated significantly with poor overall survival ($P = .010$), as indicated in Figure 2B.

TABLE 4
Correlation Between Nuclear Chromosomal Region Maintenance/Exportin 1/Xpo1 With Cyclooxygenase-2 Expression and Pathologic Factors in Patients With Invasive Ovarian Carcinoma

	No. of patients (%)			
Characteristic	All patients	CRM1 cytoplasmic negative	CRM1 cytoplasmic positive	*P*
All carcinomas	74 (100)	32 (43.2)	42 (56.8)	
COX-2 expression				.234
Negative	55 (100)	26 (42.3)	29 (57.7)	
Positive	19 (100)	6 (31.6)	13 (68.4)	
Pathologic tumor classification				.043
pT1	17 (100)	11 (64.7)	6 (35.3)	
pT2/pT3	57 (100)	21 (36.8)	36 (63.2)	
Histologic grade				.011
1/2	43 (100)	24 (55.8)	19 (44.2)	
3	31 (100)	8 (25.8)	23 (74.2)	

CRM1 indicates chromosomal region maintenance/exportin 1/Xpo1; COX-2, cyclooxygenase-2.

No association with progression-free survival was observed (data not shown). COX-2 expression was related significantly to poor overall survival for patients with ovarian cancer ($P = .026$). Other prognostic factors were residual tumor ($P = .003$) and histologic grade, which had borderline significance ($P = .076$). In an exploratory multivariate analysis that included histologic grade, tumor stage, postoperative residual tumor, age, and nuclear CRM1 expression, only residual tumor size had independent prognostic significance.

Expression of CRM1 in Ovarian Cancer Cell Lines

We determined the expression of CRM1 in 11 ovarian cancer cell lines and in HOSE cells. Figure 3 demonstrates that CRM1 protein expression was detected in various cancer cell lines (OVCAR-3, A2780, ES-2, MDAH2744, CAOV-3, SKOV-3, OAW42, EFO27, EFO21, and FU-OV-1). In contrast, HOSE cells and PA-1 cells showed reduced protein levels of CRM1.

Effects of LMB on COX-2 Expression and Cell Proliferation

COX-2 expression is associated with relevant tumor signaling pathways. Therefore, we incubated OVCAR-3 cells with inhibitors of 2 important pathways: LY294002, a PI3K-inhibitor, and PD98059, an MEK inhibitor, to examine any effect on COX-2 expression. In addition, we applied LMB, which is a specific inhibitor of CRM1. In Western blot analysis, as shown in Figure 4A, COX-2 expression was highly inducible by IL-1β. LY294002 and PD98059 did not influence COX-2 expression at the protein level. In contrast, LMB inhibited IL-1β-induced COX-2 expression in OVCAR-3 cells. Treatment of OVCAR-3 cells with LMB (5 ng/mL) revealed a significant reduction in cell proliferation after 24 hours and 48 hours as measured by XTT assay ($P < .05$; Student *t* test) (Fig. 4B).

Effects of LMB on Cell Cycle and Apoptosis

To determine whether the inhibition of CRM1 by LMB affects cell cycle progression, OVCAR-3 cells were cultured in the absence or presence of LMB at 3 concentrations (2.5 ng/mL, 5 ng/mL, and 10 ng/mL) for 48 hours and were analyzed by flow cytometry. An example of a histogram is shown in Figure 5A. The inhibition of CRM1 decreased the percentage of cells in G_1 phase. In addition, we observed an increased sub-G_0/G_1 peak that represented apoptotic cells. Induction of apoptosis was confirmed by using different assays. First, OVCAR-3 cells were analyzed with a double-labeling technique using annexin V and PI staining and then flow cytometry (FACS). A representative example is shown in Figure 5B. After 48 hours, LMB had induced apoptosis in approximately 15% of the cell population. The percentage of apoptotic cells determined for 3 different inhibitor concentrations and measured in 3 separate experiments is indicated in Figure 5C. Induction of apoptosis was confirmed by measurement of caspase-3 activity after 72 hours (Fig. 5D). The presence of apoptotic cells on the right in Figure 5D is demonstrated by increased fluorescence intensity (M2 gate) compared with the control. Finally, we detected apoptotic cells by labeling of DNA breaks with FITC-deoxyuridine triphosphate by using the Apo-Direct Kit (data not shown).

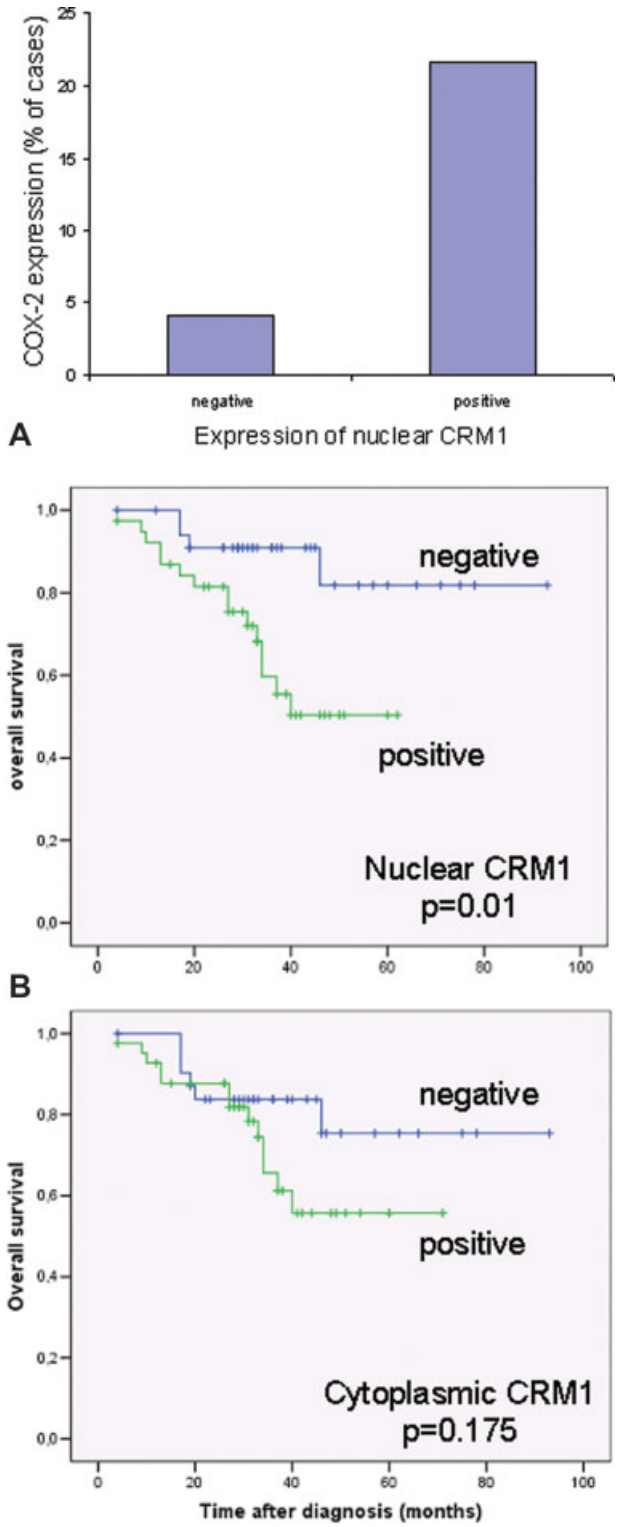

FIGURE 2. (A) Correlation of cyclooxygenase-2 (COX-2) and chromosomal region maintenance/exportin 1/Xpo1 (CRM1) expression ($P = .002$; chi-square test). (B) Univariate Kaplan-Meier survival analysis according to nuclear and cytoplasmic expression of CRM1. Significantly reduced overall survival was observed in patients who had ovarian carcinomas that expressed nuclear CRM1.

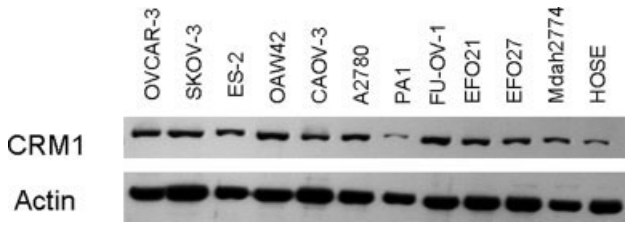

FIGURE 3. Expression of chromosomal region maintenance/exportin 1/Xpo1 (CRM1) on protein levels in ovarian carcinoma cell lines and human ovarian surface epithelial (HOSE) cells. Expression of CRM1 and β-actin was investigated by immunoblotting. One of 3 independent experiments is shown.

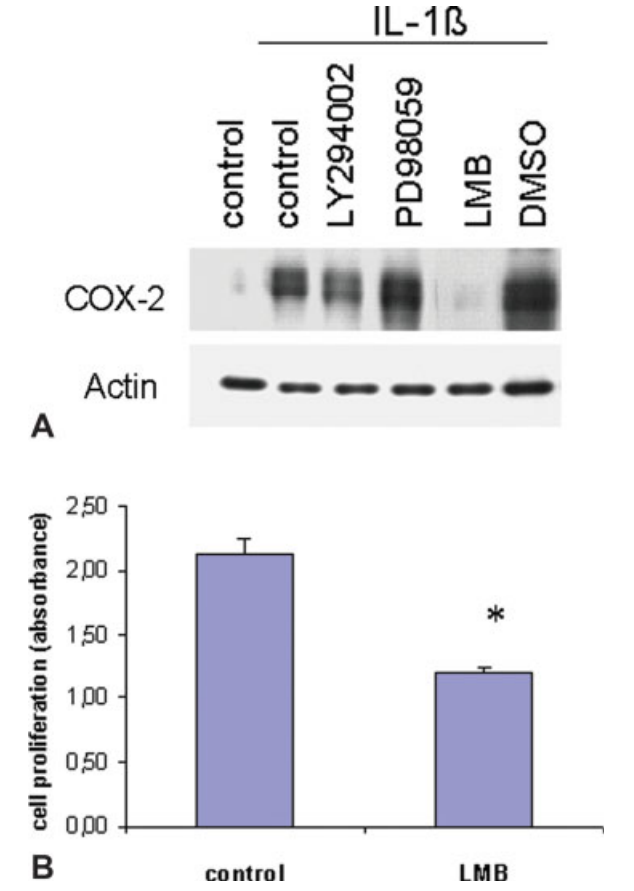

FIGURE 4. Effects of leptomycin B (LMB) in OVCAR-3 cells. (A) Cyclooxygenase-2 (COX-2) protein is highly inducible after treatment with interleukin-1β (IL-1β) in OVCAR-3 cells. The phosphoinositide-3 kinase inhibitor LY294002 and the mitogen-activated protein kinase inhibitor PD98059 did not affect COX-2 expression. In contrast, incubation with LMB resulted in COX-2 suppression at the protein level. Dimethyl sulfoxide (DMSO) served as a solvent control. (B) OVCAR-3 cells were incubated with LMB (5 ng/mL), and cell proliferation was measured by using an XTT assay. The means and standard deviations of 4 measurements after 24 hours (performed in triplicate) are shown. The asterisk indicates a statistically significant difference in cell proliferation ($P < .05$; 2-sided t test).

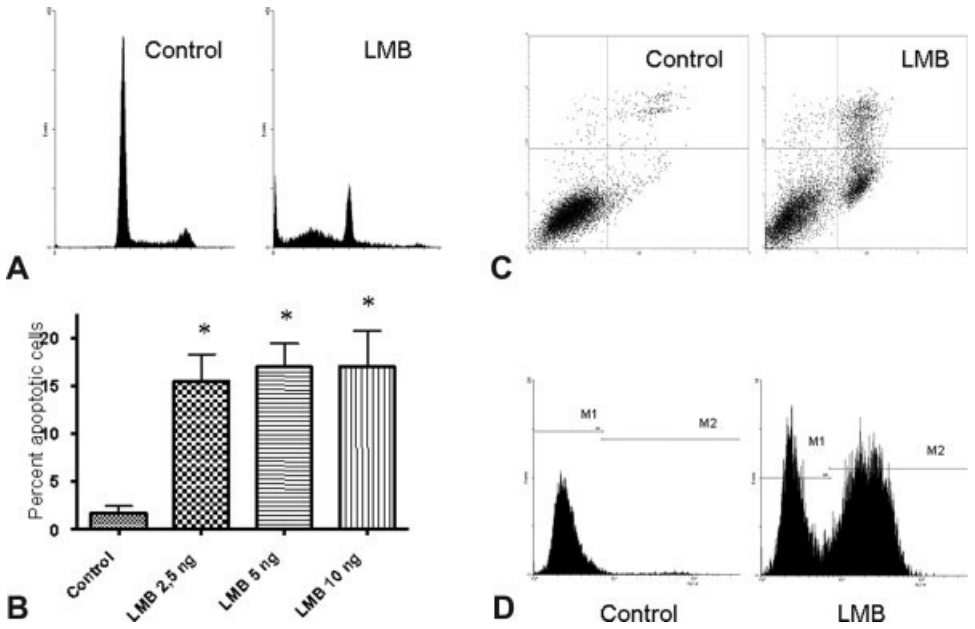

FIGURE 5. Leptomycin B (LMB) induces apoptosis in OVCAR-3 cells. (A) Cells were cultured in the absence (left) and presence (right) of LMB for 48 hours (10 ng/mL). Histograms display DNA content (x-axis; propidium iodide [PI] fluorescence) versus counts (y-axis). Treated cells showed an increased sub-G_0/G_1-peak and an decreased G_1-phase compared with untreated cells. Data shown are from 1 of 3 independent experiments. (B) Detection of apoptosis using double-labeling system and flow cytometry. A representative dual-parameter dot plot of annexin V fluorescence (x-axis) versus PI fluorescence (y-axis) is shown. OVCAR-3 cells were treated with LMB for 48 hours (10 ng/mL). (C) The effects of different concentrations of LMB on apoptosis were analyzed by PI/annexin V staining. The mean and standard deviation of 3 separate experiments is indicated. A significant difference was observed in the percentage of apoptotic cells compared with the percentage of untreated cells ($P < .05$; 2-tailed Student *t* test). (D) Flow cytometry was used to analyze caspase-3 activity in control cells and in LMB-treated cells. The apoptotic cell population increased, and there were detectable levels of active caspase-3 (represented by the M2 gate on the right; x-axis, active caspase-3 fluorescein isothiocyanate; y-axis, counts).

DISCUSSION

In this study, we investigated the expression of the nuclear export protein CRM1 in primary ovarian carcinomas and ovarian cancer cell lines. CRM1 demonstrated both nuclear (52.7%) and cytoplasmic (56.8%) expression patterns in ovarian cancer tissues. CRM1 expression was increased significantly in malignant tumors compared with the expression in borderline tumors and benign lesions of the ovary. To our knowledge, this is the first study investigating the immunohistochemical expression pattern of CRM1 in human tumor tissue. Our data from Western blot analysis support the enhanced expression of CRM1 in ovarian cancer cells compared with immortalized HOSE cells.

To our knowledge to date, there have been no studies regarding the prognostic significance of CRM1 in cancer patients. In the current study, we observed that cytoplasmic CRM1-positive ovarian carcinomas were at a more advanced tumor stage, were poorly differentiated, and had a higher mitotic rate. Nuclear CRM1 expression was associated significantly with poor overall survival. On the basis of these findings, CRM1 may be an interesting biomarker for assessing the prognosis of patients with ovarian cancer. However, it should be emphasized that there are certain limitations of this study. For example, all immunohistochemical-based studies have observer bias, because as the grade and type of tumors can be determined during the analysis and cannot be blinded. Furthermore, the interpretation concerning the prognostic role of CRM1 expression in ovarian carcinomas in this first study is preliminary. Additional large-scale prospective and retrospective studies will be needed to investigate whether it is practical to use CRM1 expression as a prognostic predictor.

We and others have demonstrated that LMB, a specific inhibitor of CRM1, blocks COX-2 expression in cancer cells.[8,25] Several groups have demonstrated that cyclooxygenases such as COX-2[26] and also COX-1[27,28] are overexpressed in ovarian cancer, suggesting that these enzymes may be interesting therapeutic targets. In our study cohort, increased COX-2 expression in ovarian carcinomas was related significantly to poor overall survival. It is noteworthy that we

observed a significant association of the nuclear CRM1 expression with increased COX-2 expression. The combination of functional data and in vivo data suggests that CRM1 may play a role in the regulation of COX-2 in ovarian cancer. Altered CRM1 seems to be responsible for COX-2 overexpression during the nuclear export of COX-2 mRNA, which is an important control point of COX-2 gene expression. LMB had no effect on COX-2 transcription, suggesting a posttranscriptional inhibition. Furthermore, it was demonstrated previously that LMB inhibits the time-dependent COX-2 mRNA export into the membrane-bound polysomal compartment at the endoplasmic reticulum.[8]

Although our results indicate that COX-2 and CRM1 are linked in ovarian cancer in vivo and in vitro, we cannot exclude the possibility that other proteins that are dependent on nuclear-cytoplasmic transport are regulated by CRM1. In fact, this seems very likely considering the central role of nuclear-cytoplasmic export in cell biology. In addition to COX-2 mRNA, CRM1 regulates nuclear-cytoplasmic transport of NES-bearing proteins, which play a role in relevant tumor signaling pathways, such as p53, AKT1, HDAC5, and EGFR. This raises the hypothesis that dysregulation of nuclear-cytoplasmic export may be crucial in malignant transformation and, thus, may be a promising field for future therapeutic approaches.

The doses of LMB in our study were well in line with those used in other studies that have investigated nuclear-cytoplasmic transport. LMB is a specific inhibitor of CRM1 based on several observations[17,29,30]: 1) LMB covalently and selectively binds the sulfhydryl group of Cys-529 of CRM1; 2) LMB inhibits nuclear export by inhibition of the interaction of CRM1 with the nuclear export sequence; 3) CRM1 is the major protein that binds LMB in vitro and in vivo; 4) to our knowledge, there are no reports on other targets of LMB.[17,29] We cannot exclude the possibility that there may be other unknown cellular targets that may be partly inhibited by LMB. However, although many research groups are using LMB as a tool to investigate nuclear-cytoplasmic export, to date, these targets have not been identified.

It also has been reported that LMB has potent antiproliferative and antitumor properties in murine experimental tumors and in first clinical trials for anticancer therapy.[3,8] LMB causes a G1 cell cycle arrest in mammalian cells,[3] which is in line with our observation in ovarian cancer cells. Moreover, we observed that LMB induces apoptosis in ovarian cancer cells as measured by different assays. Other studies have reported LMB-induced apoptosis in cancer cells (eg, prostate cancer and leukemia cells) by nuclear accumulation of NES-containing p53, BCR-ABL, or the proapoptotic bcl2 member, BOK.[13,31] Experiments with U937 leukemia cells indicated that the apoptosis by LMB seemed to be related to cytochrome c release, activation of caspases, and selective down-regulation of myeloid cell leukemia 1 and X-linked inhibitor of apoptosis. Otherwise, it also is conceivable that CRM1 overexpression inhibits or delays LMB apoptosis.[31]

In fact, inhibiting the nuclear protein export of elements of signaling pathways that are important for tumor progression, such as p53, COX-2, and others, may play an important role in anticancer treatment; and substances such as LMB appear to be promising candidates for therapeutic interference with these cellular functions. Because strong toxic side effects have been encountered with LMB during clinical trials for cancer, many efforts have focused on novel CRM1 antagonists; and some agents, such as PKF050-638 or 5219668, recently have been identified.[10]

The results of the current study demonstrated that CRM1 expression is associated with advanced ovarian carcinomas that have an aggressive behavior. Thus, CRM1 expression is related to poor patient outcome. Furthermore, we demonstrated a correlation between CRM1 and COX-2 expression that may explain the unfavorable prognosis of patients with ovarian cancer. Supporting this point, we demonstrated that treatment of ovarian cancer cells with LMB revealed a reduction of COX-2 expression. Because it has been demonstrated that LMB acts as a specific inhibitor of CRM1 in other cellular models, this finding suggests that CRM1 may be an interesting biomarker for assessing patients' prognosis and a molecular target for anticancer treatment.

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