

Osteopontin as a Potential Diagnostic Biomarker for Ovarian Cancer

Jae-Hoon Kim, MD

Steven J. Skates, PhD

Toshimitsu Uede, MD

Kwong-kwok Wong, PhD

John O. Schorge, MD

Colleen M. Feltmate, MD

Ross S. Berkowitz, MD

Daniel W. Cramer, MD, ScD

Samuel C. Mok, PhD

OVARIAN CANCER RANKS AS the fifth leading cause of death from cancer and has the highest mortality rate among the gynecologic malignancies in the United States.¹ Every year approximately 25 000 ovarian cancer cases are newly diagnosed and approximately 15 000 deaths occur secondary to the malignancy.² This high case-fatality ratio reflects the fact that most ovarian cancer cases are detected at stage III or IV, at which the 5-year survival rate is below 30% compared with 95% with stage I.³ To improve survival, it would be desirable to develop tumor markers that could be used to detect the early stages of the disease.

The development of high-throughput complementary DNA (cDNA) microarray techniques offers great potential for identifying novel and specific biomarkers for the early detection of cancer.⁴⁻⁶ Using these techniques, potential serum markers for several cancers have been identified.⁶⁻⁹ In our study with the MICROMAX (PerkinElmer Life Sciences [previously NEN Life Sciences], Boston, Mass) cDNA microarray system using RNA isolated from several ovarian cancer cell

Context Development of new biomarkers for ovarian cancer is needed for early detection and disease monitoring. Analyses involving complementary DNA (cDNA) microarray data can be used to identify up-regulated genes in cancer cells, whose products may then be further validated as potential biomarkers.

Objective To describe validation studies of an up-regulated gene known as osteopontin, previously identified using a cDNA microarray system.

Design, Setting, and Participants Experimental and cross-sectional studies were conducted involving ovarian cancer and healthy human ovarian surface epithelial cell lines and cultures, archival paraffin-embedded ovarian tissue collected between June 1992 and June 2001, and fresh tissue and preoperative plasma from 144 patients evaluated for a pelvic mass between June 1992 and June 2001 in gynecologic oncology services at 2 US academic institutions. Plasma samples from 107 women selected from an epidemiologic study of ovarian cancer initiated between May 1992 and March 1997 were used as healthy controls.

Main Outcome Measures Relative messenger RNA expression in cancer cells and fresh ovarian tissue, measured by real-time polymerase chain reaction as $2^{-\Delta\Delta CT}$ (a quantitative value representing the amount of osteopontin expression); osteopontin production, localized and scored in ovarian healthy and tumor tissue with immunohistochemical studies; and amount of osteopontin in patient vs control plasma, measured using an enzyme-linked immunoassay.

Results The geometric mean for $2^{-\Delta\Delta CT}$ for osteopontin expression in 5 healthy ovarian epithelial cell cultures was 4.1 compared with 270.4 in 14 ovarian cancer cell lines ($P=.03$). The geometric mean $2^{-\Delta\Delta CT}$ for osteopontin expression in tissue from 2 healthy ovarian epithelial samples was 9.0 compared with 164.0 in 27 microdissected ovarian tumor tissue samples ($P=.06$). Immunolocalization of osteopontin showed that tissue samples from 61 patients with invasive ovarian cancer and 29 patients with borderline ovarian tumors expressed higher levels of osteopontin than tissue samples from 6 patients with benign tumors and samples of healthy ovarian epithelium from 3 patients ($P=.03$). Osteopontin levels in plasma were significantly higher ($P<.001$) in 51 patients with epithelial ovarian cancer (486.5 ng/mL) compared with those of 107 healthy controls (147.1 ng/mL), 46 patients with benign ovarian disease (254.4 ng/mL), and 47 patients with other gynecologic cancers (260.9 ng/mL).

Conclusions Our findings provide evidence for an association between levels of a biomarker, osteopontin, and ovarian cancer and suggest that future research assessing its clinical usefulness would be worthwhile.

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lines and healthy human ovarian surface epithelial (HOSE) cell cultures, we identified a spot with an ovarian cancer/HOSE ratio of 184 that corresponded to a protein called osteopontin.⁶ We present the experimental and clinical stud-

Author Affiliations and Financial Disclosures are listed at the end of this article.

Corresponding Author and Reprints: Samuel C. Mok, PhD, Laboratory of Gynecologic Oncology, Division of Gynecologic Oncology, Department of Obstetrics, Gynecology, and Reproductive Biology, Brigham and Women's Hospital, BLI-447, 221 Longwood Ave, Boston, MA 02115 (e-mail: scmok@rics.bwh.harvard.edu).

ies addressing the potential clinical usefulness of this marker.

METHODS

Cell Lines and Cultures

All ovarian cancer cell line material was obtained either by scraping the surfaces of healthy ovaries, recovery of material from ascites, or procurement of explanted tissue from solid tumors as described previously.¹⁰ Five healthy HOSE cell cultures used in this experiment were HOSE695, HOSE697, HOSE713, HOSE726, and HOSE730. Fourteen ovarian cancer cell lines were used: ALST, CAOV3, DOV13, OVCA3, OVCA420, OVCA429, OVCA432, OVCA433, OVCA633, and SKOV3 originated from serous cystadenocarcinoma; RMUG-L and RMUG-S originated from mucinous cystadenocarcinoma; and RMG-1 and ES-2 originated from clear-cell carcinoma. All cell cultures and cell lines were established in the Laboratory of Gynecologic Oncology, Brigham and Women's Hospital, Boston, Mass, except OVCA3, SKOV3, RMUG-L, RMUG-S, RMG-1, and ES-2, which were purchased from the American Type Culture Collection (Rockville, Md) and the Japanese Collection of Research Bioresources (Tokyo, Japan), respectively.

Biosamples

All patient-derived specimens were collected and archived under protocols approved by the institutional review boards (IRBs) of the parent institutions. Paraffin blocks (n=99) of ovarian cancer and healthy tissue from the Brigham and Women's Hospital pathology department archives were collected between June 1992 and June 2001, representing a cross-section of histologic types and grades seen at the institution. The blocks were collected under a separate "discarded material" IRB protocol allowing study of such material provided there was linkage only to diagnostic details such as tumor stage and grade. A separate bank of plasma and fresh frozen tissue, representing a different group of patients and for which written informed consent was required, was obtained from

144 women evaluated for a pelvic mass at the practices of 2 oncologists (J.O.S. and R.S.B.) at the Brigham and Women's Hospital between June 1992 and June 2001, and at the University of Texas Southwestern Medical Center, Dallas, between December 2000 and June 2001. The patients represent a cross-section of women seen in these practices and were selected from those women who consented to the protocol and had non-emergent surgery when technical support to collect the specimens was available. The 144 case patients were divided into those with epithelial ovarian tumors (n=51), other gynecologic cancers (n=47), and benign disease (n=46). All tumor tissue contained less than 20% healthy tissue and was collected from the primary ovarian sites and, if possible, metastatic sites. Control plasma specimens (n=107) were derived from an IRB-approved epidemiologic study of ovarian cancer initiated between May 1992 and March 1997. These controls were selected to match 5-year age groups in cases from 496 plasma specimens still available from an original total of 523 from the epidemiologic study and involved women selected from the general population of Massachusetts and New Hampshire.^{11,12} A match for all 144 case patients was not sought but the number of controls was chosen to be at least twice as large as the number of individuals in the separate case groups.

For fresh-frozen sections, fresh specimens were embedded in Tissue Tek OCT medium (Miles, Elkhart, Ind), snap-frozen in liquid nitrogen, and stored at -80°C until use. The archival tissues in paraffin blocks were retrieved from pathology files in the Laboratory of Gynecologic Oncology at the Brigham and Women's Hospital. The plasma samples were centrifuged at 2000g at 4°C for 15 minutes. The separated plasma was removed, aliquoted, and frozen at -80°C for future analysis.

Laser Capture Microdissection

Tissues stored in Tissue Tek OCT (Miles) medium at -80°C were sectioned at 7 µm in a cryostat (Leica, Allendale, NJ). Sections were mounted on

uncoated glass slides and immediately fixed in 70% and 50% ethanol for 30 seconds in each, stained with hematoxylin-eosin, dehydrated in alcohol solutions of increasing concentration, and cleared in xylene for 5 minutes in each. After being air-dried for 3 minutes, the sections were laser microdissected using the PixCell II (Arcturus, Calif). Tissue with morphologically healthy ovarian epithelial cells and malignant epithelial ovarian cancer cells was procured, representing 27 cases with ovarian cancer tissue and 2 cases with healthy ovarian tissue. These samples were selected from enrolled cases with sufficient material for study, representing a range of histologic types.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was used for analysis of cell cultures and lines and microdissected fresh tissues. Total RNA extraction and cDNA synthesis were performed as described previously.¹³ For the quantitative RT-PCR studies, a total of 2 µL of cDNA was used in a 23-µL PCR mix containing 1X SYBR PCR buffer, 3 mM MgCl₂, 0.8 mM dNTP, and 0.025 U/µL AmpliTaq Gold (PE Applied Biosystems, Foster City, Calif). Amplification was then performed in duplicate using primer sets, which we developed for osteopontin and were manufactured by and purchased from Sigma Genosys (Woodland, Tex) (forward primer: 5'-AAGCGAGGAGTTGAATGGTGCAT-3'; reverse primer: 5'-TGTGGGTTTCAGCACTCTGCTTCAT-3') and a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a control in an ABI PRISM 5700 Sequence Detector (PE Applied Biosystems) with denaturation for 10 minutes at 95°C followed by 40 PCR cycles of denaturation at 95°C for 15 seconds and annealing or extension at 60°C for 1 minute. The ABI5700 system software monitored the changes in fluo-

rescence of SYBR Green I dye in every cycle. The threshold cycle (C_T) value for each reaction, reflecting the amount of PCR needed to identify a target gene, and the relative level of osteopontin for each sample were calculated as described.¹³⁻¹⁵ Briefly, *GAPDH* was used for the normalization of the quantity of RNA used. Its C_T value was then subtracted from that of the osteopontin gene to obtain a ΔC_T value. The difference ($\Delta\Delta C_T$) between the ΔC_T values of the samples for the gene target and the ΔC_T value of the calibrator (HOSE697 for cell lines or 741A for tissue) was determined. These calibrators were chosen because they had the lowest expression values for cell lines and tissues, respectively. The relative quantitative value was expressed as $2^{-\Delta\Delta C_T}$, representing the amount of osteopontin expression (normalized to a reference [endogenous]), relative to the calibrators.¹⁵ Specimens were run in duplicate and the values averaged. To confirm the specificity of the PCR reaction, PCR products were electrophoresed on a 1.2% agarose gel.

Immunohistochemistry

The paraffin block specimens used in this experiment were derived from archived tissue (not representing samples from patients contributing fresh specimens) and consisted of tissue from 3 healthy ovaries, 6 benign ovarian tumors (1 serous, 4 mucinous, and 1 mixed), 29 borderline ovarian cancers (13 serous, 14 mucinous, 1 clear cell, and 1 mixed), and 61 invasive ovarian cancers (21 serous, 21 mucinous, 6 endometrioid, 8 clear cell, 2 undifferentiated, and 3 mixed). The archived paraffin blocks were derived from patients having surgery at the Brigham and Women's Hospital. Blocks were selected to represent a cross-section of histologic types and grades, overselecting for early-stage cases. There were 42 blocks containing International Federation of Gynecology and Obstetrics (FIGO) stage I/II tumor and 33 blocks with stage III/IV tumor included in immunohistochemical studies.

Immunohistochemical studies were performed using the avidin-biotin

method, as described previously.¹³ Sections were incubated with primary mouse monoclonal antibody directed against osteopontin (2 $\mu\text{g}/\text{mL}$) for 60 minutes at 37°C (Immuno-Biological Laboratories [IBL], Gumma, Japan). The control sections were treated in parallel but incubated with normal mouse serum (as a negative control) instead of the primary antibody. All sections were incubated in a moist chamber. Sections were then incubated with a biotinylated secondary antibody for 30 minutes (Vector Laboratories, Burlingame, Calif). After 30 minutes of incubation in avidin-biotin complex, the reaction product was visualized by 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories). Finally, sections were dehydrated and cleared in ethanol and xylene and mounted in SP15-500 Permount (Fisher Scientific, Pittsburgh, Pa). Representative photomicrographs were recorded by a digital camera (Optronix, Goleta, Calif). Intensity of staining was quantified using a semiquantitative scoring system as described.¹⁶

Enzyme-Linked Immunosorbent Assay

Levels of osteopontin in plasma samples were measured with a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) using a commercially available kit, which has interassay coefficient of variation varying from 0.7% to 2.0% and intraassay coefficient of variation varying from 3.7% to 4.7% depending on the level of the marker (Code No. 17158, IBL). Microplates were first precoated with anti-human osteopontin rabbit IgG (100 μL of 20 $\mu\text{g}/\text{mL}$ in 0.1-M carbonate buffer; pH, 9.5) and blocked with 1% bovine serum albumin and 0.05% Tween 20. Plasma and standard osteopontin samples were diluted with 1% bovine serum albumin and 0.05% Tween 20 in phosphate-buffered saline and incubated for 1 hour at 37°C. After 7 washes with 0.05% Tween 20 in phosphate buffer, horseradish peroxidase-labeled conjugated anti-human osteopontin (10A16) mouse monoclonal an-

tibody (100 μL of 2 ng/mL) was added and incubated for 30 minutes at 4°C. After 9 washes, 100 μL of tetramethyl benzidine buffer was added and the signal was allowed to develop for 30 minutes at room temperature. The reaction was stopped with 100 μL of 1 N sulfuric acid. The absorbance at 450 nm was measured by an automatic ELISA reader (Biorad, Hercules, Calif).

Results were converted from the mean absorbance of duplicate wells after subtraction of background values. Recombinant human osteopontin protein (IBL) was used as a standard. The standard curve was prepared simultaneously with the measurement of test samples. Reagent blank, test sample blank, and internal controls of plasma samples were used to normalize osteopontin values obtained from each experiment.

Statistical Analysis

The relative measures of osteopontin expression, $2^{-\Delta\Delta C_T}$, as measured using RT-PCR in cancer and healthy cells or cancer and healthy tissue were compared with unpaired *t* tests on log-transformed values. Immunohistochemistry scores were compared using the nonparametric Kruskal-Wallis test for overall group differences. Osteopontin has plasma levels that range over multiple orders of magnitude; therefore, a logarithmic transformation was used to change to an arithmetic scale. Simultaneously, the transformed data complied more accurately with the assumptions of a Gaussian distribution for residuals in general linear models. Data were summarized with the number of observations, the geometric mean (*P* values were applied appropriately to differences in the log [osteopontin] levels), the 95% confidence intervals (CIs) for the geometric mean, and the range of the data. The general linear model was used to determine differences in means among groups (eg, diagnostic groups or within ovarian cancers for the histologic groups) and age was added to the model to adjust for any age effects on osteopontin levels. The ELISA levels were compared using the Kruskal-Wallis test

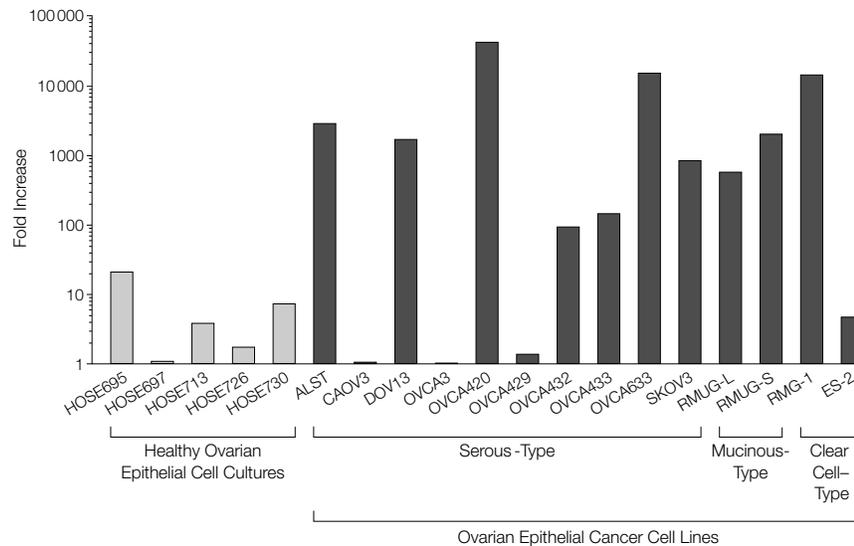
or Mann-Whitney *U* test among groups. The level of critical significance was considered to be $P < .05$.

For reporting specificity and sensitivity estimates, there are a variety of arbitrary choices, including present-

ing the full receiver operating characteristic curve, or fixing sensitivity at an arbitrary value and estimating the specificity, or fixing specificity at an arbitrary value and estimating sensitivity. The latter choice was made for this re-

port with a choice of specificity of 80%. First, the nearest cutoff for osteopontin corresponding to this level of specificity is determined, then the sensitivity is estimated as the number of patients with disease for a given subgroup with osteopontin levels exceeding the cutoff divided by the number of patients with disease in the given subgroup. The actual specificity may vary slightly from the target value ($80\% = 4/5$) because the number of case patients is not (in this study) divisible by 5. All analyses were performed using SPSS version 9.0 (SPSS Inc, Chicago, Ill) and S-PLUS (Insightful Inc, Seattle, Wash).

Figure 1. Relative Quantitation of Osteopontin in Healthy Ovarian Epithelial Cell Cultures and Malignant Ovarian Epithelial Cancer Cell Lines

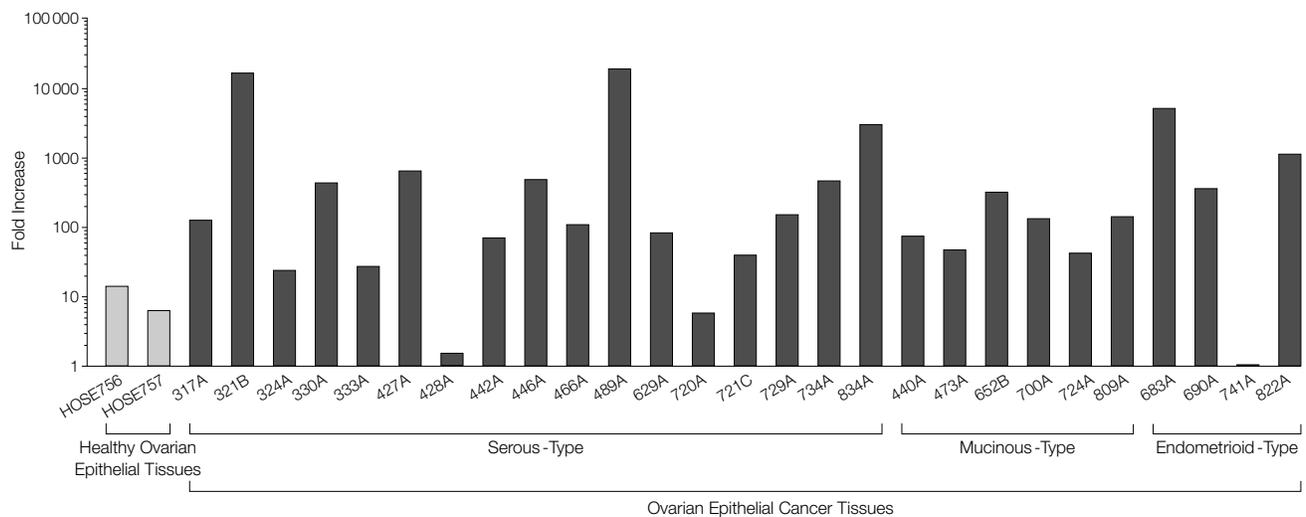


Statistically significant differences were obtained between 5 healthy human ovarian surface epithelial (HOSE) cell cultures, 10 serous-type ovarian cancer cell lines, 2 mucinous-type ovarian cancer cell lines, and 2 clear cell-type ovarian cancer cell lines by unpaired *t* test ($P = .03$). Each value was expressed as the mean of duplicate. The referent was HOSE697, considered to have a value of 1.

RESULTS

To validate overexpression of osteopontin in ovarian cancer, real-time RT-PCR was applied to an expanded series of ovarian epithelial cell cultures and cancer cell lines and tissues. Based on the $\Delta\Delta C_T$ relative to the healthy cell culture, HOSE697, the relative expression levels of osteopontin messenger RNA (mRNA) in other cell lines were calculated (FIGURE 1). The geometric means of healthy and cancer cell lines were 4.1 and 270.4 ($P = .03$). Except for CAO3, OVCA3, OVCA429, and ES-2,

Figure 2. Relative Quantitation of Osteopontin in Healthy Ovarian Epithelial Tissues and Malignant Ovarian Epithelial Cancer Tissues



Expression differences were obtained between samples of 2 healthy ovarian epithelial tissues, 17 serous-type ovarian cancer tissues, 6 mucinous-type ovarian cancer tissues, and 4 endometrioid-type ovarian cancer tissues by unpaired *t* test ($P = .06$). Each value was expressed as the mean of duplicate. The referent was 741A, considered to have a value of 1.

the other 10 ovarian cancer cell lines showed high levels of osteopontin expression. There was no significant difference in osteopontin expression among cell lines derived from different histologic subtypes (Figure 1).

Osteopontin expression in microdissected ovarian cancer tissues was also examined using real-time RT-PCR with 741A as the reference. Higher osteopontin expression in the 27 ovarian cancer tissues was observed compared with expression in the 2 samples of healthy ovarian surface epithelium. The geometric mean of the 2 groups was 9.0 and 164.0 with logarithmic transformation, respectively. The statistical difference was nonsignificant, perhaps due to the limited number of healthy samples examined ($P = .06$). There was no significant difference in osteopontin expression among tumors of different subtypes (FIGURE 2).

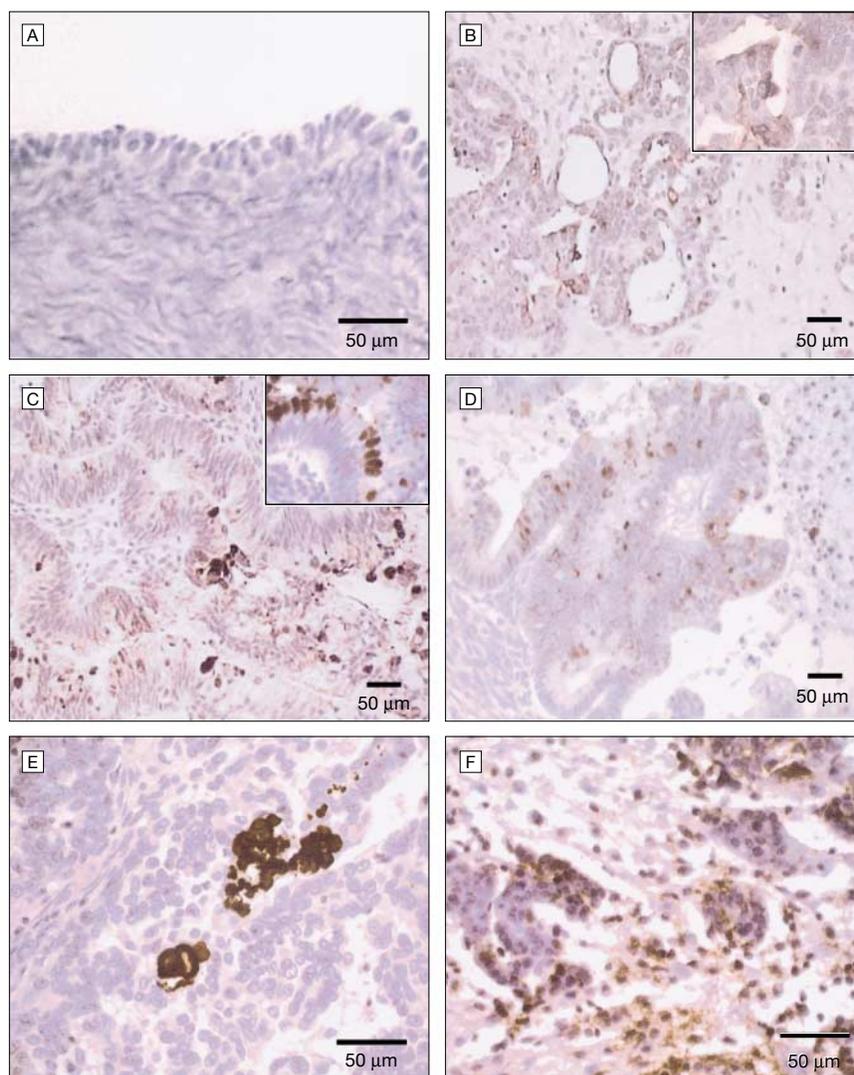
Osteopontin protein levels in paraffin block sections of 3 healthy tissues, 6 benign ovarian tumors, 29 borderline ovarian cancers, and 61 invasive ovarian cancers were assessed via immunohistochemistry studies. Osteopontin immunoreactivity was not observed in epithelium and stroma of the healthy ovarian tissues (score 0). Most positive staining observed in cancer tissues was localized to the cellular membrane and cytoplasm, extracellular matrix component, or psammoma bodies with some variations by histologic subtypes. Serous types mainly showed immunopositivity in psammoma bodies with occasional or focal cytoplasmic staining. Immunopositivity in mucinous type was localized to the cytoplasmic, especially pericytoplasmic, region. Abundantly stained extracellular materials suggested that osteopontin was secreted from mucinous ovarian cancer cells. Endometrioid and clear cell subtypes showed focal cytoplasmic localization (FIGURE 3).

The immunostaining scores in tissue sections from healthy ovary, benign ovarian tumor, borderline ovarian tumor, and invasive ovarian cancer were 0.0 (95% CI, 0.0-0.0), 0.7 (95% CI, 0.0-1.8), 2.2 (95% CI, 1.5-2.9), and 2.1 (95% CI, 1.7-

2.6), respectively (TABLE 1). The difference among diagnostic groups was statistically significant ($P = .03$) as well as the differences among histologic groups ($P = .01$) with mucinous cases having higher expression. Among the cancer (borderline and invasive) group, there was no significant difference in osteopontin immunoreactivity among different grades and stages.

Finally, we examined osteopontin levels in plasma samples obtained from 107 healthy controls, 46 patients with benign disease, 47 patients with gynecologic cancers other than ovarian cancers, and 51 patients with ovarian cancer using ELISA. The mean ages for the groups by category were 50.8 years for healthy, 54.6 years for benign disease, 50.9 years for other

Figure 3. Immunolocalization of Osteopontin in Healthy and Malignant Ovarian Tissues



Osteopontin expression is indicated by brown staining and was immunolocalized using an anti-osteopontin monoclonal antibody and an avidin-biotin peroxidase complex method with diaminobenzidine as the chromogen. The sections were counterstained with hematoxylin. A, Healthy ovarian surface epithelial cells with no expression of osteopontin. B, Endometrioid cystadenocarcinoma with osteopontin expression in cancer cells. C, Borderline mucinous tumor with osteopontin expression in cell membrane and cytoplasmic area. D, Invasive mucinous cystadenocarcinoma. E, Serous cystadenocarcinoma with osteopontin expression in psammoma body. F, Serous cystadenocarcinoma with osteopontin expression in cancer cells.

Table 1. Expression of Osteopontin in Relation to Histopathologic Characteristics in Immunohistochemical Analyses*

Characteristics	No. of Patients	Scores		P Value
		Geometric Mean (95% Confidence Interval)	Range	
All patients	99	2.0 (1.7-2.4)	0-6	
Diagnostic category				.03
Healthy	3	0.0 (0.0-0.0)	0-0	
Benign	6	0.7 (0.0-1.8)	0-2	
Borderline	29	2.2 (1.5-2.9)	0-6	
Invasive	61	2.1 (1.7-2.6)	0-6	
Histology of cancer				.01
Serous	34	1.3 (0.8-1.9)	0-4	
Mucinous	35	3.0 (2.4-3.6)	0-6	
Endometrioid	5	2.2 (0.0-4.4)	0-5	
Clear cell	10	2.3 (1.1-3.5)	0-6	
Undifferentiated	2	1.0 (0.0-6.0)	0-2	
Mixed	4	2.3 (1.5-3.1)	2-3	
Tumor differentiation†				.45
Borderline	28	2.2 (1.5-2.9)	0-6	
Well	11	2.6 (1.2-4.1)	0-6	
Moderate	17	2.1 (1.2-3.0)	0-6	
Poor	19	1.5 (0.9-2.2)	0-4	
FIGO stage†				.16
I	36	2.2 (1.7-2.8)	0-6	
II	6	2.7 (0.0-5.3)	0-6	
III	30	2.0 (1.3-2.6)	0-6	
IV	3	0.0 (0.0-0.0)	0-0	

*FIGO indicates International Federation of Gynecology and Obstetrics. The scoring system used corresponded to the sum of both staining intensity (3+, strong positive stain in most cells; 2+, moderate stain in cells; 1+, weak stain in cells; 0, no evidence of stain) and percentage of positive cells (3+, most of described cells stained; 2+, half of cells stained; 1+, few cells stained; 0, no cells stained).¹⁶ Differences between groups were evaluated by the sum of intensity and cell count scores in each. P values were for overall group differences.

†There were 15 cases that could not be categorized.

gynecologic cancers, and 55.4 years for ovarian cancer. Because the age difference between the ovarian cancer patients and healthy controls was marginally significant ($P = .04$), P values for the differences among mean (logarithm) osteopontin levels are presented from linear models that include a term for age.

The mean osteopontin level for all of the patients was 230.9 ng/mL (95% CI, 130.2-409.3 ng/mL) and in the categories of healthy controls, benign disease, other gynecologic cancers, and ovarian cancer, the mean levels were 147.1 ng/mL (95% CI, 99.4-217.7 ng/mL), 254.4 ng/mL (95% CI, 148.2-436.7 ng/mL), 260.9 ng/mL (95% CI, 127.8-532.7 ng/mL), and 486.5 ng/mL (95% CI, 315.0-751.4 ng/mL), respectively (FIGURE 4). The difference in osteopontin level between ovarian cancer cases and healthy controls was

statistically significant ($P < .001$) (TABLE 2). There were no significant differences among histologic types ($P = .11$), different histologic grades ($P = .26$), and stages ($P = .17$) of ovarian cancer (Table 2).

Using a cutoff value of 252 ng/mL, the specificity was 80.4% and the sensitivity point estimates for the detection of early-stage (I/II) and late-stage (III/IV) ovarian cancer were 80.4% and 85.4%, respectively.

COMMENT

Because of the tendency for ovarian cancer to produce clinical symptoms at a late stage, there is a need for better early detection methods. Although a number of tumor markers have been identified and studied for ovarian cancer,¹⁷⁻²² a useful screening marker for ovarian cancer has not been clearly established. The most widely re-

searched marker, CA125, has shown some value in postmenopausal women in pilot screening studies but sensitivity for early-stage disease before clinical detection remains questionable.²³ Thus, a marker of advanced disease may not translate well to identification of early asymptomatic disease.

One approach to meeting the need for new markers is the use of cDNA microarray technology to identify up-regulating genes in ovarian cancer cells or tissues. Previously, we described the cDNA MICROMAX microarray system to identify up- or down-regulated genes in ovarian cancer using RNA pooled from several healthy HOSE cell cultures and ovarian cancer cell lines.⁶ We described herein the additional studies necessary to establish the potential clinical relevance of one of the markers identified, osteopontin.

Osteopontin is an acidic, calcium-binding glycoposphoprotein that is found in all body fluids and in the extracellular matrix components.²⁴⁻²⁹ The molecular weight is 44 kd to 66 kd, depending on species and cell types.²⁴⁻²⁹ Osteopontin has been referred to as pp69, 44-kd bone phosphoprotein, 2ar, Eta-1, transformation associated phosphoprotein 1, and 2B7.²⁴⁻²⁹ It can function both as a cell adhesion protein and as a cytokine for several integrins and CD44.^{30,31} Furthermore, it has been shown to be involved in inflammation, especially in regulation of macrophages,³²⁻³⁷ tumorigenesis,^{28,38-45} and dystrophic calcification.^{39-41,46}

To further examine osteopontin's clinical potential, it was first necessary to show that osteopontin is expressed in individual cell lines and in actual cancer tissue. A technique involving real-time RT-PCR was used to measure gene expression in cells or tissue. Our study demonstrated that osteopontin was expressed in a number of ovarian cancer cell lines different from the ones previously used in the microarray experiment.⁶ We next used real-time RT-PCR with actual ovarian cancer tissue to demonstrate that expression was not an artifact associated with the culturing of ovar-

ian cancer cells in vitro. Real-time RT-PCR applied to microdissected ovarian cancer tissue also demonstrated increased osteopontin mRNA expression in the cancer tissue compared with healthy tissue, though the difference was not statistically significant. Another method for demonstrating expression is to use immunohistochemical studies to examine protein expression assessed through staining of tumor or healthy ovarian tissue. This technique offers the advantage of being able to use material archived in tumor blocks but does require the existence of an antibody to the overexpressed protein, which in this case was available.

Our immunohistochemistry results suggested that different histologic subtypes of ovarian cancer have different osteopontin expression patterns. In serous ovarian cancer, high levels of osteopontin were localized to the psammoma bodies, which are laminated calcified concretions commonly found in serous cancers. Curiously, the cancer cells expressed low levels of osteopontin protein, yet the real-time RT-PCR data on cell lines and microdissected tumors showed relatively high levels of osteopontin mRNA in the majority of samples of serous cancer cells. The discrepancy between osteopontin mRNA and protein levels may be explained by the fact that osteopontin protein may be actively secreted by serous epithelial cancer cells and, subsequently, translocated to the psammoma bodies resulting in calcium phosphate deposition. Recently, osteopontin mRNA has also been detected in CD68 protein macrophages infiltrating serous ovarian cancer tissues,⁴⁰ suggesting that tumor-infiltrating macrophages may also secrete osteopontin and are involved in the generation of psammoma bodies in serous cancers. A recent study of osteopontin expression in 16 serous borderline ovarian tumors and 14 serous invasive ovarian carcinomas showed a significantly higher level of expression in borderline tumors than carcinomas, suggesting that osteopontin may be impor-

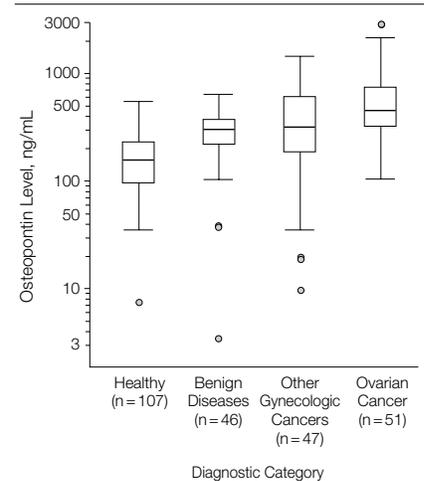
tant for the pathogenesis of serous borderline tumors.⁴¹

In contrast to serous-type ovarian cancer, higher osteopontin protein levels were detected in the cytoplasm of mucinous-type ovarian cancer cells, suggesting active osteopontin protein synthesis in these cells. Different osteopontin expression patterns in different histologic subtypes of cancer have also been described in lung and esophageal cancers.^{47,48} Osteopontin overexpression has been demonstrated in immunohistochemical studies of a number of other cancers including cancer of prostate, breast, and stomach.⁴¹⁻⁴⁵

Clearly, any proposed tumor marker must be identifiable in blood or other readily accessed biological samples. Using ELISA, we measured osteopontin levels in preoperative plasma from patients with epithelial ovarian cancer and plasma from healthy controls. Preoperative osteopontin levels were significantly higher in women with ovarian cancer compared with those in women not having ovarian cancer or those with benign pelvic disease. In addition, osteopontin levels were significantly

higher in patients with ovarian cancer than in patients with other types of gynecologic cancers (cervix and endometrium). We also observed no significant difference in osteopontin levels as

Figure 4. Osteopontin Levels in Case Patients Within 4 Diagnostic Categories



The box is bounded above and below by the 75th and 25th percentiles and the median is the line in the box. Whiskers are drawn to the nearest value not beyond a standard span from the quartiles; points beyond (outliers) are drawn individually, where the standard span is 1.5 × (interquartile range).

Table 2. Preoperative Osteopontin Levels in Patients With Ovarian Cancer vs Those of Controls and Patients With Benign or Other Gynecologic Disease*

Characteristics	No. of Patients	Osteopontin Level, ng/mL		P Value
		Geometric Mean (95% Confidence Interval)	Range	
All patients	251	230.9 (130.2-409.3)	3.4-2859.8	
Diagnostic category				<.001
Healthy	107	147.1 (99.4-217.7)	7.5-547.9	
Benign disease	46	254.4 (148.2-436.7)	3.4-640.8	
Other gynecologic cancers	47	260.9 (127.8-532.7)	9.7-1453.4	
Ovarian cancer	51	486.5 (315.0-751.4)	105.1-2859.8	
Histology of cancer				.11
Serous	31	451.6 (293.1-682.8)	105.1-1588.2	
Mucinous	3	384.0 (290.6-507.5)	274.6-640.2	
Endometrioid	5	379.4 (261.2-551.1)	210.1-843.9	
Clear cell	1	2130.5 (NA)	2130.5-2130.5	
Undifferentiated	3	538.0 (342.5-845.1)	250.4-1068.4	
Mixed	8	526.5 (357.2-776.1)	297.9-2166.0	
Tumor differentiation				.26
Borderline/well	10	373.9 (280.3-498.7)	210.1-843.9	
Moderate/poor	41	493.6 (321.6-757.5)	105.1-2166.0	
FIGO stage				.17
I/II	10	369.7 (279.8-488.5)	210.1-843.9	
III/IV	41	520.1 (329.0-822.3)	105.1-2859.8	

*NA indicates not applicable; FIGO, International Federation of Gynecology and Obstetrics. P values were calculated with analysis of variance performed on the logarithms of plasma osteopontin level data, including a term for age.

assessed via ELISA among serous, mucinous, clear cell, and endometrioid types of ovarian cancer, suggesting that osteopontin may be useful in detecting most subtypes of ovarian cancer. We believe preoperative sensitivity estimates may be sufficiently high to encourage pilot clinical screening studies, which would include osteopontin in a panel of putative early detection markers to obtain estimates of screening sensitivity and specificity. The crucial issue to be addressed is the sensitivity of a panel of biomarkers for detecting early-stage ovarian cancer or for screening subjects who would have been clinically detected in late-stage disease. Sensitivity estimates based on preoperative samples from clinically detected patients, either early- or late-stage disease, are no substitute for estimates obtained from prospectively conducted clinical trials and are at best a guide as to which markers to test in such a trial.

In conclusion, this investigation has demonstrated the potential value of the cDNA microarray analysis in identifying overexpressed genes in ovarian cancer and its subsequent link to a protein measurable in plasma. The findings suggest evidence for an association between plasma levels of osteopontin and ovarian cancer, pointing to a rationale for further research assessing potential clinical usefulness.

Author Affiliations: Department of Obstetrics and Gynecology, Saint Vincent Hospital and Catholic University of Korea, Suwon, Kyong-Ki-Do, Korea (Dr Kim); Department of Obstetrics, Gynecology, and Reproductive Biology, Brigham and Women's Hospital and Harvard Medical School (Drs Kim, Feltmate, Berkowitz, Cramer, and Mok), Massachusetts General Hospital Biostatistics Center and Harvard Medical School (Dr Skates), Dana-Farber Cancer Institute (Drs Skates, Berkowitz, and Cramer), and Dana-Farber Harvard Cancer Center (Drs Skates, Berkowitz, Cramer, and Mok), Boston, Mass; Institute for Genetic Medicine, Hokkaido University, Sapporo, Hokkaido, Japan (Dr Uede); Department of Pediatrics, Texas Children's Cancer Center, Baylor College of Medicine, Houston (Dr Wong); Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center, Dallas (Dr Schorge).

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Author Contributions: Study concept and design: Kim, Wong, Mok.

Acquisition of data: Kim, Uede, Schorge, Feltmate, Berkowitz, Cramer, Mok.

Analysis and interpretation of data: Kim, Skates, Berkowitz, Cramer, Mok.

Drafting of the manuscript: Kim, Skates, Uede, Berkowitz, Cramer, Mok.

Critical revision of the manuscript for important intellectual content: Skates, Wong, Schorge, Feltmate, Berkowitz, Cramer, Mok.

Statistical expertise: Kim, Skates.

Obtained funding: Cramer, Mok.

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It is through contact with living nature that we are reminded of the nonmechanical aspects of all living organisms, including ourselves, and can sense the independence, the unpredictableness, and the mystery of the living as opposed to the mechanical. Only by recognizing in man those characteristics which he shares with all living creatures can we base a recognition of his dignity.

—Joseph Wood Krutch (1893-1970)