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Molecular Profiling of Circulating Cytokine Levels in Human Ovarian Cancer Patients

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Abstract. *Background: Growing evidence suggests that cytokines not only are associated with ovarian cancer development, drug resistance and metastasis, but also may provide valuable markers for ovarian cancer diagnosis and prognosis. Here, we determined the expression profiles of 43 plasma cytokines in ovarian cancer patients using this high throughput protein array technology developed in our laboratory. Materials and Methods: The expression of 43 cytokines from 13 ovarian cancer patients and 12 normal women was determined simultaneously using human cytokine antibody microarray technology. The differential expression of cytokines was analyzed using the Student's t-test and two-way hierarchical cluster analysis approach. Results: Our data showed that 22 cytokines were significantly increased in the plasma of ovarian cancer patients compared to normal women (t-test, two-tailed, $p<0.05$). The results from cytokine antibody array assays were in agreement with the published data, but also revealed a new group of cytokines whose expression levels were altered in ovarian cancer. Cluster analysis suggested an interesting link between cytokine profile and ovarian cancer. Conclusion: Human cytokine antibody arrays are a valuable tool to profile cytokine expression from patients' specimen. The cytokine profile may prove to be of diagnostic and prognostic significance in ovarian cancer.*

Ovarian cancer is one of the leading causes of cancer death among females (1, 2) in the United States and Europe. The diagnosis of ovarian cancer in the later stages was the primary case of mortality in most cases. Early diagnosis is the key to reducing ovarian cancer mortality rates . But even in late stages of the disease, the outcomes are highly variable. Clinicians and pathologists have repeatedly attempted to predict the biology of the tumor and, thus, the course of the disease, in individual patients to adjust therapy accordingly. Well-established conventional prognostic markers include International Federation of Gynecology and Obstetrics (FIGO) stage, grade, patient age and residual tumor. Although these markers are useful, the required information is difficult to obtain in earlier stages. When the markers become obvious, the tumors already are in a later stage. Therefore, it is imperative that markers be developed for early diagnosis, prognosis and personalized medicine.

Cytokines mediate a wide range of physiological processes. Accumulating evidence suggests that a complex cytokine network is involved in ovarian cancer (3, 4). Published data on the study of individual cytokines have suggested that a number of autocrine and paracrine cytokine loops influence the biology of this tumor (5, 6). Cytokines not only provide defense against cancer cells, but also promote cancer cell growth at every stage of cancer development (7). Alteration of cytokine levels is associated with cancer progression (8, 9), response to chemotherapy (10, 11) and metastatic status (12, 13). Therefore, cytokines will provide new insight into cancer biology, identify new molecular targets for cancer treatment and discover new biomarkers for diagnosis and prognosis of disease.

Recent studies have shown that multiple cytokines are secreted by ovarian cancer cells (4). Alteration of cytokines indeed may have prognostic and diagnostic value (5, 14, 15). However, the limitations of currently available technology have only allowed the measurement of a single or a few cytokines simultaneously in previous studies. This greatly limits our understanding of the roles of these factors in ovarian cancer and the potential application in clinical diagnosis and prediction of clinical outcome. Recently, we developed a high throughput protein array technology system that simultaneously detects expression levels of multiple cytokines (16-19). Here we

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investigated the expression levels of 43 cytokines in plasma from13 ovarian cancer patients and 12 healthy women using high throughput protein array technology. Our results indicated that 22 cytokines and chemokines are significantly increased in ovarian cancer patients ($p<0.05$). To the best of our knowledge, this is the first report on the detection of the multiple cytokine expression levels in ovarian cancer plasma using protein array technology.

Materials and Methods

All antibodies used in this study were purchased either from BD PharMingen (San Diego, CA, USA) or R&D (Minneapolis, MN, USA). All cytokines were obtained from Peprotech (Rochy Hill, NJ, USA). Cy3-conjugated streptavidin was purchased from Rockland (Gilbertsville, PA, USA).

Blood samples. Blood samples were collected as previously described in the study of the correlation between glycodelin levels and gynecological cancer (20). Briefly, approximately 10 ml of venous blood was drawn from the patients after getting their consent. Plasma was collected and stored at -80˚C until use.

Antibody array on hydrogel substrate. Arrays were printed onto HydroGel™ pads slides (PerkinElmer Life Science, Meriden, CT, USA). Capture antibodies were diluted to a concentration of 200 μg/ml in 1 mg/ml of bovine serum albumin (BSA) and were printed at a volume of 350 pl per spot using a BioChip Arrayer™ (PerkinElmer Life Science). For each antibody, four replicates were printed at a pitch of 500 mm in the layout illustrated in Figure 1.

Antibody array assays. Printed slides were blocked with 1.0 % BSA for 1 hour or overnight. After being dried by low speed centrifugation, arrays were incubated with 50 μl of patient's or normal plasma. Incubations were carried out for 1 hour at room temperature in gasketed and sealed arrays on a rotating shaker. The slides were then washed two times for 10 minutes in 0.5%Tween/ phosphate-buffered solution (PBS) followed by a wash in PBS. The slides were dried by low speed centrifugation. Fifty μl of all 43 biotinylated detection antibodies at levels optimized for this system were loaded on to the slides, incubated for 1.5 hour at room temperature and washed and centrifuged as in the previous step. Finally, the slides were incubated for 1 hour at room temperature in 50 μl of Cy5-conjugated streptavidin. The slides were again washed and dried by the above procedure. When the volume of the sample permitted, the arrays were repeated at least once.

Imaging and data analysis. Arrays were imaged in the Cy5 channel using the ScanArray 5000 cofocal slide scanner (PerkinElmer Life Science). Within a test of plasma between cancer samples and normal samples, all slides were scanned using the same PMT and laser power settings. Images were analyzed using Quant-Array™ software (PerkinElmer Life Science). Data were analyzed using *t*-test (two-tailed). *p*-values less than 0.05 were considered statistically significant. Cluster analysis was performed using publicly available software Clusfavor 6.0 (http://mbcr.bcm.tmc.edu/genepi/). Scatter plot and Pearson's correlation were analyzed using the BRB ArrayTools software package 2.0 (NCI, Bethesda, MD, USA) and SPSS 8.0 computer program (SPSS Inc., Chicago, Illinois, USA).

ELISA. Conventional ELISA was performed according to the manufacturer's instructions (BD PharMingen). Essentially, 96-well ELISA plates were coated overnight at 4˚C using 100 μl of 4 μg/ml capture antibodies. 1% BSA/PBS was used as a blocking buffer. One hundred μl of 2-fold diluted plasma from cancer patients and different concentrations of standard cytokines were added to each well in duplicate. The plates were incubated for 3 hours at room temperature or overnight at 4˚C. Unbound materials were washed out with PBS/0.05% Tween. One hundred μl of 1 μg/ml of the appropriate biotinylated anti-cytokine detection antibody were added to each well. The plates were incubated for 1 hour at room temperature. After washing, 100 μl of streptavidin-HRP conjugated antibodies were added to the wells and incubation was continued for 30 additional minutes at room temperature. After extensive washing, color development was performed by incubation with substrate solution containing ethylbenzthiazoline sulphonate (Sigma, St. Louis, MO, USA). O.D. at 405 nm was determined by a microplate reader. Standard curves were generated by Sigma plot and the concentrations of different samples were determined from the standard curves.

Results

Previously we have shown that the cytokine antibody array system developed in our laboratory could be used to detect cytokine expression levels from real biological samples. In this study, we applied this novel technology to determine the expression profiles of 43 cytokines (Figure 1) in the plasma from ovarian cancer patients and normal subjects. Cytokines in this study included anti-inflammatory cytokines, pro-inflammatory cytokines, growth factors, angiogenic factors or chemotactic cytokines and others. Some of these cytokines reportedly are altered in ovarian cancer patients.

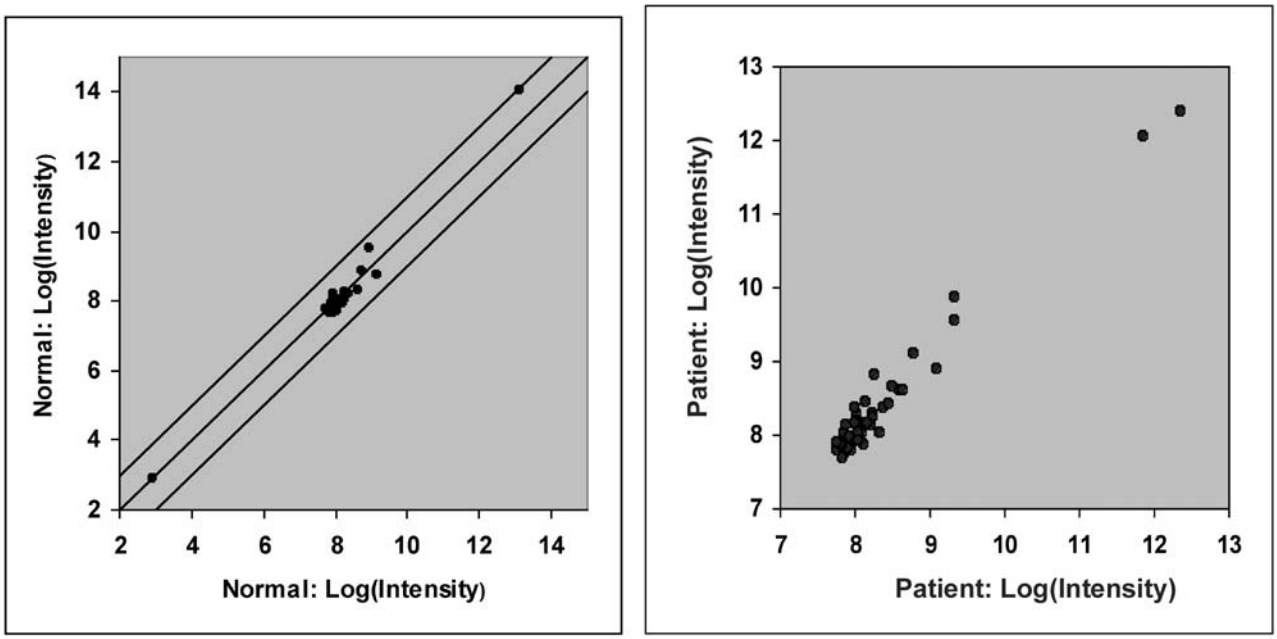
First we further determined the reproducibility of the assay. The logarithm (base 2) normalized intensity was performed by scatter plot analysis. For each graph, the results of experiment 1 are plotted on the x-axis, and the results of experiment 2 are plotted on the y-axis. If all results were in perfect agreement, the point would fall on the 45˚ identity line. As shown in Figure 2, the Pearson correlation coefficients for intra-slides and inter-slides were 0.9990 and 0.9646, respectively, suggesting high reproducibility of the assay.

Next, a total of 13 ovarian cancer patients' plasma plus 12 normal controls were assayed for expression levels of 43 cytokines with the goal of discovering new diagnostic markers for ovarian cancer. These plasma samples were used in our previous study of the expression of glycodelin in ovarian cancer (20). The characteristics of the patients included in this study are summarized in Table I. Cytokine antibody chips were incubated with 50 μl of plasma. To avoid potential variations, all slides were assayed and scanned using the same conditions and setting. Images were analyzed using Quant-Array software. The representative raw images are shown in Figure 3. Every capture antibody

43 Cytokine Antibody Array Map

Row \ Spot	1	2	3	4	5	6	7	8
1	cy5 (1/20)	cy5 (1/100)	cy5 (1/500)	cy5 (1/2500)	cy3 (1/20)	cy3 (1/100)	cy3 (1/500)	cy3 (1/2500)
2	BSA	TNF-α	IFN-γ	SDF-1	IL-2	TNF-β	GM-CSF	ENA-78
3	IL-4	IL-6	GCSF	I-309	IL-7	OSM	IL-3	MCP-1
4	IL-10	PDGF	IL-5	MCP-2	IL-12	ANG	SCF	MCP-3
5	IL-13	EGF	TPO	MDC	IL-15	IGF-1	GRO	MIP-1β
6	Leptin	VEGF	GRO-α	MIP-1γ	IL-1α	MCSF	IL-8	TARC
7	IL-1β	TGF-β1	MIG	RANTES	Biotin-IgG	Biotin-IgG	Biotin-IgG	Biotin-IgG

Figure 1. *Location of 43 cytokines spotted onto HydroGel chips. Only one quarter of the map was shown. The other three quarters were the duplicate of this map.*



1. Intra-slide (a Pearson's correlation coefficience of r^2=0.999). **2. Inter-slide (a Pearson's correlation coefficience of r^2=96.46).**

Figure 2. *Scatter plots of normalized intensity from the plasma intra- or inter-slide on log (base 2) scale. The log base 2 values of the signal intensities for duplicates experiments are plotted.*

was spotted in four replicates; therefore, four intensity readings for every cytokine were obtained and averaged. The CVs for all 43 cytokines were compared and summarized in Table II. Most of the CVs are smaller than 10%, suggesting the high reproducibility of the assay. The averages were used to compare the differential expression among normal and patients' plasma.

The comparison of cytokine expression in ovarian cancer patients' plasma and normal subjects' plasma revealed that 22 cytokines are significantly increased in ovarian cancer patients (Figure 4 and Table III). Among them, 14 cytokines were increased in ovarian cancer patients with *p* values less than 0.01, including IL-8, TNFβ, IL-13, TNFα, MIP-1β, IL-2, IL-6, MCP-1, Rantes, IL-3, IL-15, GROα,

Table I. *Classification and characteristics of normal and tumor subjects.*

	Normal (female)	Ovarian cancer
Number	Total: 12	Total: 13
Age (median range)	50.9 (29-86)	62.5 (33-85)
Premenopausal	2	4
Premenopausal	10	9
Stage		I-1 III-8 IV-3 NA-1
Histology		Serous: 1 Papillary: 6 Mucinous: 1 Endometroid: 1 Granulosa cell: 1 Metastatic: 1 MMMT: 1 Borderline: 1

IFN-γ and ENA-78. In addition, 8 cytokines were expressed highly in ovarian cancer patients with *p* values between 0.01 and 0.05. They were PDGF, TGF-β1, IL-12, OSM, TARC, IL-1α, MCSF and IL-5. Furthermore, the mean levels of SCF, IL-1α, GRO, MCP-2, MCP-3, GCSF are not very different between cancer patients and controls (the ratios between patients and normal subjects are less than 1.5-fold). The mean levels of GM-CSF, ANG and MIP-1γ in cancer patients are lower than normal, but they are $p>0.05$ (Table III).

To validate the array results, we selected two cytokines (PDGF and EGF) for ELISA assay. As shown in Figure 5, the relative expression levels of PDGF and EGF are similar between arrays and ELISA with *p* values (one-way ANOVA) of 0.563 and 0.391, respectively, suggesting the reliability of our array approach.

A major goal of our research is to discover novel biomarkers and expression patterns that may serve as markers. As a first step, cluster analysis was used to organize the cytokines with *p* values less than 0.01 into categories related to disease status. As shown in Figure 6, although no perfect separation between normal samples and malignancies was found, in most cases there were clear indications that a pattern of cytokine expression may be used to distinguish between normal subjects and cancer patients. Among the 12 normal subjects, 8 samples were clustered in one cluster. There is another major cluster, which contains 8 ovarian cancer samples plus one normal subject sample.

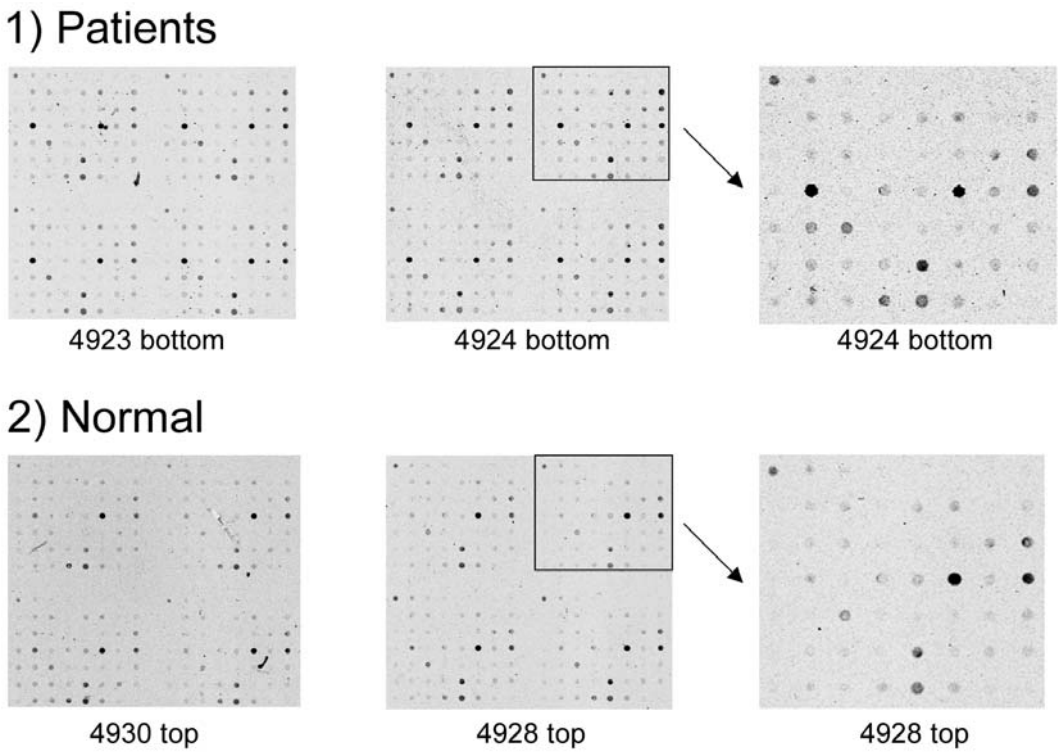


Figure 3. *A representative of raw images of human cytokine antibody microarrays for normal subjects and ovarian cancer patients.*

Table II. *Variability of human cytokine antibody arrays.*

Cytokine	Intra-arrays		Inter-arrays			Intra-arrays		Inter-arrays	
	SD	CV%	SD	CV%		SD1	CV%	SD	CV%
TNF-α	42.4	8.5	3.97	1.20	MCP-3	207.0	18.4	197	25.0
IFN-γ	38.0	10.5	9.47	3.09	IL-13	32.8	10.3	50.4	17.2
SDF-1	31.2	10.4	20.4	8.69	EGF	123.4	10.9	46.1	14.6
IL-2	71	14.9	11.6	4.04	TPO	72.4	11.2	46.4	9.06
TNF- β	50.4	10.9	0.05	0.01	MDC	40.9	12.0	14.9	6.90
GM-CSF	26.3	6.01	3.22	1.31	IL-15	30.0	9.01	32.8	12.6
ENA-78	168.4	10.8	32.3	8.43	IGF-1	27.2	9.00	4.41	1.90
IL-4	35.6	7.03	3.67	1.68	GRO	29.0	7.68	8.79	3.28
IL-6	35.2	6.81	3.21	1.20	MIP-1β	45.9	10.0	1.36	0.447
GCSF	39.0	10.3	4.26	1.75	Leptin	62.0	8.23	7.46	3.25
I-309	46.7	10.5	12.8	5.71	VEGF	123.3	13.3	20.1	7.21
IL-7	60.4	9.82	9.95	4.31	GRO-α	36.7	7.92	2.00	0.758
OSM	83.8	13.0	15.7	6.04	MIP-1γ	45.2	9.90	15.2	6.00
IL-3	51.6	9.51	2.12	0.61	IL-1α	166.3	14.8	77.1	11.0
MCP-1	66.1	11.3	81.4	16.4	MCSF	33.1	9.6	3.64	1.47
IL-10	30.6	7.43	10.5	4.58	IL-8	50.3	8.80	0.631	0.221
PDGF	1091	20.9	373	9.38	TARC	51.7	10.1	43.6	14.8
IL-5	31.4	8.72	19.3	7.81	IL-1β	29.3	9.88	12.9	5.67
MCP-2	56.7	11.2	37.1	13.1	TGF-β1	34.3	8.39	16.5	6.04
IL-12	56.1	13.8	31.6	12.5	MIG	35.8	9.36	20.0	7.37
ANG	4853	13.0	77.6	1.46	RANTES	58.6	12.7	7.53	1.92
SCF	25.8	9.95	98.7	26.1					

Discussion

In this paper we intended to identify differential cytokine expression in plasma from ovarian cancer patients and normal subjects. Using cytokine antibody arrays we had developed (5, 14, 15), we screened the expression levels of 43 cytokines in plasma samples from 13 ovarian cancer patients and 12 normal subjects. We found that 22 cytokines showed significant differential expression between those two groups with p values less than 0.05. Our data are well in agreement with data reported in the literature (Table III). Previous studies reported higher expression levels of IL-6 (21-23), TNFα (21), IL-8 (24;25), IL-1β (26), MCSF (27), TGF-β1 (28), MCP-1 (29) and IL-1α (26) in women with ovarian cancer. Moradi *et al.* reported (21), in serum, TNFα and IL-6 were significantly increased in primary ovarian cancer patients when compared with control subjects ($p<0.007$). Suzuki M *et al.* (27) analyzed serum macrophage colony-stimulating factor (MCSF) levels in 69 patients with ovarian cancer and 634 healthy individuals, including 398 women, using an ELISA. The average serum MCSF level was 754.4±153.9 U/mL in healthy females. Serum MCSF levels were significantly elevated in patients with ovarian cancer (average 1460.5±1006.2 U/mL; $p<0.001$). Zeisler *et al.* (26) measured serum IL-1α and IL-1β levels by ELISA in 75 ovarian cancer patients and 50 healthy controls. Both serum IL-1α and IL-1β levels were elevated in ovarian cancer patients compared with healthy controls (Chi-square test, both $p<0.001$). Mean serum IL-1α and β levels decreased significantly after surgical intervention (paired t-test, $p=0.0001$ and $p=0.0002$, respectively). In addition, we also found that TNFβ, IL-13, MIP-1β, IL-2, Rantes, IL-3, IL-15, GROα, IFN-γ, ENA-78, PDGF, IL-12, OSM, TARC and IL-5 were increased in ovarian cancer patients ($p<0.05$, Table III and Figure 4).

The potential for cytokines to play important roles as biomarkers for the diagnosis and prognosis of ovarian cancer has resulted in extensive investigations to determine the various levels of circulating cytokines. To date, most of the data collected in ovarian cancer were generated by enzyme-linked immunosorbent assay (ELISA). The requirement of large volumes of sample and high cost greatly limit the measurement of multiple cytokine expression using ELISA. However, it is becoming clear that a complex cytokine network does exist (3, 4) and multiple cytokines are often deregulated simultaneously in ovarian cancer (4). Therefore, it is urgent to develop a multiplexed assay for simultaneous detection of multiple cytokines. We have developed several cytokine antibody arrays for simultaneous detection of multiple cytokine expression (16, 17, 19, 30, 31). Such cytokine antibody arrays have been successfully used in the study of molecular mechanisms

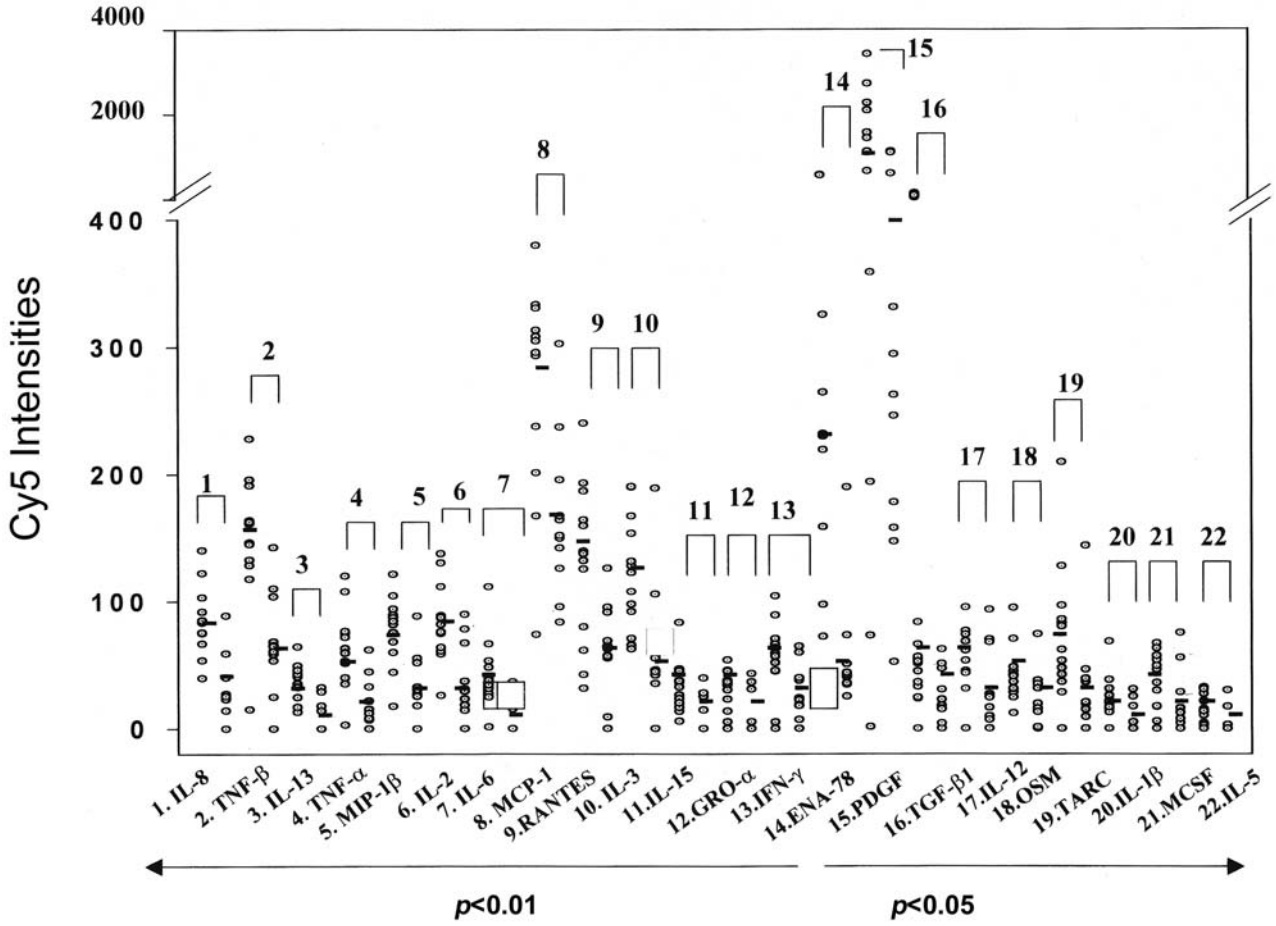


Figure 4. *Distribution of cytokine expression in plasma of ovarian cancer patients and normal subjects with statistical significance. Plasma from ovarian cancer patients (left panel) and normal subjects (right panel) was used to determine the expression levels of 43 cytokines using human cytokine antibody microarrays. The relative expression levels of individual cytokines were compared analyzed with student t-test between ovarian cancer patients and normal subjects. Among 43 cytokines, 22 showed significant changes in ovarian cancer patients compared with normal subjects.*

involved in cancer suppression (18), identification of potential targets of vitamin E (32) and PPARγ (33). To the best of our knowledge, this is the first attempt to identify potential biomarkers or classification of ovarian cancer using cytokine antibody array technology.

Potential applications of simultaneous measurement of expression levels of multiple cytokines may be to classify normal subjects and cancer patients or to provide markers for diagnosis and prognosis. Recently, cDNA microarrays have been used to classify tumors according to gene expression patterns (34-37). Our approach, if successful, will have an even more profound effect and practical significance than the cDNA microarray approach since analyses using protein arrays can be performed using plasma or other fluids. Cluster analysis shows a potential distinction between ovarian cancer patients and normal subjects using cytokine antibody array technology. It is interesting to see whether cytokine antibody arrays can accurately predict ovarian cancer by using higher-density cytokine antibody arrays and/or more advanced analysis tool (38, 39).

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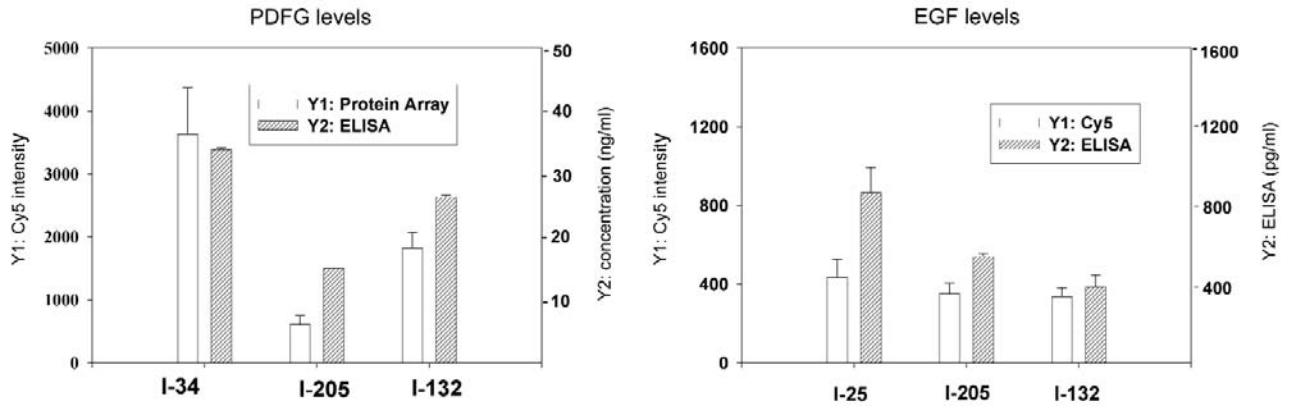

Figure 5. *Comparison of expression levels of PDFG and EGF from several samples determined by ELISA and cytokine antibody arrays. In general, the expression levels are similar between the two approaches.*

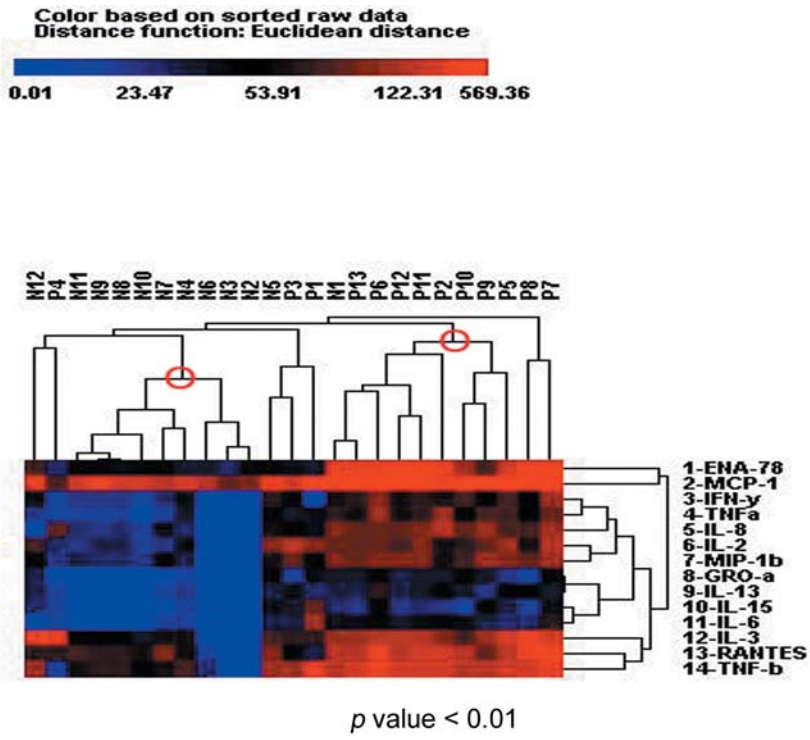

Figure 6. *Clustering analysis of cytokine expression levels between normal subjects and ovarian cancer patients. Hierarchical clustering based on a group of cytokines whose expressions were significant changes with p value less than 0.01 was performed on samples from 13 ovarian cancer patients and 12 normal subjects.*

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Table III. *Summary of plasma cytokine expression in ovarian cancer.*

	Patient/ Normal	T test (p value)	Our Array	Literature		Patient/ Normal	T test (p value)	Our Array	Literature
Il-6	4.75	<0.001	↑*	↑* [1,2,11]	MCP-1	1.66	<0.001	↑*	↑* [7]
ENA-78	4.00	0.010	↑*	N	I-309	4.66	0.075	↑	N
TNF-α	3.69	<0.001	↑*	↑* [1,11]	IL-7	2.01	0.223	↑	N
IL-13	3.50	<0.001	↑*	N	VEGF	1.99	0.154	↑	↑* and → [8,9]
IL-15	3.47	0.003	↑*	N	IGF-1	1.88	0.119	↑	→
GRO-α	3.19	0.003	↑*	N	IL-10	1.87	0.122	↑	N
IL-5	3.13	0.040	↑*	N	MDC	1.85	0.470	↑	N
IL-8	3.07	<0.001	↑*	↑* [3,4]	TPO	1.74	0.062	↑	N
IL-1β	2.98	0.025	↑*	↑* [5,11]	MIG	1.50	0.502	↑	N
IL-2	2.57	<0.001	↑*	N	SCF	1.43	0.422	↑	N
PDGF	2.79	0.012	↑*	↑* [6]	IL-1a	1.30	0.115	↑	↑* and → [1,5,11]
MIP-1β	2.46	<0.001	↑*	N	GRO	1.29	0.667	↑	N
TNF-β	2.39	<0.001	↑*	N	MCP-2	1.23	0.561	↑	N
TARC	2.39	0.024	↑*	N	MCP-3	1.2	0.230	↑	N
IFN-γ	2.30	0.004	↑*	N	SDF-1	6.62	0.051	↑	N
RANTES	2.25	0.001	↑*	N	IL-4	4.88	0.164	↑	N
IL-3	2.25	0.002	↑*	N	Leptin	1.78	0.239	↑	N
MCSF	2.17	0.032	↑*	↑* [13]	GCSF	1.15	0.846	↑	N
OSM	2.07	0.015	↑*	N	ANG	0.708	0.131	↓	→ [9]
IL-12	2.06	0.015	↑*	N	GM-CSF	0.609	0.686	↓	N
TGF-β1	2.01	0.013	↑*	↑* [12]	MIP-1γ	0.72	0.545	↓	N

Notice: ↑ : Increased (average), ↓ : Decreased (average), → : No changed (average), N: No published data, * : $p<0.05$.

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