Short Communication

Multiplexed Immunobead-Based Cytokine Profiling for Early Detection of Ovarian Cancer

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Abstract

Early detection of ovarian cancer might improve clinical outcome. Some studies have shown the role of cytokines as a new group of tumor markers for ovarian cancer. We hypothesized that a panel comprised of multiple cytokines, which individually may not show strong correlation with the disease, might provide higher diagnostic power. To evaluate the diagnostic utility of cytokine panel, we used a novel multianalyte LabMAP profiling technology that allows simultaneous measurement of multiple markers. Concentrations of 24 cytokines (cytokines/chemokines, growth, and angiogenic factors) in combination with cancer antigen-125 (CA-125), were measured in sera of 44 patients with early-stage ovarian cancer, 45 healthy women, and 37 patients with benign pelvic tumors. Six markers, i.e., interleukin (IL)-6, IL-8, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), and CA-125, showed significant differences in serum concentrations between ovarian cancer and control groups. Out of this group, IL-6, IL-8, VEGF, EGF, and CA-125, were used in a classification tree analysis that resulted in 84% sensitivity at 95% specificity. The receiver operator characteristic curve created using the combination of markers produced sensitivities between 90% and 100% in the area of 80% to 90% specificity, whereas the receiver operator characteristic curve for CA-125 alone resulted in sensitivities of 70% to 80%. The classification tree analysis for discrimination of benign condition from ovarian cancer used CA-125, granulocyte colony-stimulating factor (G-CSF), IL-6, EGF, and VEGF resulting in 86.5% sensitivity and 93.0% specificity. The presented data show that simultaneous testing of a panel of serum cytokines and CA-125 using LabMAP technology may present a promising approach for ovarian cancer detection. (Cancer Epidemiol Biomarkers Prev 2005;14(4):981–7)

Introduction

Ovarian cancer represents the third most frequent cancer of the female genital tract. The majority of early-stage ovarian cancers are asymptomatic, and over three-quarters of clinical diagnoses are made at a time when the disease has already established regional or distant metastases. Despite aggressive cytoreductive surgery and platinum-based chemotherapy, the 5-year survival for patients with clinically advanced ovarian cancer is only 15% to 20%, in striking contrast to the cure rate for stage I disease, which is usually >90% (1). These statistics provide the primary rationale to improve ovarian cancer screening and early detection.

Due to the low prevalence of spontaneous ovarian cancer in the general population, a screening strategy must have sensitivity of at least 80% in early-stage disease and near-perfect specificity of at least 99.6% (2). At present, there are two screening tests for ovarian cancer: serologic screening for tumor antigen using cancer antigen-125 (CA-125), and imaging using transvaginal sonography (2-6). However, with a cutoff of 30 to 35 units/mL, serum CA-125 has been shown to have a sensitivity of only 50% to 60% with the specificity of >98%, for early-stage disease (4, 7, 8). Transvaginal sonography alone or combined with Doppler and morphologic indices, are only sensitive and specific for established tumors, and are, therefore, not suitable for early diagnostics of ovarian cancer (6, 9). Recently, a novel technology, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry has been offered for early detection of ovarian cancer (10). This technology was reported to allow for discriminating serum protein profiles with 100% sensitivity and 100% specificity (11). However, in two other studies of early detection of ovarian cancer using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, the results were less optimistic, demonstrating 72.8% to 95.7% sensitivity and 82.6% to 94.9% specificity (12, 13). Therefore, at present, proteomic profiling, whereas promising, does not possess the required diagnostic discrimination for primary ovarian cancer screening. Additional approaches are necessary to provide the required high level of specificity and positivity for an effective high-throughput screening for ovarian cancer.

During the last two decades, a large number of serologic tumor markers have been evaluated for their ability to detect early-stage epithelial ovarian cancer. Biomarkers that have a shown association with ovarian cancer include cancer antigens, differentiation markers, antibodies to mutated oncogenes, and cytokines (reviewed in ref. 14). Cytokines are a diverse group of proteins comprised of hematopoietic growth factors, interferons, lymphokines, and chemokines (15). Serum cytokines that possess diagnostic value in ovarian cancer include interleukin (IL)-6, IL-8, macrophage colony-stimulating

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factor (M-CSF), MCP-1, tumor necrosis factor receptor (TNFR), and vascular endothelial growth factor VEGF (refs. 16-21). Cytokines are implicated in many aspects of tumor growth (reviewed in ref. 22). Tumor cells express and produce various angiogenic factors, such as VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), IL-6, IL-8 (23-26), and other cytokines, such as MCP-1, granulocyte CSF (G-CSF), M-CSF, tumor necrosis factor-α (TNFα), IL-1α, and IL-1β (24, 27-29). Tumor-produced cytokines could bind to their receptors expressed by endothelial and hematopoietic/lymphoid cells and trigger production of additional types of cytokines (22). This leads to the accumulation of high concentrations of these factors locally (ascites) as well as systemically (in the blood). Cytokine profiles could be cancer-specific since malignant cells of different histologic types could produce different patterns of proangiogenic factors, growth factors, and chemokines. Thus, cytokine panels could serve as cancer biomarkers that can be used for early diagnosis and assessment of therapy response. Previous publications showed that none of these markers, used alone, is sufficiently diagnostic of malignancy (14, 30). In some studies, combinations of several markers have been evaluated for early detection of ovarian cancer using conventional ELISA assays. Analysis of the diagnostic power of individual serologic markers in combination with CA-125 resulted in increased sensitivity and specificity (3, 14, 30-39). Due to the limitations of ELISA (which is expensive, time-consuming, and each assay encompasses only one marker at a time), none of the tested marker combinations thus far was sufficiently comprehensive and achieved the required characteristics for diagnosis of ovarian cancer. Therefore, further research is necessary to identify a multibiomarker panel allowing for early detection of ovarian cancer with required high sensitivity and specificity.

To test this, we have used a novel multianalyte LabMAP profiling technology (Luminex Corp., Austin, TX), that allows simultaneous measurement of multiple biomarkers in serum or plasma of ovarian cancer patients and control healthy women. In this study, a panel of 24 serologic markers including cytokines, chemokines, growth and angiogenic factors, and CA-125 was analyzed in blood sera of ovarian cancer patients (stages I and II), patients with benign pelvic disease, and control healthy women. Our studies show that a panel of cytokines in combination with CA-125 showed increased specificity and sensitivity as compared with CA-125 alone.

Patients and Methods

Patient Populations. The serum samples from 44 patients diagnosed with early-stage (I and II) ovarian cancer, 37 patients with benign pelvic masses, and 45 healthy age-matched controls were tested. Serum samples from patients with early-stage (I and II) ovarian cancer, and women with benign pelvic disease, were provided by the Gynecologic Oncology Group (Cleveland, OH). Patients were enrolled by the Gynecologic Oncology Group under their Institutional Review Board protocols. Information about gynecologic diagnoses and ovarian cancer staging as well cancer histology and grade was provided by the Gynecologic Oncology Group. No data allowing identification of patients were provided. All major types of epithelial ovarian cancer and a variety of benign pelvic conditions were represented in these series (Table 1). Control serum samples from healthy, age-matched women were received from the Allegheny County Case-Control Network. Written informed consent was obtained from each subject or from his or her guardian. Sample collection was done after approval by the Institutional Review Board and in accord with an assurance filed with and approved by the U.S. Department of Health and Human Services.

Table 1. Patient characteristics

Patient group	Age, median (range)	Histologic types
Control (n = 45)	46 (36-76)	
Early-stage ovarian cancer (n = 44)	46 (34-88)	Papillary serous carcinoma (n = 13) Carcinoma, endometrioid (n = 10) Carcinoma, mucinous (n = 7) Carcinoma, poorly differentiated (n = 6) Adenocarcinoma, serous (n = 5) Carcinoma, clear cell (n = 3)
Benign tumors (n = 37)	44.5 (28-87)	Adenofibroma, serous (n = 1) Brenner tumor (n = 1) Crystadenofibroma, serous (n = 2) Cyst, paratubal (n = 2) Cyst, serous (n = 1) Cyst, simple (n = 3) Cystadenofibroma, serous (n = 3) Cystadenoma, mucinous (n = 8) Cystadenoma, serous (n = 9) Endometriosis (n = 1) Fibrosis (n = 1) Ovary benign (n = 3) Mucinous benign (n = 2)

Collection and Storage of Blood Serum. Ten microliters of peripheral blood was drawn from subjects using standardized phlebotomy procedures. Handling and processing was similar for all three groups of patients. Samples were obtained from patients diagnosed with ovarian cancer, prior to surgery, and before administration of anesthesia. Blood samples were collected without anticoagulant into red top vacutainers and allowed to coagulate for 20 to 30 minutes at room temperature. Sera were separated by centrifugation, and all specimens were immediately aliquoted, frozen and stored in a dedicated −80°C freezer. No more than two freeze-thaw cycles were allowed for each sample.

Multiplex Analysis. The LabMAP technology (Luminex) combines the principle of a sandwich immunoassay with the fluorescent-bead-based technology allowing individual and multiplex analysis of up to 100 different analytes in a single microtiter well (40). The LabMAP serum assays were done in 96-well microplate format according to the protocol by Biosource International (Camarillo, CA). A filter-bottom, 96-well microplate (Millipore, Billerica, MA) was blocked for 10 minutes with PBS/bovine serum albumin. To generate a standard curve, 5-fold dilutions of appropriate standards were prepared in serum diluent. Standards and patient sera were pipetted at 50 μL per well in duplicate and mixed with 50 μL of the bead mixture. The microplate was incubated for 1 hour at room temperature on a microtiter shaker. Wells were then washed thrice with washing buffer using a vacuum manifold. Phycoerythrin (PE)-conjugated secondary antibody was added to the appropriate wells and the wells were incubated for 45 minutes in the dark with constant shaking. Wells were washed twice, assay buffer was added to each well, and samples were analyzed using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA). Analysis of experimental data was done using five-parametric-curve fitting.

Development of LabMAP Assays for CA-125. Assay for CA-125 was developed in our laboratory according to the protocol by Luminex. Antibody pair for CA-125 was purchased from Fitzgerald Industries International

(Concord, MA). Detection antibody were biotinylated using the EZ-Link sulfo-NHS-biotinylation kit (Pierce, Rockford, IL) according to the manufacturer's protocol. The extent of biotin incorporation was determined using the HABA assay (Pierce) and was found to be 20 mol of biotin per mole of protein for all of the biotinylation reactions. The capture antibody was covalently coupled to individual spectrally addressed carboxylated polystyrene microspheres purchased from Luminex. Covalent coupling of the capture antibodies to the microspheres was done by following the procedures recommended by Luminex. In short, microsphere stock solutions were dispersed in a sonication bath (Sonicor Instrument Corporation, Copiaque, NY) for 2 minutes. An aliquot of 2.5 $\times$ 10^6 microspheres was resuspended in microtiter tubes containing 0.1 mol/L sodium phosphate buffer (pH 6.1), to a final volume of 80 μL. This suspension was sonicated until a homogeneous distribution of the microspheres was visually observed. Solutions of *N*-hydroxy-sulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Pierce), both at 50 mg/mL, were prepared in phosphate buffer, and 10 μL of each solution was sequentially added to stabilize the reaction and activate the microspheres. This suspension was incubated for 10 minutes at room temperature and then resuspended in 250 μL of PBS containing 50 μg of antibody. The mixture was incubated at room temperature overnight in the dark with continuous shaking. Microspheres were then incubated with 250 μL of PBS-0.05% Tween 20 for 4 hours. After aspiration, the beads were blocked with 1 mL of PBS-1% bovine serum albumin-0.1% sodium azide. The microspheres were counted with a hemacytometer and stored at a final concentration of 10^6 microspheres/mL in the dark at 4°C. The coupling efficiency of monoclonal antibodies was tested by staining 2,000 microspheres with phycoerythrin-conjugated goat anti-mouse IgG (BD Biosciences, San Diego, CA). Microspheres were analyzed by Bio-Plex system, and the mean fluorescence intensity of >15,000 was accepted as an indicator of sufficient coupling efficiency. The minimum detection levels for CA-125 was <5 international units/mL. Interassay variability, expressed as a coefficient of variation, was calculated based on the average for 10 patient samples and standards that were measured in four separate assays. The interassay variability within the replicates presented as an average coefficient of variation was in the range of 5.4% to 9.1% (data not shown). Intraassay variability was evaluated by testing quadruplicates of each standard and 10 samples measured thrice. The variabilities of these samples were between 5.6% and 9.6% (data not shown). CA-125 assay was further validated in comparison with standard clinical ELISA (Centocor, Malvern, PA) and has shown 94.5% correlation. 24-plex assay for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-18, TNFα, IFNγ, GM-CSF, EGF, VEGF, G-CSF, basic fibroblast growth factor, hepatocyte growth factor (HGF), RANTES, macrophage inflammatory protein (MIP)-1α, MIP-1β, and MCP-1 purchased from Biosource International. Interassay variabilities for individual cytokines in 24-plex were in the range of 3.5% to 9.8% and intraassay variabilities were in the range of 3.6% to 12.6% (information provided by Biosource International). Each assay was validated against appropriate ELISA demonstrating 95% to 99% correlations (information provided by Biosource International).

Statistical Analysis of Data. Descriptive statistics and graphical displays (i.e., dot plots) were prepared to show the distribution of each marker for each disease state. The Wilcoxon rank-sum test was used to evaluate the significance of differences in marker expression between each disease state. Spearman's (nonparametric) rank correlation was also calculated to quantify the relationships between each pair of markers.

Discrimination of ovarian cancer status was accomplished using classification and regression trees (CART; ref. 41) implemented through S-Plus statistical software (42). Classification trees discriminate between outcome classes (e.g., cancer patients versus controls) by first searching the range of each potential predictor (e.g., a given cytokine) and finding the split that maximizes the likelihood of the given data set. Within each resulting subset (or node), the algorithm again searches the range of each variable to choose the optimal split. This process is continued until all observations are perfectly discriminated, or the sample size within a given node is too small to divide further (i.e., n = 5 or less). Only two observations in the data set had missing values for any of the markers and were excluded from the analysis. The final output of the resulting classification tree is a graphical display of decision criteria for each split and resulting predicted probabilities of being a case across the final splits (i.e., terminal nodes). Several other methods (logistic regression and neural networks) were also implemented with similar, but somewhat less optimal results (results not shown).

Ten-fold cross-validation (43, 44) was implemented to assess classification accuracy using independent data. Specifically, the data were randomly split into 10 subsets of equal size (or as equal as possible; n_k = 8-9 for these data). For each subset, a model was fit to 90% of the data outside that subset; the resulting model (or tree) was then applied to 10% of data within the given subset. The resulting estimate of classification accuracy therefore uses separate subsets of data for model fitting and validation, and thus avoids resubstitution bias. The resulting sensitivity and specificity are reported across a range of decision rules (i.e., cut-points for classifying a given predicted probability as either a case or control) to generate the receiver operator characteristic (ROC) curve. Since cross-validation produces a potentially different model for each subset of the data, however, the classification tree produced using all observations (i.e., without cross-validation) was displayed for purposes of describing the optimal model. When not otherwise stated, observations with a predicted probability >0.5 are classified as a case (or as a benign condition for the comparison of benign versus controls).

Results

LabMAP-Based Analysis of Serum Concentrations of Cytokines and CA-125 in Ovarian Cancer Patients. Concentrations of 24 different serum markers belonging to different functional groups were evaluated in a multiplexed assay using LabMAP technology, in serum samples of patients from three clinical groups: women with early stage (I and II) ovarian cancer, women with benign pelvic masses, and age-matched healthy controls (Table 1). Serum levels of IL-2, IL-4, IL-5, IL-10, IL-13, IL-15, IL-17, IL-18, TNFα, and IFNγ were undetectable in either control or patients' sera. IL-1β, IL-12p40, MIP-1α, MIP-1β, HGF, RANTES, bFGF, and GM-CSF showed measurable serum concentrations which did not differ between the control and patient groups (data not shown). Serum concentrations of IL-6, IL-8, CA-125, and VEGF were found to be significantly higher in ovarian cancer patients as compared with controls (P < 0.05 - P < 0.001; Table 2; Fig. 1). LabMAP assays showed relatively high serum concentrations of EGF (224 ± 12 pg/mL) and MCP-1 (384 ± 21 pg/mL) in the serum of healthy women (Table 2; Fig. 1). However, serum levels of EGF and MCP-1 were significantly lower (P < 0.05 - P < 0.001) in ovarian cancer patients as compared with controls (Table 2; Fig. 1).

Serum of patients with benign tumors had significantly elevated levels of VEGF (P < 0.05), G-CSF (P < 0.01), IL-6 (P < 0.001), and CA-125 (P < 0.01) as compared with controls (Table 2). In addition, patients with benign tumors had

Table 2. Levels of serum markers

Analytes/patients		Healthy controls	Ovarian cancer	Benign
EGF	mean ± SE	223.8 ± 11.46	110.7 ± 15.58***	98.6 ± 12.35***
	median (range)	238 (29.8-402.6)	74.9 (0-396.9)	94.9 (0-276.4)
IL-6	mean ± SE	8.8 ± 2.50	64.2 ± 12.72***	28.0 ± 9.3***
	median (range)	0 (0-64.1)	23.8 (0-280.2)	7.6 (0-275.3)
G-CSF	mean ± SE	21.8 ± 8.44	49.2 ± 12.04^{NS}	77.4 ± 14.04**
	median (range)	0 (0-257.6)	0 (0-290.8)	0 (0-339.1)
IL-8	mean ± SE	10.2 ± 1.68	24.0 ± 5.98**	12.4 ± 3.11
	median (range)	6 (2.3-51.4)	9.6 (2.0-180.6)	7.6 (3.0-127.8)
VEGF	mean ± SE	90.7 ± 10.52	153.5 ± 19.95*	258.8 ± 26.04*
	median (range)	67 (18-306)	106 (28-552)	218 (48-662)
CA-125	mean ± SE	10.4 ± 2.28	153.7 ± 44.04***	51.8 ± 13.23**
	median (range)	6.0 (0-87)	51.0 (0-1412)	16.0 (0-372)
MCP-1	mean ± SE	341.8 ± 21.34	210.3 ± 20.54***	196.3 ± 16.06***
	median (range)	326.8 (135.5-695.7)	172.9 (17.1-502.3)	178.2 (44.9-434.6)

NOTE: Comparison of ovarian cancer or benign patients with controls: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

significantly lower levels of EGF and MCP-1 as compared with controls ($P < 0.001$ for both; Table 2). When cytokine levels were compared between cancer and benign groups, significantly lower circulating concentrations of IL-6, IL-8, and CA-125 were observed in sera of benign cases ($P < 0.05$ for all; Fig. 1). G-CSF concentration was significantly ($P < 0.05$) higher in the benign group as compared with the cancer group (Fig. 1). Patients with benign pelvic disease did not differ from patients with early-stage ovarian cancer with regard to of concentrations of EGF, VEGF, and MCP-1 (Fig. 1).

Statistical Analysis of Serum Cytokines as Ovarian Cancer Biomarkers

Comparison of Early-Stage Ovarian Cancer versus Healthy Controls. Table 3 illustrates classification results using each individual cytokine to distinguish early-stage ovarian cancer from controls. Results show that the individual markers led to only moderately accurate prediction of early-stage cancer. Only CA-125, EGF, and IL-6 correctly classified >80% of the test set subjects (Table 3).

Figure 2A displays the classification tree using CART methodology for discriminating controls from early-stage ovarian cancer. The model in Fig. 2A used all observations in either group to fit the model (as opposed to cross-validation, which is used for subsequent estimation of classification accuracy as explained in subsequent paragraphs). The classification tree used five of the eight markers, including CA-125, EGF, VEGF, IL-6, and IL-8. The numbers specified for each of the final groups (i.e., terminal nodes) represent the probability of being a case within each subset.

Rates of classification accuracy (in discriminating controls from early-stage cancer) were then obtained using 10-fold cross-validation. Figure 2B displays the resulting ROC curve. As described in Patients and Methods, the sensitivity and specificity depend on the cut-point (i.e., predicted probability from the classification tree) used to classify each subject as either a case or control. Using the standard cut-point of 0.5 (i.e., everyone with a predicted probability >0.5 is classified as a cancer case) gives 100% sensitivity, 86% specificity, and 93% correctly classified. Fixing the specificity at 91% still leads to a very high sensitivity, at 96% (again with 93% correctly classified). Alternatively, a specificity of 95.3% corresponds to a sensitivity of 84.1% (and 90.0% correctly classified). The total area under the ROC curve was near one, at 0.966. Additionally, the ROC curve was created using only CA-125 (again based on 10-fold cross-validation; Fig. 2C). Comparing this curve to the combination of markers clearly shows a substantial gain from using multiple markers to predict cancer status. Specifically, in the area of 80% to 90% specificity (i.e., between 0.1 and 0.2 on the *x*-axis), the final model (using multiple markers) produces sensitivities between 90% and 100%, whereas CA-125 only produces sensitivities in the area of 70% to 80%.

Several models provided comparable high sensitivity and specificity for early diagnosis of ovarian cancer. Therefore, the resulting combination of cytokines should not be viewed as a

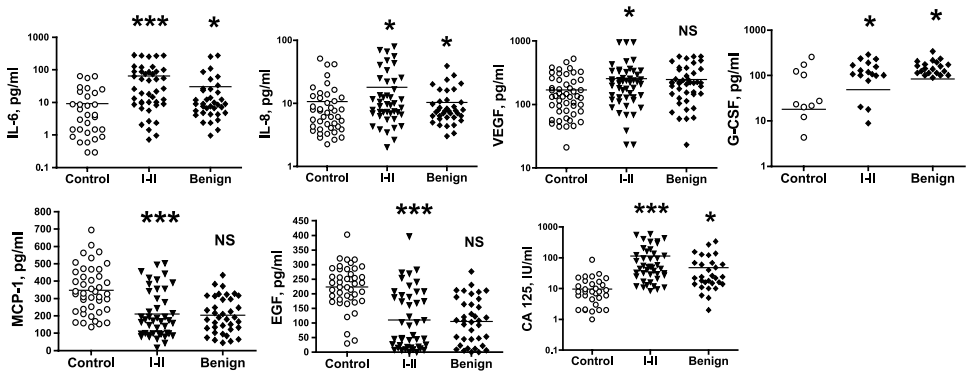

Figure 1. Distribution of serum levels of markers in the three study groups. Serum levels of cytokines and growth factors in healthy controls, ovarian cancer patients at stages I and II and patients with benign gynecologic disease. Sera were collected from 45 patients with early-stage (I and II) ovarian cancer, 44 patients with benign pelvic masses, and from 37 age- and sex-matched healthy controls. Circulating concentrations of cytokines and growth factors were measured using LabMAP technology as described in Patients and Methods. Measurements were done twice. Horizontal lines, mean values; *, statistical significance between controls and cancer patients or between patients with benign pelvic disease and patients with ovarian cancer; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3. Predictive values for single serum markers for early-stage ovarian cancer

Cytokine	% Correctly classified	Sensitivity	Specificity
CA-125	85.1	95.5	74.4
IL-6	85.1	84.1	86.0
EGF	80.5	84.1	76.7
IL-8	79.3	88.6	69.8
MCP	78.2	84.1	72.1
VEGF	73.6	79.5	67.4
G-CSF	73.6	72.7	74.4

unique subset of markers. Other models with the same number of cytokines (data not shown), often led to very similar results. For instance, all of the tested three-variable models led to very similar classification rates. The large number of possible combinations, and the computational demands of iteratively partitioning the training and test sets, prevented an exhaustive search of all possible models.

Comparison of Controls and Early-Stage Ovarian Cancer versus Benign Conditions. To assess the validity of serum biomarker panel for discrimination of benign pelvic tumors from the other groups, separate classification tree models were fit to predict (*a*) benign conditions versus early-stage cancer, and (*b*) benign conditions versus controls. The same 10-fold cross-validation procedure was used to assess classification accuracy. For the comparison of benign versus cancer, 80.2% of subjects were correctly classified, with a sensitivity of 84.1% and a specificity of 75.7%. The classification tree for comparison of benign versus cancer (data not shown) used five markers (CA-125, G-CSF, IL-6, EGF, and VEGF). For the comparison of benign versus controls, 90.0% of subjects were correctly classified, with a sensitivity of 86.5% and a specificity of 93.0%. The classification tree for comparison of benign versus controls (data not shown) used six of the eight markers, including EGF, VEGF, G-CSF, CA-125, IL-6, and IL-8.

Correlation Between Biomarkers. Analysis of correlations between individual markers using Spearman rank correlation method revealed that most of the markers were relatively uncorrelated (Table 4). Only MCP-1 and EGF ($r = 0.45$) had a correlation >0.4; IL-6 and IL-8 ($r = 0.34$) were the only other markers having a correlation >0.3. The majority of markers had a correlation of 0.10 or less (in absolute value), suggesting that marker combinations may provide complementary classification information.

Discussion

We used LabMAP technology for analyzing 24 cytokines and CA-125 antigen in sera of women with early-stage ovarian cancer in comparison with matched healthy controls and patients with benign pelvic tumors. The sensitivity of the LabMAP assay is comparable to ELISA and RIA (40). In fact, in our experiments, circulating levels of all 25 proteins in healthy women were very similar to those measured by ELISA or RIA and that reported in previously published observations (20, 45-50).

We have identified six circulating proteins that showed an association with ovarian cancer, i.e., EGF, MCP-1, CA-125, VEGF, IL-6, and IL-8. We observed two distinct patterns of changes in the sera of women with ovarian cancer: the concentrations of VEGF, IL-6, IL-8, and CA-125 were higher in patients with ovarian cancer, whereas decreased concentrations of EGF and MCP-1 were found in ovarian cancer as compared with the healthy controls. These elevated levels of VEGF, IL-6, IL-8, and CA-125 in the circulation of ovarian cancer patients have been previously reported from studies using conventional ELISA assays (48, 51-53). Increased levels of cytokines in the blood of cancer patients may be due to secretion by malignant or by normal stromal cells, i.e., immune or endothelial cells.

To the best of our knowledge, this study is the first to observe reduced levels of EGF in patients with ovarian cancer and benign pelvic disease. Lower circulating levels of MCP-1 in ovarian cancer as compared with controls were previously reported by Penson et al. (20). Lower serum concentrations of EGF were observed in patients with differentiated thyroid carcinoma (45) and breast cancer, but not in patients with pancreatic, lung, and head and neck

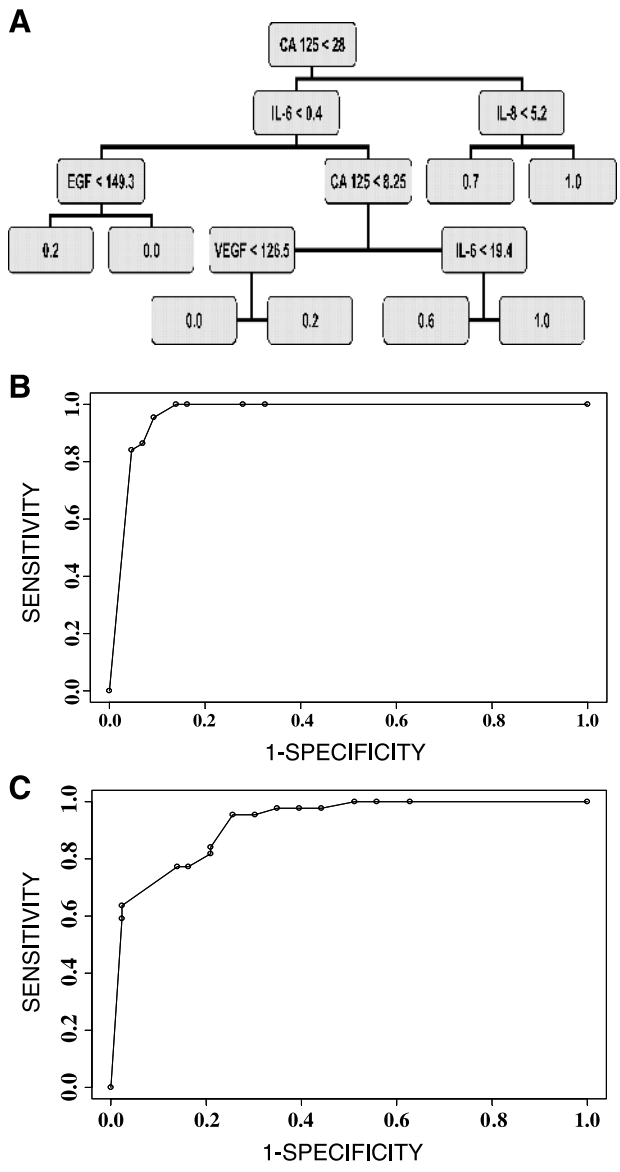

Figure 2. Classification tree and ROC discriminating early-stage ovarian cancer from healthy controls. **A.** Classification tree: rectangles, splitting nodes containing cytokine and cytokine cutoff. The range of data specified at each split represents the subset of data which is further subdivided by branches to the left. The numbers specified for each of the final groups (i.e., terminal nodes) represent the probability of being a case within each subset. **B.** ROC curve for biomarker panel. Results from 10-fold cross-validation of classification tree analysis of early-stage ovarian cancer versus healthy controls. **C.** ROC curve for CA-125 alone. Results from 10-fold cross validation of early-stage ovarian cancer versus healthy controls.

Table 4. Spearman rank correlations

Marker	EGF	V-EGF	MCP-1	G-CSF	CA-125	IL-6	IL-8	IL-12
EGF	1.00							
V-EGF	−0.10	1.00						
MCP-1	0.45	0.01	1.00					
G-CSF	−0.08	0.01	−0.04	1.00				
CA-125	−0.26	0.07	−0.16	−0.05	1.00			
IL-6	−0.07	0.02	−0.06	0.13	0.22	1.00		
IL-8	0.15	0.08	0.21	0.10	0.10	0.34	1.00	
IL-12	0.12	−0.03	0.23	0.13	−0.02	−0.05	0.01	1.00

cancers or melanoma.[6] Therefore, decreased circulating levels of EGF may be specific for particular type(s) of cancer. Ovarian cancer cells express EGF receptor, and EGF is an autocrine growth factor for ovarian cells (54, 55). We have observed the absorption of EGF and MCP-1 by ovarian tumor cells *in vitro* incubated with serum,[6] indicating that lower circulating levels of these molecules in ovarian cancer patients might be due to the consumption by ovarian tumor expressing specific receptors (56, 57).

Analysis of serum biomarkers in patients with benign pelvic masses revealed increased levels of VEGF and G-CSF that could be explained by their proangiogenic effects and stimulation of angiogenesis and formation of a blood vessel network that is essential for supporting growth not only of malignant but also benign tumors. However, in comparison with ovarian cancer serum, no significant increase in IL-8 concentrations in the serum of patients with benign tumors was observed. The serum levels of IL-6 and CA-125 were found to be elevated in patients with benign tumors but not to the same extent as in patients with ovarian cancer. Decreased concentrations of EGF and MCP-1 were found to be similar in the serum of patients with benign masses and ovarian cancer. It is possible that receptors for EGF and MCP-1 are also expressed by benign tumor cells. Expression of EGFR by normal epithelial cells has been reported (55). Discrimination of ovarian cancer from benign samples has presented a difficult problem in past studies, in which a relatively high percentage of false-positive classification of benign neoplasms has been observed (12, 58).

Statistical analysis showed that although correlation of each of the above markers with ovarian cancer was modest when evaluated alone, a combined biomarker panel showed very strong association with malignant disease, and can have potential utility for early diagnosis of ovarian cancer. Combinations of several serum markers as measured by LabMAP technology provided a sensitivity of 84% at a specificity of 95% in this sample set. Due to the low prevalence of spontaneous ovarian cancer in the general population, a screening strategy must have sensitivity of at least 80% in early-stage disease and near-perfect specificity of at least 99.6% (2). We should therefore ideally evaluate the sensitivity of a given model using a cutoff that produces very high specificity. Sensitivities are reported here for fixed specificities as high as 95%. Although reporting the model's sensitivity at higher specificities (e.g., 98% or 99%) would be preferable, such results could not be reached (with the given model and the given data sets) due to the small number of controls. We expect to improve on such results through the future collection of larger data sets and an expanded panel of ovarian-associated biomarkers. It should also be noted that the 100% sensitivity and specificity results apply to samples obtained from ovarian cancer cases already clinically diagnosed, along with healthy controls, and not from a prospective screening trial. The results in a prospective screening trial are likely to be lower than obtained with preoperative samples. These results, however, show a strong potential to accurately discriminate cancer status with only a moderate number of samples.

For an estimate of the optimal classification tree, we presented the model fit to the entire data set, which is subsequently referred to as the overall model. It should be noted that the cross-validation procedure used here produces a different model for each of the 10 training data sets. Each of these 10 classification trees, however, was either the same as, or subsets of the overall model. None of the 10 models fit through the cross-validation procedure included any markers that were not in the overall model (i.e., MCP-1 or G-CSF). Seven of the 10 cross-validation models included four of the five markers in the overall model. Although some bias may result from this cross-validation procedure, as opposed to separate training and test sets, the latter approach is not feasible unless one has very large sample sizes. With the given sample sizes, separate training and test sets would lead to more unstable estimates of sensitivity and specificity, since each observation can only be used for training or prediction. For the given data, the 10-fold cross-validation approach represents a reasonable alternative to at least partially avoid classification bias (imposed when the same data are used from both training and prediction), and estimate classification measures (e.g., sensitivity and specificity) with improved precision. However, whereas this approach substantially reduces the problem of classification bias, it does not easily allow ready calculation of confidence limits, which is a limitation of the cross-classification approach, compared with splitting the data into two independent training and test sets.

The predictive power of combined serologic markers for early-stage ovarian cancer, as determined by LabMAP technology, is at least as good as that identified in published studies for surface-enhanced laser desorption/ionization time-of-flight mass spectrometry technology (12). However, LabMAP technology is less expensive and permits a high-throughput approach. Furthermore, the flexibility of LabMAP technology allows for the addition of new markers and therefore for the opportunity to incrementally increase the diagnostic power of the combined assay. To the best of our knowledge, the reported multiplexed cytokines/CA-125 offers the highest predictive power, as compared with other published assays using defined protein serologic markers (3, 14, 30, 32-39, 59-62).

In conclusion, we show here that analysis of multiple serum biomarkers using a novel LabMAP technology is a promising approach for the development of a diagnostic assay for ovarian cancer. The predictive power of the cytokines/CA-125 panel is still lower than that required for general population ovarian cancer screening as defined by Jacobs (2). Optimization of cytokine/CA-125 panel by including additional markers with high association with ovarian cancer would likely increase the diagnostic power of the assay. Further validation of this assay in retrospective studies with a larger number of samples will allow for confirming the clinical diagnostic utility of LabMAP-based assay.

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