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Potential markers that complement expression of CA125 in epithelial ovarian cancer

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Abstract

Background. When ovarian carcinoma is diagnosed in stage I, up to 90% of patients can be cured with surgery and currently available chemotherapy. At present, less than 25% of cases are diagnosed at this stage. To increase the fraction of ovarian cancers detected at an early stage, screening strategies have been devised that utilize a rising serum CA125 level to trigger the performance of transvaginal sonography. One limitation of CA125 as an initial step in such a screening strategy is that up to 20% of ovarian cancers lack expression of the antigen. Serum tumor markers that can be detected in ovarian cancers that lack CA125 expression might improve the sensitivity for early detection.

Methods. From 296 ovarian cancers, 65 (22%) were found to have weak or absent CA125 expression on immunoperoxidase staining. Tissue expression of CA125 was compared to serum CA125 levels. Using immunoperoxidase staining of tissue arrays, we have assessed expression of 10 potential serum tumor markers in the 65 epithelial ovarian cancers with little or no CA125 expression and in ovarian cystadenomas, tumors of low malignant potential, normal ovaries, and 16 other normal tissues.

Results. Low or absent expression of CA125 in surgical specimens of epithelial ovarian cancer was associated with low levels of serum CA125 in pre-operative serum specimens. In ovarian cancers that lacked CA125, all specimens (100%) expressed human kallikrein 10 (HK10), human kallikrein 6 (HK6), osteopontin (OPN), and claudin 3. A smaller fraction of CA125-deficient ovarian cancers expressed DF3 (95%), vascular endothelial growth factor (VEGF) (81%), MUC1 (62%), mesothelin (MES) (34%), HE4 (32%), and CA19-9 (29%). When reactivity with normal tissues was considered, however, MES and HE4 showed the greatest specificity. Differential expression was also found for HK10, OPN, DF3, and MUC1.

Conclusions. At the level of tissue expression, each of 10 potential serum markers could be detected in 29–100% of ovarian cancers that had low or absent expression of CA125. Several markers exhibited more intense expression in cancers than in normal organs. Further investigation is needed to demonstrate complementary expression of markers in serum.

Keywords: Ovarian carcinoma; Tumor markers; CA125; HK6; HK10; Claudin 3; Osteopontin

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Introduction

Ovarian cancer is neither a common nor a rare disease. In 2004 some 25,580 women will be diagnosed with ovarian cancer in the United States and 16,090 will die from the disease [1]. Currently, more than 50% of the women diagnosed with ovarian cancer survive 5 years, but less than 30% of women with advanced stage disease can be cured. When diagnosed in Stage I, however, the cure rate can approach 90% with currently available cytoreductive surgery and combination chemotherapy [2]. At present less than 25% of ovarian cancer cases in the U.S. are diagnosed in stage I. The possibility that detection of the disease at an early stage in a larger fraction of cases might impact on survival has prompted evaluation of different screening strategies.

Early detection has been attempted with ultrasonography [3–5], serum markers such as CA125 [7–9] or a combination of the two modalities where a rising serum marker would trigger transvaginal sonography [6–9]. The latter approach promises to provide an acceptable positive predictive value [10] and to be cost-effective [11]. A trial is currently being conducted in the United Kingdom to compare (1) conventional surveillance, (2) annual transvaginal sonography, and (3) an annual serum CA125 determination followed by transvaginal sonography if the CA125 is rising, judged by a computer algorithm [12,13]. Whatever the outcome of this study, however, CA125 alone cannot be an optimally sensitive initial step in a two-stage strategy to detect early stage disease. Ideally, the initial stage would detect all cases of ovarian cancer in early stage and the second stage would provide the necessary specificity to prompt an acceptable number of laparotomies for definitive diagnosis. At the time of conventional diagnosis, CA125 levels are elevated (>35 U/mL) in 50–60% of patients with stage I disease. Using a computer algorithm, a rising value of CA125 within the normal range might trigger detection of early stage ovarian cancer in a higher fraction of cases, but this fraction is not likely to exceed 80%. In approximately 20% of ovarian cancers, tissue levels of CA125 are low or absent.

Greater sensitivity might be achieved with multiple serum markers than with CA125 alone. Our recent studies suggest that as few as 4–5 markers might encompass the antigenic heterogeneity of ovarian cancer at the tissue level [14]. At least 27 serum and urine markers have been studied in combination with CA125 for the identification of patients with ovarian cancer [15]. These biomarkers have included oncofetal antigens, mucin-like proteins, enzymes, co-enzymes, enzyme inhibitors, receptors, cytokines, peptide hormones, other proteins, phospholipids, and sialylated lipids. Most studies have evaluated only 2–3 markers at a time and few have included a significant fraction of patients with low serum CA125 values despite the presence of clinically evident disease. To detect markers that would complement the expression of CA125 in ovarian cancer, we have identified ovarian cancers that express little or no CA125 and examined the expression of 10 potential candidate serum markers at the tissue level using immunohistochemistry.

Materials and methods

Patients

Samples from 322 women with primary epithelial ovarian cancer who had undergone initial surgery at the University of Texas M.D. Anderson Cancer Center between 1990 and 2001 were initially included in this study. Satisfactory immunohistochemical data could be obtained in 296. Follow-up information for the 296 patients was updated through June 2003 by reviewing medical records and the U.S. Social Security Index. Demographic and survival data were entered into a comprehensive database created with Microsoft Access (version 97). Histopathologic diagnoses were based on Gynecologic Oncology Group criteria [16] and each case was assigned a disease stage according to the International Federation of Gynecology and Obstetrics (FIGO) system [17]. Analysis of tissue blocks and chart review was conducted using protocols approved by the U.T. M.D. Anderson Institutional Review Board. Preoperative serum levels of CA125 were available from 192 cases. Normal levels of CA125 were considered to be less than 35 U/mL.

Selection of markers was done by review of the literature, from our own gene expression array analysis [14] and availability of antibodies suitable for immunohistochemistry for formalin-fixed and paraffin-embedded tissue.

Construction of tissue microarrays

Tissue blocks were stored under ambient conditions at approximately 24°C. Hematoxylin-and-eosin-stained sections were reviewed by a pathologist to select representative areas of tumor from which to acquire cores for microarray analysis. Tissue microarray blocks were constructed by taking core samples from morphologically representative areas of paraffin-embedded tumor tissues and assembling them on a recipient paraffin block. For this study microarray blocks were constructed with a precision instrument (Beecher Instruments, Silver Spring, MD) that uses two separate core needles for punching the donor and recipient blocks and a micrometer-precise coordinate system for assembling tissue samples on a block. For each case, two replicate 1-mm core-diameter samples were collected and each was placed on a separate recipient block. All samples were spaced 0.5 mm apart. Five-micrometer sections were obtained from the microarray and stained with hematoxylin and eosin to confirm the presence of tumor and to assess the

tumor histology. Tumor samples were randomly arranged on the blocks.

Sample tracking was based on coordinate positions for each tissue spot in the tissue microarray block; the spots were transferred onto tissue microarray slides for staining. This sample tracking system was linked to a Microsoft Access database containing demographic, clinicopathologic, and survival data on the subjects who provided the samples, thereby allowing rapid links between histologic data and clinical features. The array was read according to the given tissue microarray map; each core was scored individually and the results were presented as the mean of the two replicate core samples. Cases in which no tumor was found or no cores were available were excluded from the final data analysis.

Four different microarrays were constructed. The first tissue microarray contained 322 cases of ovarian carcinoma distributed over 4 blocks. The first pair (blocks 1a and b) contained duplicates of 158 spots, and the second pair (2a and b) contained duplicates of 164 spots. A total of 296 cases could be interpreted histologically. After immunostaining against CA125 a total of 65 cases showing negative or weak expression were identified. New cores from these cases from the same paraffin block were acquired and arrayed into a new second tissue microarray block containing all 65 samples and five known positive controls. A third tissue microarray block was constructed containing five core samples from 16 different normal tissues including endometrium, colon, spleen, kidney, pancreas, endocervix, skeletal muscle, small bowel, stomach, lymph nodes, liver, fallopian tube, adrenal, lung, appendix, and skin. The fourth tissue microarray contained core samples from 7 normal ovaries, 5 serous cystadenomas, 5 mucinous cystadenomas, 10 serous low malignant potential tumors, and 10 mucinous low malignant potential tumors.

Immunohistochemical analysis

Tissue microarray slides were subjected to immunohistochemical staining. After initial deparaffinization, endogenous peroxidase activity was blocked by using 0.3% hydrogen peroxide. Deparaffinized sections were microwaved in 10 mM citrate buffer (pH 6.1) to unmask antigenic epitopes. The slides were then incubated for 1 h at room temperature with optimal dilutions of monoclonal antibodies against CA125 (1:500) [18], VEGF (1:50 Vascular Endothelial Growth Factor Ab-3, NeoMarkers, Inc., Fremont, CA) [22], claudin 3 (1:50) [14], DF3 (1:500) [20], MUC1 (1:100, Muc-1 Core Glycoprotein monoclonal antibody, Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom) [21], CA19-9 (1:500, Fujiribio, Malvern, PA) [23], and at 4°C overnight for HK6 (1:500) [19], HK10 (1:500) [19], HE4 (1:500, Pacific Northwest Research Institute, Seattle, WA) [24], osteopontin (1:500) [25], and mesothelin (1:500, Pacific Northwest Research Institute, Seattle, WA) [26]. Slides were washed and incubated with appropriate biotin-labeled anti-globulins for 20 min, and finally with a 1:40 solution of streptavidin–peroxidase for 20 min. Tissues were then stained for 5 min with 0.05% 3′,3-diaminobenzidine tetrahydrochloride that had been freshly prepared in 0.05 M Tris buffer at pH 7.6 containing 0.024% H_2O_2 and then counterstained with hematoxylin, dehydrated, and mounted. All of the dilutions of antibody, biotin-labeled secondary antibody, and streptavidin–peroxidase were made in phosphate-buffered saline (pH 7.4) containing 1% bovine serum albumin. Conditions for each marker where optimized using the microarray block containing various normal tissue samples and whole sections from ovarian carcinomas. Antigen retrieval methods used were as follows: microwave, trypsin digestion, pressure cooker, and none at all. Increasing dilutions were tested for each marker starting at 1:50 to 1:1000 for 1, 2, and 4 h at room temperature and 4°C overnight. Negative controls were also made by replacing the primary antibody with phosphate-buffered saline. All controls gave satisfactory results.

The immunostained tissue microarray slides were scored using computerized digital analysis (Ariol SL-50, Applied imaging, California). The total cytoplasmic stained area was expressed in pixels, and total integrated optical density was expressed in arbitrary optical density units. For statistical analysis, all cases displaying total integrated optical density (mean ± SE) where then group together in a 0–3 scale. Negative staining was defined as absence of cytoplasmic stain and given a score of "0". Weak staining was given a score value of "1", intermediate a score of "2", and strong a score of "3". Counting criteria and software settings were identical for all slides. Quantitation was done blinded to clinicopathologic information. Normal ovarian epithelial cells and normal tissues were used as a comparison for intensity and pattern of staining. The mean of the results for the two replicate core samples from each tumor specimen was considered for each case.

Statistical analysis

Basic descriptive statistical analysis and tables were created using the Statistica Software Package Version 6.0 (Statsoft, Tulsa, OK). Correlation analysis for CA125 serum marker and immunohistochemical expression levels was done using Spearman correlation coefficient and differences were evaluated by chi-square analysis considering the results statistically significant at $P < 0.05$.

Results

CA125 expression is decreased in a fraction of ovarian cancers

CA125 expression was measured immunohistochemically in a tissue microarray that contained 296 cases of

primary ovarian carcinoma. Overall, 65 ovarian cancers (22%) exhibited decreased expression of CA125, including 38 cases (13%) that were negative and 27 cases (9%) with weak expression of the marker. CA125 was found mainly in the cytoplasm of epithelial tumor cells (Fig. 1A). In some cases staining was found in patches (Fig. 1B). Clinicopathological characteristics are compiled in Table 1. None of the 14 low-grade tumors exhibited loss of CA125 expression by immunohistochemical staining. Grade 2 and grade 3 ovarian cancers had decreased CA125 levels in 16% and 24% of cases, respectively, but this trend did not achieve statistical significance ($P = 0.2$). Decreased CA125 expres-

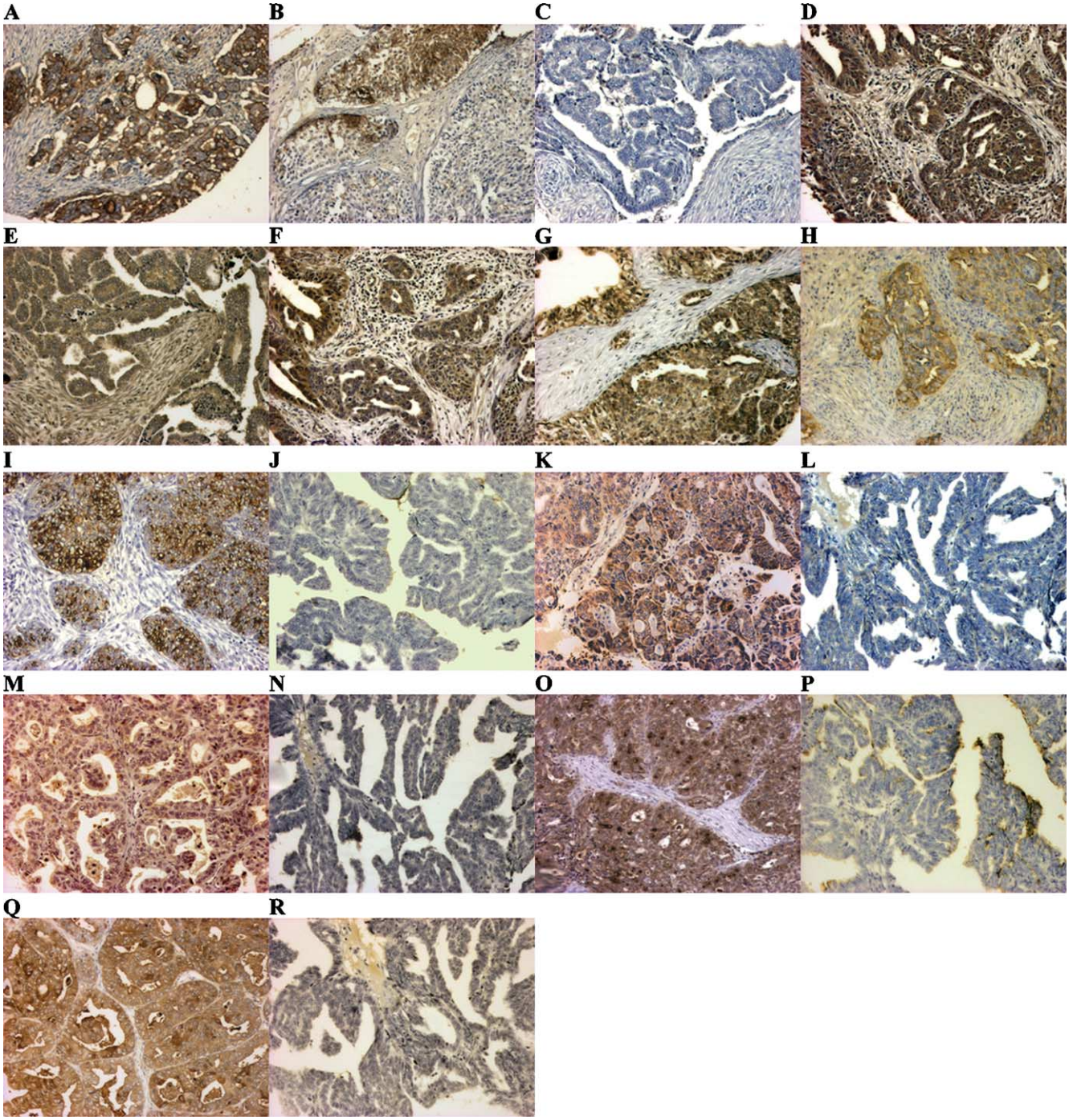

Fig. 1. Examples of immunohistochemical stains of biomarkers. (A) Strong staining; (B) patchy; and (C) negative staining for CA125 (20×). Positive immunostainings for (D) HK6, (E) HK10, (F) osteopontin, (G) claudin 3, and (H) DF3 (20×). (I) Positive immunostaining for MUC1, (J) negative staining for MUC1, (K) positive immunostaining for VEGF, (L) negative immunostaining for VEGF, (M) positive immunostaining for mesothelin, (N) negative immunostaining for mesothelin, (O) positive immunostaining for HE4, (P) negative immunostaining for HE4, (Q) positive immunostaining for CA19-9, and (R) negative immunostaining for CA19-9 (20×).

Table 1
Clinicopathological characteristics associated with CA125 level of expression in ovarian cancers on tissue microarrays

	0	1	2 + 3	Total
Tumor grade				
1	0 (0%)	0 (0%)	14 (100%)	14
2	3 (12%)	1 (4%)	21 (84%)	25
3	35 (13.6%)	26 (10.1%)	196 (76.3%)	257
P value		0.2		
Stage				
Stage I	10 (35.7%)	1 (3.6%)	17 (60.7%)	28
Stage II	1 (4.5%)	2 (9.1%)	19 (86.4%)	22
Stage III	21 (11.2%)	19 (10.2%)	147 (78.6%)	187
Stage IV	6 (10.2%)	5 (8.5%)	48 (81.4%)	59
P value		0.01		
Relapse				
Unknown	1 (20%)	0 (0%)	4 (0%)	5
No	14 (16.7%)	6 (7.1%)	64 (76.2%)	84
Progressive disease	8 (17.4%)	7 (15.2%)	31 (67.4%)	46
Yes	15 (9.3%)	14 (8.7%)	132 (82%)	161
P value		0.1		
Histotype				
Clear cell carcinoma	7 (50%)	1 (7.1%)	6 (42.9%)	14
Endometrioid adenocarcinoma	7 (22.6%)	2 (6.5%)	22 (71%)	31
Poorly differentiated carcinoma	2 (25%)	0 (0%)	6 (75%)	8
Malignant mixed mullerian tumor	4 (57.1%)	2 (28.6%)	1 (14.3%)	7
Serous carcinoma	13 (6.1%)	21 (9.9%)	178 (84%)	212
Mucinous adenocarcinoma	4 (66.7%)	0 (0%)	2 (33.3%)	6
Peritoneal papillary serous carcinoma	0 (0%)	0 (0%)	12 (100%)	12
Serous surface carcinoma	0 (0%)	1 (25%)	3 (75%)	4
Transitional cell carcinoma	1 (50%)	0 (0%)	1 (50%)	2
P value		<0.0001		
Level of cytoreduction achieved				
Suboptimal	14 (10.4%)	14 (10.4%)	106 (79.1%)	134
Optimal	24 (14.8%)	13 (8%)	125 (77.2%)	162
P value		0.45		
Age				
<55	11 (9.6%)	11 (9.6%)	93 (80.9%)	115
>55	27 (14.9%)	16 (8.8%)	138 (76.2%)	181
P value		0.4		
Total	38 (12.8%)	27 (9.1%)	231 (78%)	296

P values calculated using chi-square test of independence.

sion was found in a larger fraction of early stage than late stage ovarian cancers ($P = 0.01$). As in earlier studies, CA125 expression correlated with histotype ($P < 0.00001$). Serous cancers had decreased CA125 expression in only 16%, whereas decreased expression was observed in a higher fraction of malignant mixed mullerian tumors (MMMT) (86%), mucinous (66%), and clear cell (57%) histotypes. Primary peritoneal carcinomas and serous ovarian cancers that involved mainly peritoneal surfaces tended to have higher levels of CA125 expression than did their solid counterparts, although the number of cases is small.

Preoperative serum levels of CA125 correlate with tissue expression of the antigen in primary ovarian cancers

Pre-operative serum CA125 values were available from 192 cases. Serum levels of CA125 measured by immunoassay were compared to tissue levels of CA125 detected by immunohistochemistry using image analysis (Fig. 2). Overall, serum CA125 was elevated pre-operatively in 177 cases (92%). A high correlation between serum CA125 levels and immunohistochemical CA125 expression was found (Spearman rank correlation 0.28; $P \leq 0.0005$). Interestingly, serum CA125 was mildly elevated (>35 U/mL) in 20 of 27 cases (74%) where CA125 was not detected in tissue sections, consistent with expression of CA125 by normal mesothelial cells in the presence of ascites and metastatic implants. Median serum CA125 levels increased significantly from CA125-negative cancers (151 U/mL), to cancers that had weak expression of CA125 (878 U/mL), and to those cancers with strong expression of the antigen at a tissue level (1223 U/mL) (Table 2).

Several biomarkers complement CA125 at the tissue level

The 65 CA125 negative cases were then resampled and arrayed into a new block along with five known CA125-positive controls. Sections of the tissue arrays were then stained with antibodies against each of 10 potential serum markers, chosen from the available literature and from our own gene expression array analysis [14]. The expression of biomarkers in CA125-deficient cases is shown in Table 3 and in Fig. 3. Expression was graded as negative, weak, intermediate, and strong. HK10, HK6, OPN, and CLDN3 were expressed at some level by all CA125-deficient ovarian cancers (100%). DF3 expression was found in 95% of CA125-deficient cancers and the fraction of cancers stained strongly by anti-DF3 antibody exceeded the fraction of cancers stained strongly by antibodies against osteopontin or claudin 3. A smaller fraction of CA125-deficient ovarian cancers expressed VEGF (81%), MUC1 (62%), MES (34%), HE4 (32%), and CA19-9 (29%).

The intensity of the staining pattern was similar for HK10 and HK6, and this was stronger than for OPN, CLDN3, and DF3. While HK6 was more consistently expressed in the cytoplasm of epithelial cells, HK10 also stained very strongly the tumor stroma (Figs. 1D and E). OPN was found in the cytoplasm of epithelial tumor cells and in the stroma, staining fibroblasts and vessels, particularly in those areas with more active stromal response (Fig. 1F). CLDN3 was expressed in all 62 cases with a strong cytoplasmic stain, in some cases with reinforcement of the cytoplasmic membrane (Fig. 1G). DF3 and MUC1 were detected in the cytoplasm, and in a few cases only apically (Figs. 1H and J–R). VEGF, MUC1, MES, HE4, and CA19-9 were found in a smaller number of cases (Figs. 1J–R), but each complemented CA125.

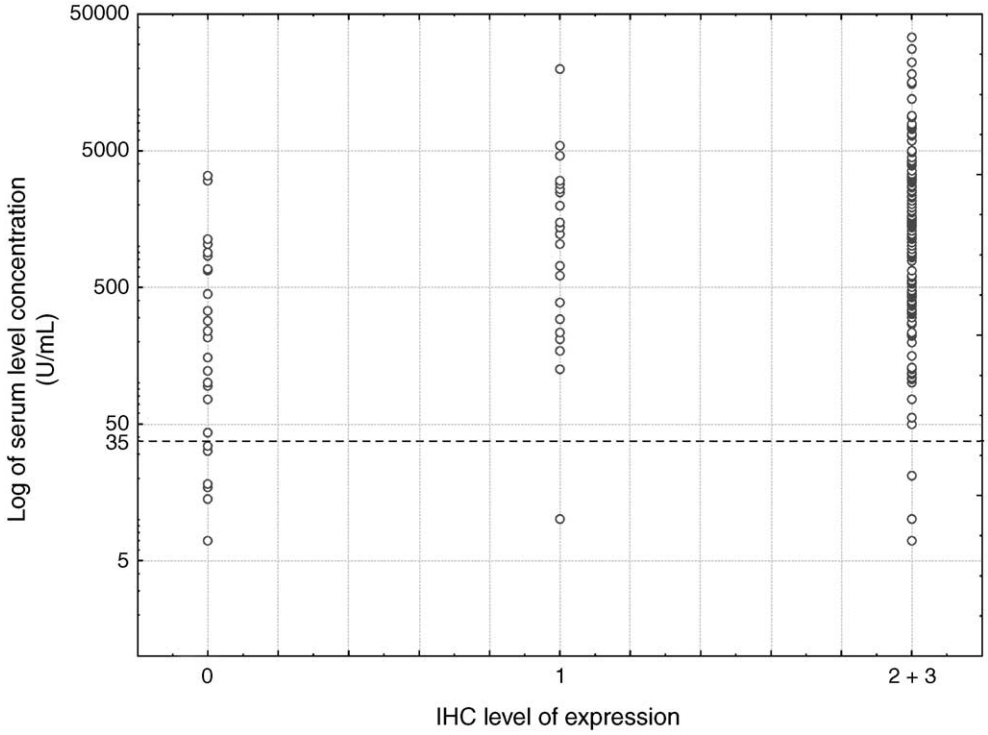

Fig. 2. Comparison of CA125 serum levels and immunohistochemical expression in tissue sections.

Biomarkers that complement CA125 are expressed by normal ovaries, ovarian cystadenomas, and tumors of low malignant potential

Expression of the 10 biomarkers was measured in tissue arrays that contained normal ovaries, benign ovarian cystadenomas, and ovarian tumors of low malignant potential. Intensity of expression was estimated on a scale of 0–3+ that ranged from negative to weak, intermediate, and strong. To permit comparison across tissues, a weighted average staining intensity was calculated for each antibody by summing the staining intensities (0–3) for each specimen and dividing by the total number of specimens assayed (Table 4). HK10 and HK6 exhibited intermediate expression in normal ovaries and the remaining markers were only weakly expressed, as was CA125. When marker expression in benign ovarian cystadenomas was compared to that in normal ovaries, HK10, HK6, CLDN3, DF3, MUC1, MES, and CA19-9 were upregulated by a full unit or more, OPN and CA125 did not change, and VEGF and HE4 exhibited an intermediate increase. Tumors of low malignant potential had higher levels of OPN, VEGF, and CA125 than did cystadenomas, but little change was noted in the other markers.

Biomarkers that complement CA125 are expressed by normal tissues. Each of the 10 biomarkers was evaluated in arrays that contained 80 specimens from 16 normal tissues including skin, endocervix, endometrium, fallopian tube, stomach, small intestine, appendix, colon, pancreas, liver, spleen, lymph node, adrenal, kidney, lung, and skeletal muscle. A weighted average staining index was calculated across these different tissues (Table 4). Little, if any, expression of MES and HE4 was detected in normal tissues (staining intensity 0.00–0.09), comparable to the expression of CA125 on the same array (0.17). Greater average staining

Table 2
Correlation between expression of CA125 in ovarian cancer tissues by immunohistochemistry (IHC) and serum CA125 levels

IHC expression	Serum CA125 Levels				
	<35 (U/mL)	>35 (U/mL)	Mean (U/mL)	Median (U/mL)	SD (U/mL)
0	7 (26%)	20 (74%)	523	151	860
1	1 (5.5%)	17 (94.5%)	2270	878	4616
2 + 3	7 (4.8%)	140 (95.2%)	2794	1223	4889

Spearman: 0.28, $P < 0.00005$

Table 3
Expression of biomarkers in 65 CA125-deficient cases

	Level of biomarker expression			
	0	1	2	3
HK10	0 (0%)	0 (0%)	11 (18%)	51 (82%)
HK6	0 (0%)	1 (2%)	6 (10%)	53 (88%)
OPN	0 (0%)	8 (14%)	33 (55%)	19 (31%)
VEGF	11 (18%)	22 (35%)	17 (28%)	12 (19%)
CLND3	0 (0%)	14 (23%)	1 (2%)	45 (75%)
DF3	3 (5%)	3 (5%)	37 (60%)	18 (30%)
MUC1	24 (38%)	1 (2%)	24 (39%)	13 (21%)
MES	40 (66%)	1 (2%)	3 (5%)	16 (27%)
HE4	42 (68%)	5 (8%)	5 (8%)	10 (16%)
CA19-9	43 (72%)	5 (8%)	9 (15%)	3 (5%)

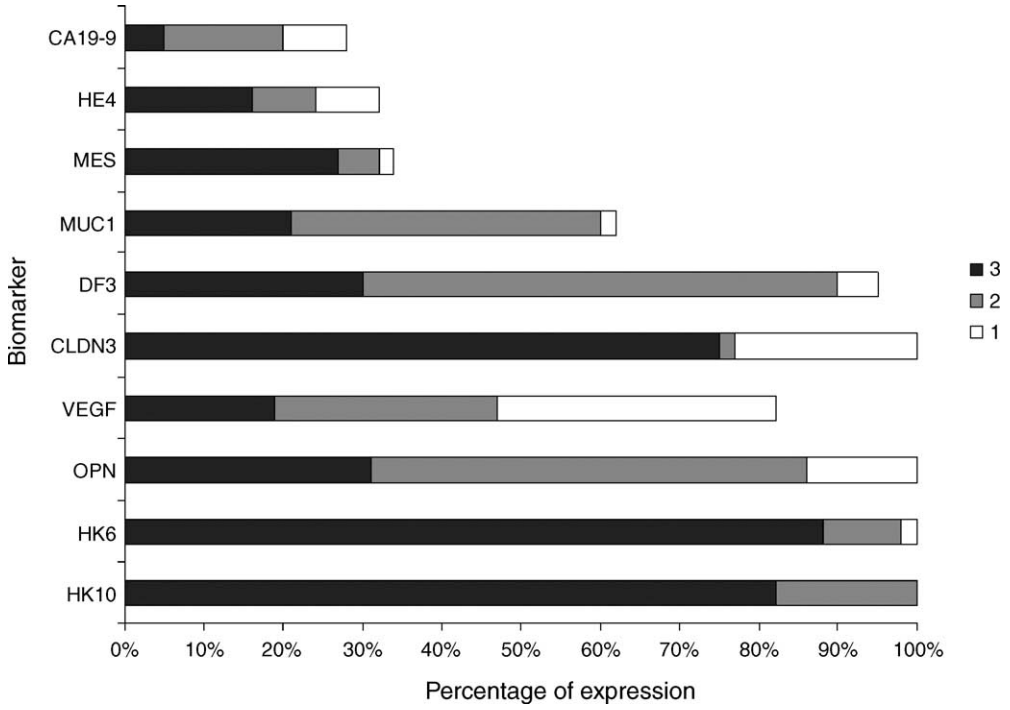

Fig. 3. Expression of biomarkers in 65 CA125-deficient cases.

intensity was found in the CA125-deficient invasive ovarian cancers (0.81–0.91) where 32–34% of cancers expressed MES or HE4. By contrast, HK6 and CA19-9 were expressed as strongly in normal tissues as in the ovarian cancers, arguing against their potential utility. Other markers had higher expression in normal tissue than did CA125, MES, or HE4, but several of these had substantially higher staining indices in the cancers than in normal tissues (1.67- to 2.33-fold), including HK10, OPN, DF3, and MUC1.

Discussion

At a tissue level, 22% of 296 ovarian cancers were found to have weak or absent CA125 expression on immunoperoxidase staining, consistent with previous observations [27,28].

Among clinicopathological features, CA125 expression varied with histotype. More consistent expression of CA125 was found in serous and endometrioid ovarian cancers. Conversely, more frequent loss of expression was observed in malignant mixed mullerian tumors, clear cell, and mucinous ovarian cancers. Within the serous histotype, low-grade cancers consistently expressed CA125, whereas medium and high-grade lesions had a higher rate of CA125 loss. Primary peritoneal and serous cancers that spread across the peritoneal surface also tended to express CA125. Decreased expression of CA125 in early stage cancers may relate to the larger fraction of non-serous histotypes in these lesions.

When CA125 was measured pre-operatively, the level of serum CA125 correlated with the intensity of tissue expression. Serum CA125 could, however, be elevated when CA125 had been lost at a tissue level. In part, this may relate to expression of CA125 by mesothelial cells of the parietal and visceral peritoneum in the presence of ascites or around metastatic implants. Other possible explanations include loss of CA125 in tissues during formalin fixation and paraffin embedding, or heterogeneity within cancers and between different metastases. Breitenecker et al. [23] found that 54% of the cases had discordant results for CA125 staining in different tumor metastases. Therefore, assessment of CA125 by immunohistochemical technique requires ample sampling of tumor tissue.

The primary aim of this study is to identify potential markers that can complement CA125 to detect ovarian carcinoma. Complementary expression of different markers,

Table 4
Biomarker weighted average staining intensity

	OSE	Cystadenomas	LMPs	CA125-deficient cancers	Other normal tissues
HK10	2.00	2.67	2.88	2.82	1.68
HK6	2.00	3.00	2.94	2.87	3.00
OPN	0.00	0.50	1.25	2.18	1.06
VEGF	1.00	1.75	3.00	1.48	0.93
CLDN3	1.00	2.17	2.53	2.75	2.10
DF3	1.12	2.17	2.60	2.14	1.13
MUC1	1.00	2.75	2.42	1.42	0.61
MES	1.00	2.28	1.50	0.91	0.00
HE4	1.00	1.83	1.92	0.81	0.09
CA19-9	1.00	2.50	2.33	0.53	0.61
CA125	1.00	1.20	2.24	–	0.17

OSE: ovarian surface epithelium; LMP: low malignant potential tumor.

where results are considered positive when any marker is elevated, might increase sensitivity. More than 20 different markers have been found elevated in the serum, plasma or urine of individual ovarian cancer patients whose CA125 values are within normal limits. While most reports have studied a limited number of markers in a limited number of serum, plasma, or urine specimens, the present study measures expression of 10 of the most promising markers in 65 CA125-deficient ovarian cancers selected from some 296 cases. In ovarian cancers that lacked CA125, all 65 specimens (100%) expressed HK10, HK6, osteopontin, and claudin 3. A smaller fraction of CA125-deficient ovarian cancers expressed DF3 (95%), VEGF (81%), MUC1 (62%), mesothelin (34%), HE4 (32%), and CA19-9 (29%).

When expression of the 10 biomarkers was measured in normal tissues, MES and HE4 appeared quite specific. Mesothelin is an antigen present in normal mesothelium, mesotheliomas, ovarian carcinomas, and some squamous cell carcinomas [29]. Similarly, mesothelin/megakaryocyte-potentiating factor mRNA was upregulated on expression arrays [30] and the mesothelin protein has been detected in sera from ovarian cancer patients [31]. Recently, serum mesothelin has been shown to complement serum CA125 in that a combination of the two markers produced an improved ROC curve relative to either marker alone [26].

Immunostaining of HE4 may have been suboptimal in that only 32% of the cases were positive. When HE4 was measured in serum, 6 of 7 patients with early stage disease (86%) and 24 of 30 patients with late stage disease (80%) had HE4 levels that exceeded those in 96% of 65 healthy individuals [24]. The serum assay demonstrated sensitivity similar to CA125, but produced fewer false-positive values with non-malignant ovarian disease [24]. In this regard it was surprising that in our present study, higher HE4 levels were observed at the tissue level in benign cystadenomas than in normal ovaries. Tumors of low malignant potential did not have higher levels of HE4 judged by immunohistochemistry than did the benign cystadenomas. By contrast, CA125 levels were minimally increased in the benign tumors, but substantially upregulated in the borderline tumors.

HK6 and HK10 were strongly expressed in 98–100% of CA125-deficient cancers. Recently, 12 new members of the human kallikrein (hK) family have been cloned [32]. Aside from their kininogenase activity, tissue kallikreins have been implicated in the processing of growth factors and peptide hormones. To date only 3 of the 15 kallikreins have been assigned a specific biological function. HK6 levels are elevated in a majority of early and late stage borderline and invasive ovarian tumors. Using expression array analysis, the NES-1 gene (HK10) was found upregulated in mRNA from ovarian cancers when compared to normal ovarian epithelial cells. Kallikrein 10 encoded by this message was expressed by 91% of serous ovarian cancers, 73% of non-serous ovarian cancers, and 73% of primary peritoneal cancers in tissue section [33]. Additionally, recent micro-array studies confirmed the overexpression of HK6, HK10, and other kallikreins in ovarian cancer [34,35,36]. Serum HK6 levels are elevated in about 66% of patients with ovarian cancer [37]. Similarly, serum kallikrein 10 levels were elevated in 56% of patients with ovarian cancer [38]. In our present study, differential expression of HK10, but not HK6, was found when marker levels were compared in CA125-deficient ovarian cancers and in normal tissues. This may reflect the limitations of extrapolating from apparent tissue expression to the detection of shed antigen in the blood. Alternatively, the relative specificity of HK10 might permit greater sensitivity for detecting early stage disease.

OPN, claudin 3, and DF3 were also expressed by 95–100% of CA125-deficient cancers. OPN is an integrin-binding protein believed to be involved in a variety of cellular functions [39]. It has been shown to play an important role in tumorigenesis, tumor invasion, and metastasis in breast, lung, prostate, and colon cancers [40–45]. The physiological functions of OPN are best documented in the bone where this secreted adhesive glycoprotein seems to be involved in osteoblast differentiation and bone formation, as well as in the anchorage of osteoclasts to bone and consequent bone resorption [46,47], immune function [48], and in vascular remodeling [49]. OPN has shown promise as a potential biomarker for both primary and recurrent ovarian cancer [25,50]. In our present study, relatively low expression of OPN was found in benign ovarian lesions, suggesting that it might distinguish malignant from benign disease.

Claudin proteins represent a large family of integral membrane proteins crucial for tight junction (TJ) formation and function. Claudins have been shown to be upregulated in various cancers and have been suggested as possible biomarkers and targets for cancer therapy. Normal cells typically express multiple claudin proteins, but some family members exhibit highly tissue-specific expression patterns [51]. The exact function of claudin proteins within TJs is still unclear, but they appear to be important in TJ formation and function. In a comprehensive study using RT-PCR analysis, immunoblotting, and immunohistochemistry of 70 cases of ovarian carcinoma, Rangel et al. [52] found that CLDN3 was overexpressed in 80–84% of the cases, mostly located at the membrane or in the cytoplasm, and was not associated with TJ integrity. In addition, the expression of CLDN3 was only present in 28–37% of the cystadenomas [52], consistent with our current study. Whether sufficient quantities of CLDN3 are shed from ovarian cancers to provide a useful serum marker remains to be determined. Expression of claudin in multiple normal tissues may limit its specificity.

Three mucin markers complemented CA125. DF3 antigen was expressed by 95% of CA125-deficient ovarian cancers, MUC1 by 62%, and CA19-9 by 29%. The murine monoclonal antibody designated DF3 reacts with a 300-kDa mammary epithelial antigen, an underglycosylated precursor of the DF3/MUC1 mucin-like glycoprotein [20]. DF3

antigen is elevated in sera from 47% of patients with ovarian carcinoma and in 15% of non-gynecologic malignancies [53]. In an immunohistochemical analysis of metastatic adenocarcinomas, DF3 did not show better specificity or sensitivity to detect ovarian tumors than CA125 alone [53]. MUC1 was found to be aberrantly expressed in 86–100% of all breast carcinomas and in serum in 21% [54,55]. On gene expression array analysis, MUC1 was frequently associated with ovarian cancers [14]. Immunohistochemical examination reveals that MUC1 is overexpressed primarily in serous carcinomas (90%), but also in benign lesions and in normal epithelium [56]. CA19-9 is a monosialoganglioside associated with mucins in gastrointestinal adenocarcinomas. In ovarian cancers, CA19-9 is most frequently associated with the mucinous histotype. As there were relatively few mucinous cancers in the tissue array used in this study (Table 1), we may have underestimated the value of CA19-9, particularly for detecting early stage disease where a greater fraction of mucinous cancers can occur. On the other hand, the staining index of CA19-9 in normal tissue was comparable to that in ovarian tumor tissue.

VEGF is an important angiogenic cytokine with a critical role in tumor angiogenesis and in increasing vascular permeability. High levels of VEGF have been detected in serum, cyst fluid, and ascites of ovarian carcinoma patients [57,58]. In ovarian carcinoma patients, statistically higher VEGF levels were detected in tumor effusions than in corresponding sera [57]. The prognostic value of the pretreatment concentration of serum growth factor, VEGF, is controversial. VEGF levels correlated with a poor prognosis of ovarian cancer in multivariate analysis in some [59,60], but not in all studies [58]. In addition to neovascularization and angiogenesis, VEGF also reflects the presence of ascites and tumor progression in ovarian cancer [61,62].

Overall from our study, OPN, HK6, and HK10 arise as promising potential markers than might complement CA125 in ovarian carcinomas. These markers showed a positive staining pattern in 100% of the cases that were CA125-deficient and in all five CA125-positive controls (data not shown). Furthermore, no staining was observed for OPN in normal ovarian surface epithelium and low levels in cystadenomas and in tumors of low malignant potential. Human kallikreins 6 and 10 expression levels were slightly lower in normal ovarian surface epithelium compared to most normal tissues, ovarian cystadenomas, and tumors of low malignant potential.

Expression of biomarkers in tissue may not reflect levels of antigen in serum. In some cases, such as CA125, serum assays may have greater sensitivity than would be predicted from tissue levels, but the opposite trend might be observed with other antigens that were less readily shed. Multiple markers are currently being evaluated against a standard panel of sera in a collaboration between four institutions funded by National Cancer Institute Specific Programs of Research Excellence led by investigators from the Fred Hutchinson Research Center. Ultimately, multiplexed assays may be required to evaluate multiple markers with limited amounts of serum that have been saved from screening trials.

When multiple markers are combined to improve sensitivity, specificity generally declines. Specificity is a particularly important issue in screening for ovarian cancer. Given the prevalence of ovarian cancer among postmenopausal women of 1 in 2500, a screening strategy must exhibit a sensitivity of at least 75% for early stage disease and a specificity of 99.7% to achieve a positive predictive value of 10%, i.e., no more than 10 laparotomies per case of ovarian cancer diagnosed. Mathematical techniques have been developed that permit multiple markers to be combined, increasing sensitivity without sacrificing specificity [63,64].

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