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Identification of Epithelial Cell Adhesion Molecule Autoantibody in Patients with Ovarian Cancer

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ABSTRACT

The epithelial cell adhesion molecule (Ep-CAM) exhibited an ovarian cancer:normal human ovarian surface epithelium ratio of 444. For validation studies, real-time quantitative PCR analysis and immunohistochemistry were performed in normal and malignant ovarian epithelial cell lines and tissues. To evaluate the potential of the Ep-CAM autoantibody as a tumor marker, we examined the amount of Ep-CAM autoantibody in serum samples obtained from ovarian cancer patients and normal controls by an ELISA. Real-time quantitative PCR analysis revealed significant overexpression of Ep-CAM mRNA in cancer cell lines ($P <$ 0.001) and microdissected cancer tissues ($P <$ 0.05), compared with that in cultured normal human ovarian surface epithelium and microdissected germinal epithelium, respectively. Immunolocalization of the Ep-CAM autoantibody showed that the sera of ovarian cancer patients expressed higher levels of Ep-CAM autoantibody than benign tumor patients and normal controls ($P <$ 0.05). The levels of Ep-CAM autoantibody found were as follows: 0.132 in 52 patients with ovarian cancer, 0.098 in 26 cases with benign gynecologic disease, and 0.090 in 26 normal women. This investigation has shown that the Ep-CAM autoantibody was found to be associated with ovarian cancer and suggested that future research assessing its clinical usefulness would be worthwhile.

INTRODUCTION

Ovarian cancer has the highest mortality rate among all of the gynecologic malignancies. Every year, 25,000 ovarian cancer cases are newly diagnosed in the United States, and ~15,000 deaths, secondary to the malignancy, occur annually (1).

Despite intense efforts with cytoreductive surgeries and combined chemotherapeutic modalities, most advanced-stage ovarian cancer patients experience relapses and eventually die from disease (2). There have been continuous efforts in developing new drugs and treatment modalities. Nevertheless, the prognosis for advanced and recurrent ovarian cancers has not substantially changed (3). More than 70% of the patients are in stage 3 or stage 4 at the time of diagnosis (4). In present screening modalities, transvaginal sonography can detect early stage disease with great sensitivity, but it is expensive and has low specificity. Although serum marker assays could provide a less expensive and more convenient initial screening test, the sensitivity of assays is low and variable. Measurement of serum CA 125 with ultrasound screening confers high specificity but detects only 60% of early stage ovarian cancer (5). To improve survival, therefore, it is necessary to develop specific tumor markers that can be used to detect early disease.

In our previous cDNA microarray study, we identified a spot corresponding with a protein called Ep-CAM[2] (6). Because most ovarian cancers are of epithelial cell origin, deregulated epithelial antigens may be ideal candidate markers.

A known biological role of Ep-CAM is its relationship to homophilic cell adhesion (7). Like other adhesion molecules, Ep-CAM is known to be involved in the signaling cascade related to proliferation, differentiation, and apoptosis. The effect of Ep-CAM as a regulator of cadherin-mediated functions is to promote invasion and metastasis (8–10). However, it has also been shown to play a role as an adhesion molecule that suppresses metastasis by preventing cell scattering (11). Therefore,

[2] The abbreviations used are: Ep-CAM, epithelial cell adhesion molecule; HOSE, human ovarian surface epithelial cell; DNP, dinitrophenyl; CI, confidence interval; ROC, receiver operating characteristic.

Ep-CAM may have a bidirectional effect in the progression of malignancy.

Here, we show the use of microarray technology and subsequent validation studies to identify overexpression of Ep-CAM transcript and protein in ovarian cancer cells and tissues, and provide evidence that Ep-CAM autoantibody may be associated with ovarian cancer.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. All of the cell lines and cultures were maintained at 37°C in a humidified 5% CO_2 ambient air atmosphere. They were grown in Medium 199 and MCDB 105 (1:1; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA). Normal HOSE cultures were established by scraping the surface of the ovary, as described previously. (12) Eight normal HOSE cells used in this experiment were HOSE 17, HOSE 36, HOSE 642, HOSE 695, HOSE 697, HOSE 713, HOSE 726, and HOSE 730. Ovarian cancer cell lines were established either by recovery from ascites or explanted from solid tumors as described previously. (12) Ten ovarian cancer cell lines were used: OVCA3, OVCA420, OVCA429, OVCA432, OVCA433, OVCA633, CAOV3, DOV13, and ALST, as well as SKOV3. All of the cell cultures and cell lines were established in the Laboratory of Gynecologic Oncology, Brigham and Women's Hospital, except OVCAR-3 and SKOV-3, which were purchased from American Type Culture Collection (Rockville, MD).

Tissue and Serum Samples. All of the patients were treated at the Brigham and Women's Hospital between 1992 and 2000. We retrieved patients with ovarian tumors with different histological types and grades based on the WHO and the International Federation of Gynecology and Obstetrics criteria. All of the patient-derived biological specimens were collected and archived under protocols approved by the Brigham and Women's Human Subjects Committee or studied as an approved use of discarded human materials. All of the tumor tissues were collected from the primary ovarian sites from patients undergoing surgery. They contained less than 20% of normal tissue. In this experiment, 136 primary ovarian tumors were used.

For fresh-frozen sections, fresh specimens collected at the operating room were placed in tissue culture medium, Medium 199 and MCDB 105 (1:1) with 10% fetal bovine serum, and transported to the laboratory. After removing the nontumorous tissue, the specimens were immediately embedded in Tissue Tek OCT medium (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −80°C until use.

The archival tissues in paraffin blocks were collected from pathology files in the Laboratory of Gynecologic Oncology at the Brigham and Women's Hospital.

Preoperative serum samples from women with ovarian cancer and benign gynecologic disorders, and serum samples from nondiseased normal were obtained between 1999 and 2000. These specimens were stored at −80°C without any incident of thawing.

Laser Capture Microdissection. Tissues stored in Tissue Tek OCT medium at −80°C were sectioned at 7 μm in a cryostat (Leica, Allendate, NJ). Sections were mounted on uncoated glass slides and immediately fixed in 70% and 50% ethanol for 30 s in each, stained with H&E, dehydrated in an increased series of alcohol, and cleared in xylene for 5 min in each microdissection. Once air-dried for 3 min, the sections were laser microdissected with the PixCell II system (Arctarus, CA). Morphologically normal ovarian epithelial cells and malignant epithelial ovarian cancer cells were procured.

Microarray Probe and Hybridization. The MICRO-MAX human cDNA system I (NEN Life Science Products, Inc., Boston, MA), which contains 2400 known human cDNA on a $1 \times 3''$ slide, was used in this study as described (6). Biotin-labeled cDNA was generated from 3 μg total RNA, which was pooled from HOSE17, HOSE36, and HOSE642. DNP-labeled cDNA was generated from 3 μg total RNA that was pooled from ovarian cancer cell lines OVCA 420, OVCA 433, and SKOV3. Before the cDNA reaction, an equal amount of RNA controls (nonhuman RNA from Arabidopsis and bacteria) were added to each batch of the RNA samples for normalization. The biotin-labeled and DNP-labeled cDNA were mixed, dried, and resuspended in 20 μl hybridization buffer, which was added to the cDNA microarray and covered with a coverslip. Hybridization was carried out overnight at 65°C inside a hybridization cassette (Telechem, Inc., Sunnyvale, CA).

After hybridization, the microarray was washed with 30 ml $0.5 \times$ SSC, 0.01% SDS, and then 30 ml $0.06 \times$ SSC, 0.01% SDS, and finally, $0.06 \times$ SSC alone. The hybridization signal from biotin-labeled cDNA was amplified with streptavidin-horseradish peroxidase and Cy5-tyramide, whereas hybridization signal from DNP-labeled cDNA was amplified with anti-DNP-horseradish peroxidase and Cy3-tyramide. After the posthybridization wash, the cDNA microarray was air-dried and signal amplification was detected with a laser scanner.

Laser detection of the Cy3 signal (derived from ovarian cancer cells) and Cy5 signal (derived from HOSE cells) on the microarray was acquired with a confocal laser reader, ScanArray3000 (GSI Lumonics, Watertown, MA). Separate scans were taken for each fluor at a pixel size of 10 μm. cDNA derived from the control RNA hybridized to 12 specific spots within the Microarray. Cy3 and cy5 signals from these 12 spots should theoretically be equal and were used to normalize the different efficiencies in labeling and detection with the two fluors. The fluorescence signal intensities and the Cy3:Cy5 ratios for each of the 2400 cDNAs were analyzed by the software Imagene 3.0 (Biodiscovery Inc., Los Angeles, CA).

Real-Time Quantitative Reverse Transcription-PCR. Total RNA extraction and cDNA synthesis were performed as described previously (13). mRNA was extracted from normal ovarian epithelial cell cultures (HOSE 695, HOSE 697, HOSE 713, HOSE 726, and HOSE 730), ovarian carcinoma cell lines (OVCA3, OVCA420, OVCA429, OVCA432, OVCA433, OVCA633, CAOV3, DOV13, SKOV3, and ALST), 3 normal ovarian epithelial tissues, and 13 ovarian cancer tissues.

Real-time PCR was performed in duplicate using primer sets specific for Ep-CAM (forward primer: 5′-CGTCAAT-GCCAGTGTACTTCAGTTG-3′; reverse primer: 5′-TCC-AGTAGGTTCTCACTCGCTCAG-3′) and a housekeeping
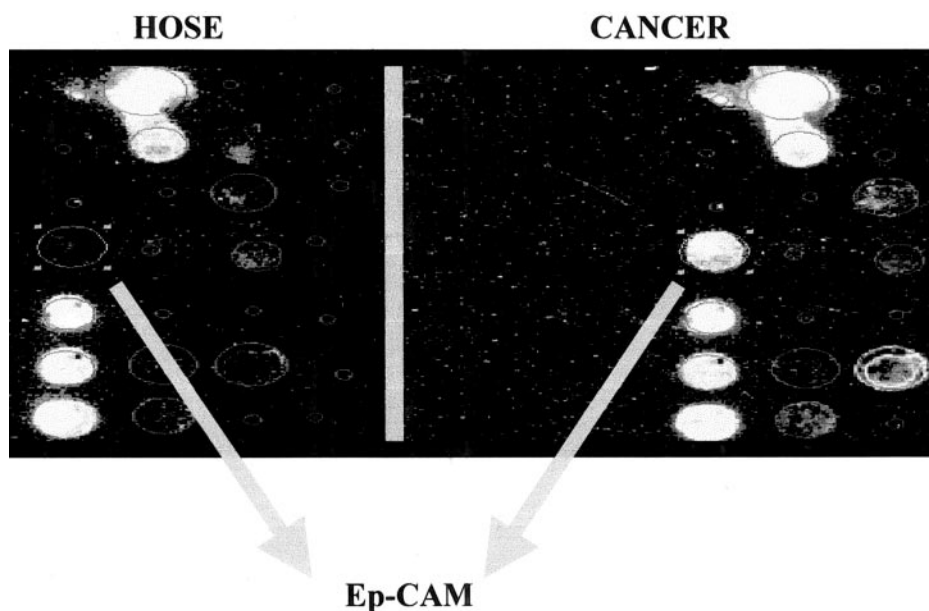
Fig. 1 Microarray analysis using pooled RNA isolated from 3 normal HOSE cultures and 3 ovarian cancer cell lines. *Arrows* indicate spots on two microarrays, which correspond to Ep-CAM.

gene, *GAPDH*, in an ABI PRISM 5700 Sequence Detector. cDNA was generated from 1 μg total RNA using the TaqMan reverse transcription reagents containing 1× TaqMan reverse transcription buffer, 5.5 mM MgCl$_2$, 500 μM dNTP, 2.5 μM random hexamer, 0.4 units/μl RNase inhibitor, and 1.25 units/μl MultiScribe reverse transcriptase (PE Applied Biosystems, Foster City, CA) in 100 μl. The reaction was incubated at 25°C for 10 min, 48°C for 30 min, and finally at 95°C for 5 min. A total of 0.5 μl of cDNA was used in a 20 μl PCR mix containing 1× SYBR PCR buffer, 3 mM MgCl$_2$, 0.8 mM dNTP, and 0.025 units/μl AmpliTaq Gold (PE Applied Biosystems). Amplification was then performed with denaturation for 10 min at 95°C, followed by 40 PCR cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The ABI5700 system software monitored the changes in fluorescence of SYBR Green I dye in every cycle, and the threshold cycle for each reaction was calculated as described (13).

Immunohistochemistry. Specimens used in this experiment consisted of 5 normal ovaries, 17 benign ovarian tumors, 52 borderline ovarian cancers (29 serous, 21 mucinous, 1 endometrioid, and 1 clear cell), and 67 invasive ovarian cancers (31 serous, 20 mucinous, 12 endometrioid, and 4 clear cell).

Immunostaining was performed by the avidin-biotin method, as described previously (13). Sections were incubated with mouse monoclonal antibody GA733 against Ep-CAM (2.35 μg/ml, 1:100 dilution; DAKO, Carpinteria, CA) for 60 min at room temperature. The negative control sections were treated in parallel but incubated with normal mouse serum instead of the primary antibodies. All of the sections were incubated in a moist chamber. Sections were then incubated with a biotinylated goat antimouse IgG antibody for 30 min (Vector Laboratories, Burlingame, CA). After incubation in avidin-biotin complex (Vector Laboratories) for 30 min, the reaction product was visualized by

3,3-diaminobenzidine tetrahydrochloride (Vector Laboratories). Finally, sections were dehydrated in ethanol, cleared in xylene, and mounted in SP15–500 Permount (Fisher Scientific).

Representative photomicrographs were recorded by a digital camera (Optronics, Goleta, CA). To evaluate the result, we established a score corresponding to the sum of both staining intensity (strong positive staining in most of cells, 3+; moderate staining in cells, 2+; weak staining in cells, 1+; and no evidence of staining, 0), and percentage of positive cells (most of cells demonstrating staining, 3+; half of cells demonstrating staining, 2+; few cells demonstrating staining, 1+; and no cells staining, 0), as described (14). Differences between groups were evaluated by the sum of intensity and cell count score. The slides were scored in the absence of any clinical data, and the final score reported was the average of the three observers.

ELISA. ELISA performed Immunodetection of Ep-CAM autoantibody, as described (15). Flat-bottomed microtiter ELISA plates (α Diagnostic, San Antonio, TX) were incubated at 4°C overnight with 100 μl purified Ep-CAM (2.5 μg/ml), purified with monoclonal antibody GA 733 as described (16), in 0.05 M carbonate buffer (pH 9.7). After washing three times with 5 mM Tris buffer (pH 7.8) with 0.15 mol of NaCl, 1 mmol of MgCl$_2$ and 8 mM of sodium azide, the wells were blocked for 1 h at 37°C with 200 μl 50 mmol/liter Tris buffer (pH 7.8) with 10 g of BSA/liter and washed three times. Serum samples were diluted 1:50 in 50 mmol/liter Tris Buffer (pH 7.8) with 60 g BSA and 0.5 g of sodium azide, and incubated overnight at 4°C. After washing six times, the wells were incubated for 2 h at room temperature with 100 μl horseradish peroxidase-conjugated goat antihuman IgG (Pierce, Rockford, IL) diluted 1:20,000 in 50 mmol/liter Tris buffer (pH 7.8) containing 60 g of BSA and 0.5 g of sodium azide/liter. After washing six times, 100 μl

Fig. 2 A, relative quantitation of Ep-CAM mRNA in normal and malignant epithelial ovarian cancer cell lines. Statistically significant difference was obtained between normal (HOSE 695, HOSE 697, HOSE 713, HOSE 726, and HOSE 730) and cancer cell lines (ALST, CAOV3, DOV13, OVCA3, OVCA 420, OVCA429, OVCA432, OVCA433, and OVCA633) by Mann-Whitney *U* test ($P < 0.001$). *B,* relative quantitation of Ep-CAM mRNA in normal and malignant ovarian cancer tissues. Statistically significant difference was obtained between normal (756HOSE, 757HOSE, and 763HOSE) and cancer tissues (330A, 333A, 426C, 427A, 466A, 489C, 629A, 683A, 690C, 720C, 721C, 734A, and 834A) by Mann-Whitney *U* test ($P < 0.05$). Each value was expressed as the mean of duplicate determinations; *bars,* ±SD.
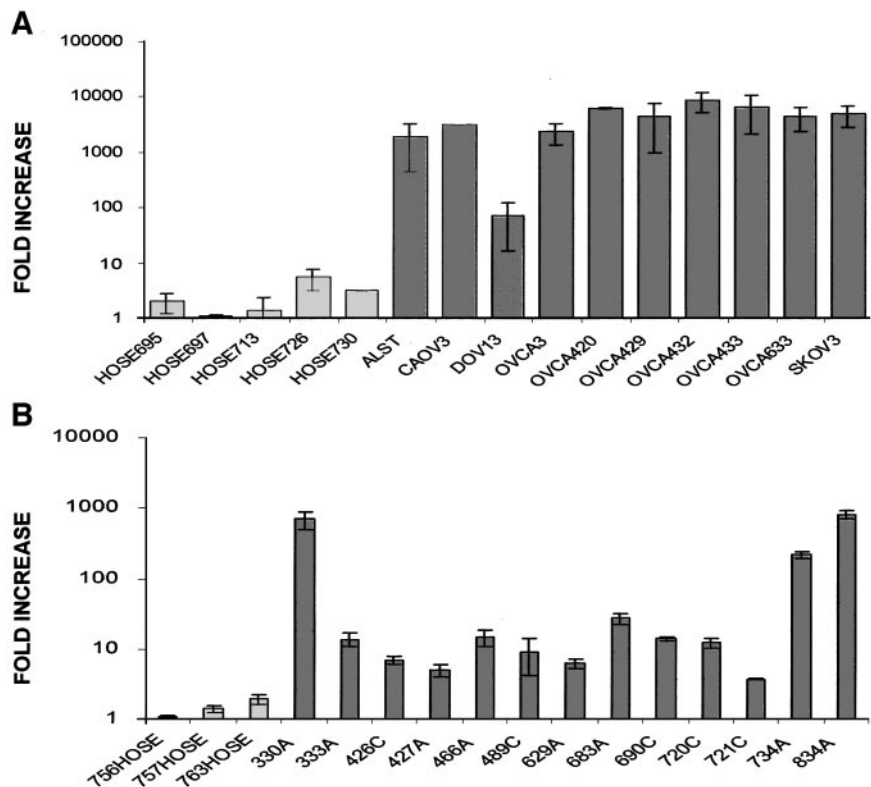
TMB (3,3,5.5′-tetramethyl-benzidine) substrate solution (α Diagnostic) was added for development at room temperature for 15 min. After the addition of stop solution, the absorbance at 450 nm was measured by an automatic ELISA reader (Bio-Rad, Hercules, CA).

The CA 125 assay was performed by an immunoradiometric assay according to the manufacturer's instructions (Abbot Diagnostics).

Results were expressed as the mean absorbance of triplicate wells after subtraction of background values. Negative controls include the elimination of purified Ep-CAM, patient serum, secondary antibody, or substrate for development in the assay.

Statistical Analysis. Data were mainly summarized as mean, SD, 95% CI, or range. Mann-Whitney *U* test was used to test statistical significance in real-time PCR. Immunohistochemistry and ELISA were tested by one-way ANOVA and Turkey's multiple comparison tests among groups.

To obtain the cutoff values, we performed ROC analysis. In cases of combined cutoff vale, the result of the logistic analysis using both Ep-CAM and CA 125 was used.

Sensitivity and specificity for serum samples were estimated nonparametrically with binomial point estimates and exact 95% CIs. Differences between two sensitivities (or specificities) on the same subjects were evaluated for significance with McNemar's test. Partial correlation coefficients adjusted by age were calculated between CA 125 and Ep-CAM autoantibody.

The level of critical significance was assigned at $P < 0.05$. All of the analyses were performed using SPSS version 9.0 (SPSS Inc., Chicago, IL).

RESULTS

Using RNA isolated from 3 normal HOSE cell lines and 3 ovarian cancer cell lines, we identified 30 genes with Cy3:Cy5 ratios >5 (5). One of these, with a Cy3:Cy5 ratio of 444, corresponded to a tumor-related protein called Ep-CAM. It is selectively illustrated in Fig. 1.

To validate the expression of Ep-CAM, real-time PCR was applied to an expanded series of ovarian cancer cell lines and tissues. On the basis of the $\Delta\Delta C_T$ relative to the normal cell line, HOSE 697, the expression of Ep-CAM in a variety of cell lines was calculated. There was a highly significant difference in the expression of Ep-CAM between 5 normal ovarian epithelial cell lines and 10 ovarian cancer cell lines ($P < 0.001$). The mean ± SD of normal and cancer cell lines were 2.63 ± 1.79 and 4265.61 ± 2522.14, respectively. Except for DOV13, the expression of EP-CAM in the other ovarian cancer cell lines was 1000-fold greater than that in HOSE 697 (Fig. 2*A*).

Ep-CAM expression in ovarian cancer tissues was also examined. We found a significant difference between the 3 normal ovarian surface epithelia and 13 ovarian cancer tissues ($P < 0.05$). The mean ± SD of the two groups was 1.68 ± 0.75 and 140.92 ± 277.91, respectively. (Fig. 2*B*). Ep-CAM immunoreactivity was not observed in the stroma of any of the specimens examined. Positive staining was mainly localized to the cellular membrane and cytoplasm of epithelial cells (Fig. 3).

The mean (and 95% CI) of immunostaining scores in normal ovary, benign ovarian tumor, borderline ovarian tumor, and invasive ovarian cancer were 0.80 (95% CI, 0.00–2.16),
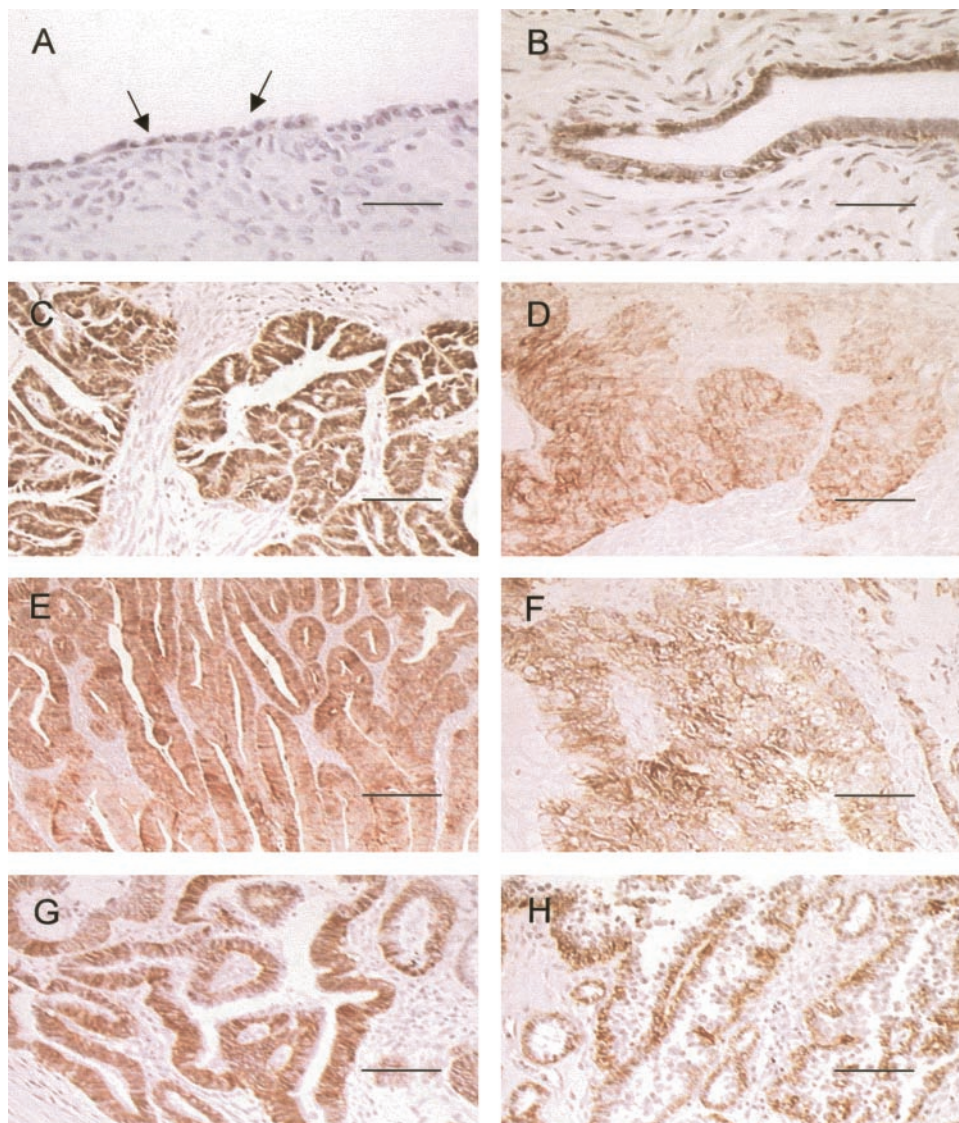
Fig. 3 Immunolocalization of Ep-CAM in normal and malignant ovarian tissues. *A,* normal ovarian surface epithelial cells (*arrowheads*); *B,* benign serous cystadenoma; *C,* serous borderline tumor; *D,* serous cystadenocarcinoma; *E,* mucinous borderline ovarian tumor; *F,* mucinous cystadenocarcinoma; *G,* endometrioid cystadenocarcinoma; *H,* clear cell cystadenocarcinoma. *Scale bar* represents 50 mm.

1.76 (95% CI, 1.07–2.46), 3.74 (95% CI, 3.27–4.21), and 3.34 (95% CI, 2.99–3.70), respectively. This difference among groups was statistically significant ($P < 0.001$). There was no statistical difference between borderline tumors and invasive cancers ($P = 0.174$; Table 1).

In the cancer group, no difference in Ep-CAM immunoreactivity among different histological types and grades was observed. It appeared that mucinous borderline cases represented relatively higher Ep-CAM expression, however, compared with any other cancer groups. Stage III and IV cases showed lower Ep-CAM expression, compared with stage I cases ($P < 0.01$; Table 1).

We examined the autoantibody of Ep-CAM by ELISA in sera of 26 normal controls, 26 patients with benign ovarian disease, and 52 ovarian cancer patients by ELISA. Normal controls matched for age with patients with benign ovarian disease and ovarian cancer with a mean age of 58 years old (range, 45–76). Reciprocal serum end point dilutions ranged

between 10 and 1000 among 3 cancer patients (Fig. 4). The schematic results are shown in Fig. 5. The mean (and 95% CI) of Ep-CAM autoantibody levels in normal controls, benign ovarian disease, and cancer patients were 0.090 (95% CI, 0.080–0.100), 0.098 (95% CI, 0.088–0.108), and 0.132 (95% CI, 0.123–0.141), respectively. The difference between cancer cases and the other cases was statistically significant ($P < 0.05$). In cancer patient serum, there were no significant difference in histological types and grades. The sera of stage IV cases showed lower levels of Ep-CAM autoantibody, compared with either stage I or II ($P < 0.05$; Table 2).

On the basis of the cutoff value as 0.140, which was defined as an absorbance >2 SDs above the mean value of the normal controls, 22 ovarian cancer cases (42.3%) were positive, whereas none of the control (0%) and 2 benign ovarian disease (7.7%) cases were positive.

We plotted a ROC to investigate optimal cutoff values and to compare Ep-CAM autoantibody with CA 125. On the

Table 1 The expression of Ep-CAM in relation to histopathologic characteristics in normal ovary, benign ovarian tumor, borderline ovarian tumor, and invasive ovarian cancer in immunohistochemical study

| Characteristics	No.	Scores[a]	95% CI[b]	Range	T[c]	P[d]
All subjects	141	3.17	2.88 to 3.46	0–6		
Diagnostic category						
Normal	5	0.80	0.00 to 2.16	0–2	a	$P < 0.001$
Benign	17	1.76	1.07 to 2.46	0–4	b	
Borderline	52	3.74	3.27 to 4.21	0–6	c	
Invasive	67	3.34	2.99 to 3.70	0–6	c	
Histology of cancer						
Serous	60	3.36	2.99 to 3.72	0–4		$P = 0.32$
Mucinous	41	3.88	3.32 to 4.43	0–6		
Endometrioid	13	3.31	2.44 to 4.18	0–5		
Clear cell/other	5	3.00	0.37 to 5.63	0–6		
Tumor differentiation						
Borderline	51	3.65	3.15 to 4.16	0–6		$P = 0.61$
Well	26	3.83	3.23 to 4.42	0–6		
Moderate	14	3.29	2.40 to 4.18	0–5		
Poor	28	3.36	2.86 to 3.85	0–6		
FIGO stage						
I	54	4.11	3.72 to 4.49	2–6	a	$P = 0.007$
II	8	4.33	2.90 to 5.77	2–6	a, b	
III	43	3.20	2.68 to 3.72	0–6	b	
IV	14	2.90	1.81 to 3.99	0–5	b	

[a] Values are given as a mean.
[b] CI, confidence interval.
[c] The same letters indicate nonsignificant difference between groups based on Tukey's multiple comparison test.
[d] Statistical significances were tested by ANOVA among groups.

Fig. 4 Absorbance values (mean ± SD) of diluted sera of 3 patients against the Ep-CAM protein. Each value was expressed as the mean of triplicate; *bars,* ±SD.
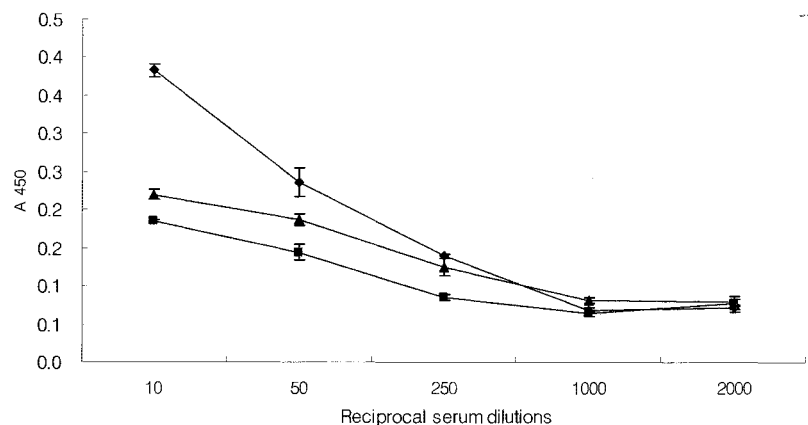
basis of the cutoff value as 0.115, 38 ovarian cancer cases (73.1%) were positive, whereas 5 normal (19.2%) and 6 benign ovarian disease cases (23.1%) were positive. Data obtained from Ep-CAM autoantibody screening showed a sensitivity of 73.1% (95% CI, 61.0–85.2), and a specificity of 80.8% (95% CI, 65.7–95.9). CA125, for which the cutoff value is 35 units/ml in accordance with the supplier, showed a sensitivity of 86.5% (95% CI, 77.2–95.8) and a specificity of 88.5% (95% CI, 76.2–99.8) in this experiment. By area test of ROC curve, significant difference was found between CA 125 (0.965; 95% CI, 0.932–0.998) and Ep-CAM autoantibody (0.851; 95% CI, 0.769–0.934; $P < 0.05$; Fig. 6). A patient with positive Ep-CAM autoantibody has 11.4-fold

risk of normal women in the diagnosis of ovarian cancer (odds ratio = 11.4; 95% CI, 3.6–36.1; relative risk = 3.8) in comparison with CA 125 (odds ratio = 49.3; 95% CI, 11.6–208.6; relative risk = 7.5). We also plotted the ROC curve in using both Ep-CAM autoantibody and CA 125.

Fig. 7 displays a bivariate plot of Ep-CAM autoantibody *versus* CA 125 for normal control subjects and epithelial ovarian cancer cases. From the results of the multiple logistic analysis using both Ep-CAM autoantibody and CA 125, the combined cutoff line was 5.23 = 33.84×Ep-CAM +0.04×CA 125, which gave the sensitivity 47 of 52 = 90.4% (95% CI, 82.4% ∼ 98.4%) and specificity 24 of 26 = 92.3% (95% CI, 82.1% ∼ 100%), respectively.
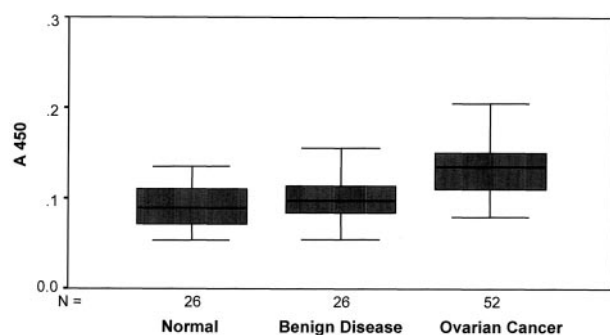
Fig. 5 Box plots of EP-CAM autoantibody levels in case patients with normal ($n = 26$), benign disease ($n = 26$), and ovarian cancer ($n = 52$). The box is bounded above and below by the 25% and 75% percentiles, the median is the *line in the box*, and the upper and lower *error bars* indicate ~99% of values. There is a significant difference among three groups by ANOVA ($P < 0.05$), and the differences are significant between cancer cases and the other groups based on Tukey's multiple comparison test.

McNemar's test for the equality of the two sensitivity estimates between the two marker combination and CA 125 or Ep-CAM autoantibody alone gives a $P = 0.50$ or $P < 0.02$, respectively, indicating a significant increase in sensitivity when combining Ep-CAM autoantibody with CA 125 *versus* Ep-CAM autoantibody alone. The simultaneous point estimate of specificity of the combined rule for CA 125 and Ep-CAM autoantibody increased to 92.3%. However, there was no significant difference between the combination and CA 125 or Ep-CAM autoantibody alone.

By the partial correlation coefficient adjusted by age, we observe a weak correlation between CA 125 and Ep-CAM autoantibody in all of the cases ($r = 0.181$; $P < 0.05$) and in ovarian cancer cases ($r = 0.076$; $P = 0.59$), respectively.

DISCUSSION

In epithelial ovarian cancer, many tumor markers have been identified and studied. However, most of these markers have not shown satisfactory sensitivity and specificity, and, therefore, are not useful as a routine screening method for ovarian cancer. CA 125 is the most extensively researched marker in ovarian cancer, but there is only preliminary evidence that ovarian cancer screening using CA 125 can reduce mortality (17). Therefore, it is of paramount importance to identify new markers, particularly serological markers, which can be used alone or in combination with CA 125 to improve the sensitivity and the specificity of the screening assay.

Multiple methods have been applied to identify tumor markers. One approach is through the identification of differentially expressed genes in ovarian cancer cells and normal ovarian surface epithelial cells. This is achieved by validation processes to determine whether the differentially expressed protein can be used as a serological marker. Methods used to identify differentially expressed genes include expression sequencing tag sequencing, serial analysis of gene expression, differential display PCR, and cDNA or oligonucleotide microarray analysis (18–21). In this study, the MICROMAX cDNA microarray system, which contains 2400 known genes with known function,

was used. This system, which requires the use of only 1 μg of total RNA, is particularly attractive when small numbers of cells, such as normal HOSE cells, are unavoidable. Among all of the genes analyzed, Ep-CAM showed the highest Cy3:Cy5 ratio, suggesting that it was highly overexpressed in ovarian cancer cells (5).

Ep-CAM is a M_r 40,000 glycoprotein encoded by the GA733-2 gene located at chromosome 4q (22, 23). Ep-CAM has been referred to as CO17-1A, MH99, AUA1, MOC31, 323/A3, KS1/4, GA733, HEA125 or KSA, EGP, EGP40, and GA733-2 (22, 24–31). GA733-1 gene product has been known as a unique homologous protein and shares 49% homology with the Ep-CAM amino acid sequence (22). Low levels of Ep-CAM are detected in all of the epithelial cells except for squamous stratified epithelium (10). Using real-time PCR and immunohistochemistry, we also demonstrate low levels of Ep-CAM mRNA and protein expression in normal ovarian surface epithelial cells.

The exact biological function of Ep-CAM is still unknown. On the basis of the presence of EGF-like repeats in the extracellular domain, Ep-CAM was proposed to function as a cell adhesion molecule or a cell surface receptor capable of signal transduction (8–10). Although the molecule is capable of mediating homophilic adhesive interactions, however, there is not sufficient evidence that EP-CAM-mediated adhesions are required for epithelial cell support. Despite the correlation with malignant proliferation in epithelial cancers, Ep-CAM-mediated cell-cell adhesions prevent cell scattering (11). This dualistic role of Ep-CAM in carcinogenesis has required additional investigation. Similar to most epithelial-derived cancers, ovarian cancers express significantly higher levels of Ep-CAM than normal and benign ovarian epithelia. We found no significant difference, however, in Ep-CAM expression in borderline and invasive ovarian tumors with different grades. These data suggest that Ep-CAM may be involved in the development of both borderline and invasive disease and may be associated with an early phase of ovarian carcinogenesis. In contrast to ovarian cancer, other cancers show a different relationship between Ep-CAM expression and degree of differentiation. For example, high-grade transitional cancer of the bladder shows significantly higher Ep-CAM expression than low-grade transitional cancer (32,33). Furthermore, Ep-CAM was expressed at higher level in high-grade cervical intraepithelial neoplasia than in low-grade cervical intraepithelial neoplasia (34).

In 5 consecutive normal ovary staining, we concluded baseline expression levels of Ep-CAM; we also found the same result in the normal epithelium of the cancer patient slides. It is expected that Ep-CAM expression varies dramatically in tumor tissue samples, because ovarian tumors have different grades and subtypes, whereas the normal surface epithelium contains a homogenous population of epithelial cells.

It is interesting to note that stage III and IV ovarian cancer show significantly lower Ep-CAM expression than stage I disease. A similar pattern has been observed in laryngeal cancer in which lower expression of Ep-CAM correlates with a high frequency of metastases (35). Furthermore, it has been shown that transfection of murine Ep-CAM into mouse colorectal cancer cells suppressed their metastatic

Table 2 Preoperative Ep-CAM autoantibody levels by selected characteristics of case patients with ovarian cancer and control subjects without ovarian cancer

Characteristics	No.	Absorbances[a]	95% CI[b]	Range	T[c]	P[d]
All subjects	104	0.113	0.106 to 0.120	0.054–0.203		
Diagnostic category						
Normal	26	0.090	0.080 to 0.100	0.054–0.133	a	$P < 0.001$
Benign disease	26	0.098	0.088 to 0.108	0.055–0.154	a	
Ovarian cancer	52	0.132	0.123 to 0.141	0.080–0.203	b	
Histology of cancer						
Serous	26	0.126	0.112 to 0.139	0.081–0.203		$P = 0.083$
Mucinous	10	0.129	0.116 to 0.141	0.104–0.149		
Endometrioid	10	0.151	0.143 to 0.178	0.139–0.201		
Clear cell/others	6	0.120	0.087 to 0.153	0.080–0.147		
Tumor differentiation						
Borderline	8	0.119	0.104 to 0.133	0.093–0.136		$P = 0.061$
Well	6	0.163	0.132 to 0.194	0.141–0.201		
Moderate	12	0.135	0.120 to 0.150	0.104–0.166		
Poor	26	0.128	0.114 to 0.142	0.080–0.203		
FIGO stage						
I	16	0.137	0.120 to 0.153	0.093–0.201	a	
II	6	0.150	0.105 to 0.195	0.108–0.203	a	
III	26	0.131	0.120 to 0.142	0.081–0.166	a, b	$P = 0.039$
IV	4	0.094	0.068 to 0.120	0.080–0.108	b	

[a] Values are given as a mean.
[b] CI, confidence interval.
[c] The same letters indicate nonsignificant difference between groups based on Tukey's multiple comparison test.
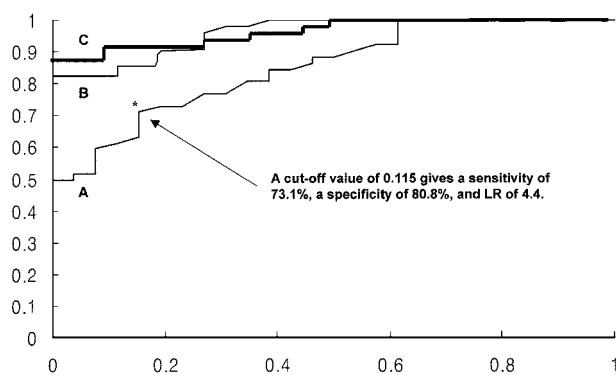[d] Statistical significances were tested by ANOVA among groups.

Fig. 6 ROCs curves of Ep-CAM autoantibody (*A*), CA 125 (*B*), and Ep-CAM autoantibody and CA 125 combined for all normal controls and ovarian cancers (*C*) for all normal controls and ovarian cancers, respectively. ∗ indicates the cutoff value of 0.115 that gives a sensitivity of 73.1%, a specificity of 80.8%, and likelihood ratio (*LR*) of 4.4 in Ep-CAM autoantibody.

Fig. 7 Bivariate plot of combined Ep-CAM autoantibody and CA 125 (on log scales) in the sera of ovarian cancer ●: normal (*n* = 26); ▲: cancer (*n* = 52). The oblique line illustrates the separation that can be achieved between case patients and control subjects by the multiple logistic analysis using both Ep-CAM and CA 125. The combined cutoff line was 5.23 = 33.84×Ep-CAM +0.04×CA 125, which gave the sensitivity 47 of 52 = 90.4% (95% CI, 82.4% ~ 98.4%) and specificity 24 of 26 = 92.3% (95% CI, 82.1% ~ 100%), respectively.

potential (11). These results may be explained by the fact that Ep-CAM also acts as an adhesion protein of which the down-regulation may facilitate the metastasis process during cancer progression (7).

Using an established ELISA, we evaluated the potential of using Ep-CAM autoantibody levels to detect ovarian cancer. Ep-CAM autoantibody levels proved to be significantly higher in ovarian cancer than normal and benign ovarian disease. Although it is less sensitive and less efficient than CA 125 as shown in this experiment (ROC curve and odds ratio), Ep-CAM autoantibody may be complementary to CA
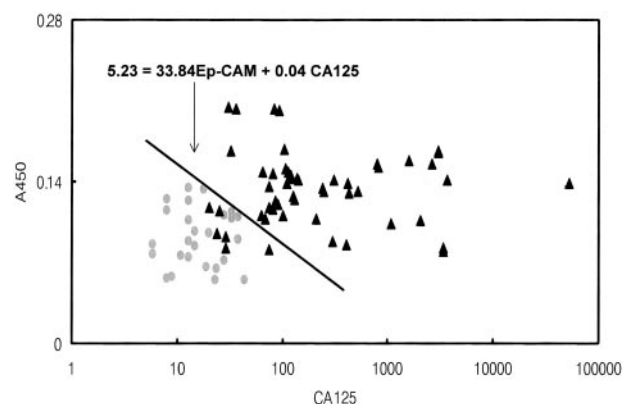
125 as suggested by the low correlation between the two. Using the combination of Ep-CAM autoantibody and CA 125, we found that the specificity was increased as compared with CA 125 alone without lowering the sensitivity. Consequently, it may be valuable to use Ep-CAM autoantibody in ovarian cancer screening. Furthermore, considering that there was no expression difference in patient sera between early and advanced disease, it may be possible to increase early

detection ratio in ovarian cancer in addition to CA 125 with unsatisfactory detection rate in early disease. CA 125 is elevated in several kinds of benign diseases, such as endometriosis and benign ovarian tumor. This experiment showed 14 cases (53.8%) positivity in CA 125 but 6 cases (23.1%) in Ep-CAM autoantibody in benign diseases experiment (data were not shown in result section). It means that false positive ratio can be decreased by way of Ep-CAM autoantibody in the screening of ovarian cancer. Nevertheless, a large study with more cases and controls needs to be performed to confirm the potential diagnostic value of Ep-CAM autoantibody.

In conclusion, this investigation has demonstrated the potential value of the cDNA microarray analysis in identifying overexpressed genes in ovarian cancer, and suggests that Ep-CAM autoantibody is associated with ovarian cancer and may be valuable biomarker after population-based research assessing.

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