Enhanced Protein Profiling Arrays with ELISA-Based Amplification for High-Throughput Molecular Changes of Tumor Patients' Plasma

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ABSTRACT

Purpose: **The purpose of this study is to develop a high-throughput approach to detect protein expression from hundreds and thousands of samples and to apply this technology to profile circulating angiogenic factor protein levels in patients with gynecological tumors.**

Experimental Design: **Analytes containing a mixture of protein are immobilized onto antibody-coated surface of support in array format. The presence of protein in analytes is detected with biotin-labeled antibody coupled with an enhanced chemiluminescence or fluorescence detection system. The exact amount of protein can be quantitatively measured. The expression levels of five angiogenic factors (angiogenin, interleukin 8, vascular endothelial growth factor, platelet-derived growth factor, and epidermal growth factor) from 157 samples were quantitatively measured using this novel protein array technology and were statistically analyzed. The expression patterns of angiogenic factors were analyzed using two-way hierarchical cluster analysis approach.**

Results: **A novel protein array technology, which can simultaneously and quantitatively measure few protein levels from hundreds and thousands of samples was developed. Only minute amounts of sample are required for the assay. This approach also features high sensitivity and specificity. Using this novel protein array approach, we analyzed the plasma expression levels of five angiogenic factors in 137 patients diagnosed with a tumor and 20 controls. Statistical analysis reveals different expression levels of angiogenic factors between patients and controls. Cluster analysis suggests a possible classification of normal subjects from patients.**

Conclusions: **Enhanced protein profiling arrays provide a high-throughput and sensitive system to detect one or few protein from hundreds and thousands of samples. Such an approach should have broad application in biomedical discovery.**

INTRODUCTION

Angiogenesis, a process leading to growth of new blood vessels, is a fundamental requirement for reproduction, embryogenesis, wound healing and tissue repair (1). The physiological control of angiogenesis involves a delicate and complex balance between angiogenic factors and angiogenesis inhibitors. At least 20 angiogenic factors have been identified, including vascular endothelial growth factor (1), interleukin (IL)-8 (2, 3), angiogenin (4), epidermal growth factor (EGF; 5), platelet-derived growth factor (6), basic fibroblast growth factor (7), glycodelin (8) and PR39 (9). Increasing numbers of angiogenesis inhibitors also have been found *in vivo*, such as endostatin (10), angiostatin (11, 12), thrombospondin-1 (13) and 2-methoxyestradiol (14). Loss of this balance may result in impaired or overactivated angiogenesis, and may lead to the development of certain diseases, including cancer (15), cardiac diseases (16), diabetes (17), endometriosis (18), and many others (19, 20).

A detailed understanding of the mechanisms of angiogenesis should lead to improved treatment of such diseases. Particularly in cancer, angiogenesis is a prerequisite for growth and metastasis of solid tumors (21, 22). Furthermore, measurement of angiogenic factors in a patient's serum may provide prognostic and diagnostic information. Indeed, the prognostic value of angiogenic factors has been linked to breast carcinoma (23–25), colorectal carcinoma (26), head and neck squamous cell carcinoma (27) and Wilms tumor (28, 29). In addition, a possible link between angiogenic factors and gynecological malignancy has been extensively investigated (30–32).

Because multiple factors may contribute to angiogenesis, it is critical to determine the expression levels of multiple angiogenic factors simultaneously. The expression levels of angiogenic factors are primarily determined and quantitated by ELISA, reverse transcription-PCR, and RNase protection. The ability of these methods to quantitatively measure multiple samples for angiogenic factors simultaneously is greatly limited because they require large sample volumes and exhibit low throughput. Protein arrays are now being designed to meet this increasing demand.

Previously, we developed several cytokine protein array

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technologies and began to apply this technology to analyze human diseases (33–36). However, it has been difficult and cumbersome to develop a quantitative system in array format. Furthermore, when comparing a limited number of factors from large sample sizes, it is much easier to array the samples in the same matrix and test for one or several factors at a time. In this article, we describe an innovative approach that detects from one to several factors simultaneously in hundreds or thousands of samples with high specificity and sensitivity. We specifically apply this technology to analyze levels of angiogenic factors in plasma samples from tumor patients.

MATERIALS AND METHODS

Materials. All antibodies were purchased from either BD PharMingen (San Diego, CA) or R&D (Minneapolis, MN). All cytokines were obtained from Peprotech (Rocky Hill, NJ). Horseradish peroxidase-conjugated streptavidin was purchased from BD PharMingen.

Blood Samples. Blood samples were collected as described previously and have been used by us in the study of the correlation between glycodelin levels and gynecological cancer (37). Briefly, ~10 ml of venous blood was drawn from patients after receiving their consent. Plasma was collected and stored at −80°C until use. The plasma samples were collected and analyzed by different investigators; therefore, the plasma samples were blinded during analysis.

Enhanced Protein Profiling Arrays. Hybond enhanced chemiluminescence membranes were soaked with corresponding antibodies for 4 h at 4°C. Membranes were then air-dried. Plasma (0.2 μl) or a like amount purified recombinant cytokine was spotted onto the membranes. After blocking with 5% BSA/Tris-buffered saline (TBS), membranes were incubated with biotin-conjugated antibody at room temperature for 2 h. After extensive washing with TBS/0.1% Tween three times and TBS twice, membranes were incubated with horseradish peroxidase-conjugated streptavidin at room temperature for 1 h. The signals were visualized using the Enhanced Chemiluminescence (ECL) system (Amersham Pharmacia Biotech Aylesbury, United Kingdom). In some cases, membranes were incubated with cy3-conjugated streptavidin and signals were imaged by laser scanner.

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 8 μg/ml capture antibodies. 1% BSA/PBS were used as a blocking buffer. One hundred μl of 2-fold diluted patients' sera and different concentrations of standard cytokines were added to each well in duplicate. The plates were incubated for 3 h at room temperature or for overnight at 4°C. Unbound materials were washed out with PBS/0.05% Tween. One hundred μl of the appropriate biotinylated anticytokine detection antibody (1 μg/ml) were added to each well. The plates were incubated for 1 h at room temperature. After washing, 100 μl of streptavidin-horseradish peroxidase conjugated antibodies were added to the wells, and incubation was continued for 30 min at room temperature. Followed by extensive washing, color development was done by incubation with substrate solution containing ethylbenz-thiazoline sulfonate (Sigma, St. Louis, MO). Optical density 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.

Data Analysis. The intensities of signals were scanned and quantitated by densitometry (Bio-Rad, Hercules, CA) using equal-sized spot. Standard curves were generated using Sigma plot (Chicago, IL). Data were analyzed using a *t* test (two-tailed). The *t* test (two-tailed) was analyzed using SPSS 8.0 computer program (SPSS Inc., Chicago, IL). A P less than 0.05 was considered statistically significant. Unsupervised cluster analyses were performed using publicly available software, Clusfavor 6.0 (http://mbcr.bcm.tmc.edu/genepi/).

RESULTS

Development of an Enhanced Profiling Protein Array System. Tissue microarrays have become a powerful means for high-throughput molecular profiling of tumor specimens. Equally important is the detecting of hundreds and even thousands of plasma, conditioned media, tissue lysate, cell lysate, and other fluids quantitatively. Two major difficulties are encountered in this type of assay. One difficulty is the considerably different properties of the antigens being analyzed; the other is the difficulty involved with detecting expression levels in minute volumes of analytes. To overcome both problems, we precoated membranes with specific antibodies before spotting analytes onto the membranes. For example, with monocyte chemotactic protein 1 (MCP-1), precoated surfaces increased detection sensitivity at least 100-fold (34). In this article, we describe the further development and refinement of this system.

First, different coating conditions were tested. Membranes were precoated with one of the following: BSA, α-MCP-1 antibody in 5% BSA, phosphate buffer, or rabbit IgG. As shown in Fig. 1*A*, precoated membranes with α-MCP-1 significantly increased detection sensitivity consistent with our previous report (34). The detection sensitivity is further increased by dissolving α-MCP-1 antibody in phosphorylated buffer. The detection sensitivity is enhanced about 400-fold compared with membranes coated with BSA.

Secondly, membranes were coated with different concentrations of antibodies followed by immunoassays for MCP-1. As shown in Fig. 1*B*, membranes coated with higher concentrations of antibodies further increased the detection sensitivity. However, increased antibody concentrations will result in high background.

Then we tested whether this could be a universal approach to enhance detection sensitivity. Membranes were coated with different capture antibodies. Coated membranes were then spotted with different concentrations of purified proteins ranging from 5 μg/ml to 50 pg/ml and were probed with corresponding biotin-labeled antibodies. Signals were then visualized with horseradish peroxidase-streptavidin coupled with enhanced chemiluminescence. The signal intensities were then compared between antibody-coated and non-antibody-coated membranes. As shown in Table 1, precoated membranes with different specific antibodies all enhanced the detection sensitivity, suggesting that this may be a general approach to enhance the detection sensitivity. In the case of IL-8, the detection sensitivity was enhanced up to 50,000-fold.

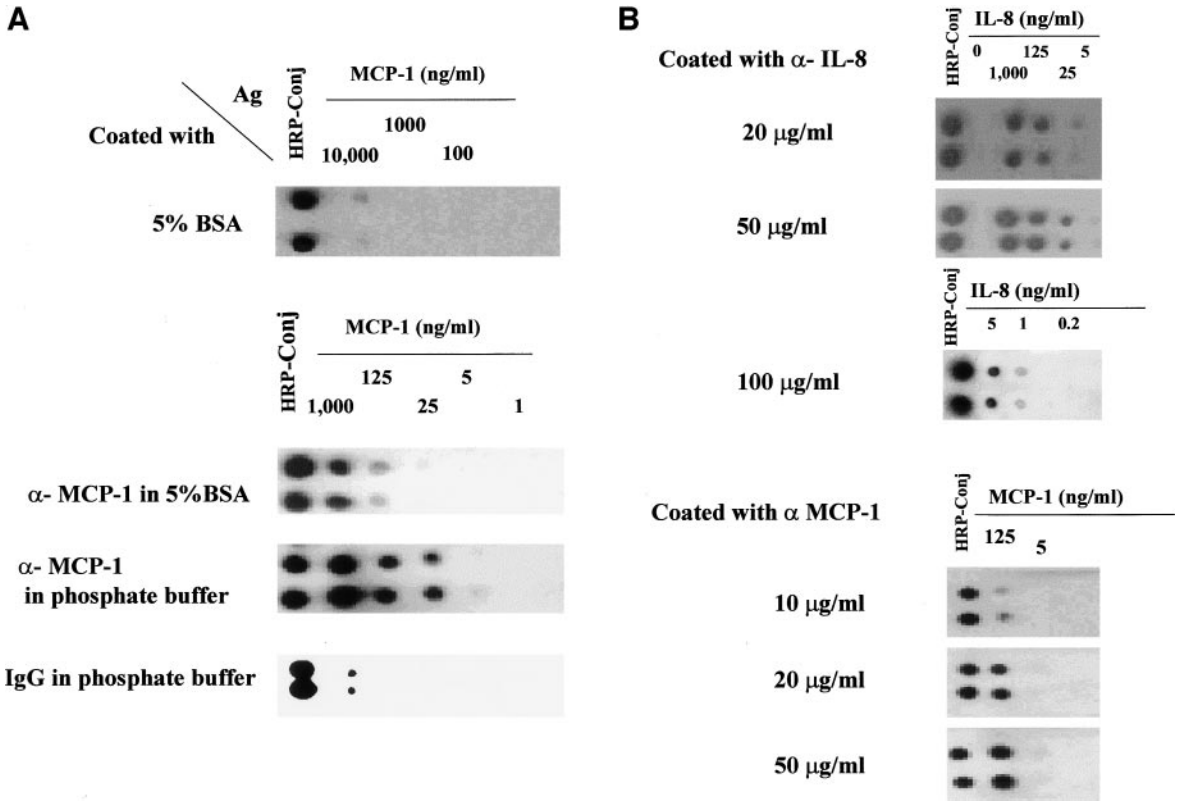


Fig. 1 Detection sensitivity was profoundly increased by using precoated substrates. *A*, membranes were coated with anti-monocyte-chemotactic-protein (α-*MCP*) antibody (20 μg/ml) in different conditions overnight; 0.2 μl of known amount of recombinant MCP-1, as indicated, was spotted onto the coated membranes. After incubation with biotin-conjugated (*Conj*) anti-MCP-1 and horseradish peroxidase-conjugated (*HRP-Conj*) streptavidin, the membranes were subjected to enhanced chemiluminescence. *B*, membranes were coated with different concentrations of anti-interleukin 8 (α-*IL-8*) or anti-MCP-1 antibodies, as indicated in the figure. Recombinant protein IL-8 or MCP-1 were spotted onto the precoated membranes. Signals were detected as described in *A*. Ag, antigen.

The intravariability was determined by comparing the signal from five duplicate spots in the same array membrane (Table 2). The variability of spots from different membranes (intervariability) was determined from four different arrays (Table 2). The CV (SD divided by the average) was usually less than 10%, suggesting the reliability of our system.

Profiling Angiogenic Factors in the Plasma of Patients with Gynecological Tumors. After the establishment of enhanced protein profiling arrays, we applied this approach to study the significance of angiogenic factors in gynecological tumors. The plasma of a total of 137 patients plus 20 normal controls was assayed for expression levels of IL-8, angiogenin, EGF, platelet-derived growth factor, and vascular endothelial growth factor using enhanced protein profiling arrays. Table 3 lists the characteristics of patients included in this study. There were 20 controls and 137 study subjects, among whom, 81 had benign diseases (leiomyoma, cyst, fibroma, endometriosis, and so forth) and 56 had malignancies (endometrial, cervical, vulvar,

Table 1 Detection sensitivity of enhanced protein profiling arrays

	Coated (ng/ml)	Non-coated (ng/ml)	Increased sensitivity (folds)
Interleukin 8	0.2	10,000	50,000
Epidermal growth factor	0.4	1,000	2,500
Monocyte chemotactioprotein 1	25	10,000	400
MIP 1α	25	10,000	400
Vascular endothelial growth factor	4	1,000	400
GROα	8	1,000	125
Angiogenin	10	1,000	100
GROβ	5	>200	ND
SCF	25	1,000	40
GROγ	5,000	>5,000	ND

[a] MIP, macrophage inflammatory protein; GRO, growth-related oncogene; SCF, stem cell factor; ND, not determined.

Table 2 Variability of enhanced protein profiling arrays

EGF (ng/ml)	Average density	SD	CV%
Intramembrane			
1800	1.016	0.026	2.560
600	0.804	0.016	1.976
200	0.494	0.049	9.884
66.67	0.212	0.025	11.575
22.2	0.166	0.001	0.770
Intermembrane			
1800	1.038	0.0306	2.946
600	0.752	0.075	9.93
200	0.460	0.048	10.397
66.67	0.213	0.000	0.350
22.2	0.170	0.005	2.806

and ovarian cancer) based on final pathology. The concentrations of individual angiogenic factors from all of the samples as shown in Figs. 2 and 3 were determined using the corresponding standard curves. To validate the array data, we measured the expression levels of angiogenic factors, platelet-derived growth factor, and EGF from several patients' plasma using conventional ELISA. As shown in Fig. 4, the levels of platelet-derived growth factor and EGF were not significantly different, as determined by both ELISA and enhanced protein profiling arrays described in this article, suggesting the reliability of our array approach.

The data were further analyzed statistically between normal subjects and patients as shown in Figs. 2 and 3 and summarized in Table 4. Different tumors had different expression patterns of angiogenic factors. Interestingly, most of the tumor patients had elevated plasma levels of certain angiogenic factors. Moreover, the elevated levels of plasma angiogenic factors differed with the types of tumor. For instance, cervical cancer patients had high levels of IL-8 and angiogenin compared with normal subjects, whereas vulvae cancer patients selectively had increased expression levels of vascular endothelial growth factor. The ability to easily measure the expression levels of multiple cytokines from a large number of samples allows us to compare numerous angiogenic factors between normal and cancerous samples. Furthermore, the expression levels are also different between benign and malignant tumors, as shown in Table 5. One major goal of our study is to discover novel biomarkers and expression patterns that may serve as markers. As a first step, cluster analysis was used to organize the angiogenic factors into categories related to disease status. Several hierarchical trees are shown in Fig. 5. Although no perfect separation between normal samples and malignancies was found, in most cases, there were clear indications that a pattern of angiogenic factors may be used to distinguish between normal subjects and tumor patients. In the case of ovarian cancer, the specificity to distinguish between normal subjects and tumor patients is 70.00% with sensitivity of 68.75%. For leiomyoma, the specificity and sensitivity were 70.00% and 61.90%, respectively.

Refinement of the Enhanced Protein Array System. We have demonstrated that enhanced protein arrays are a very powerful tool for high throughput of molecular profiling. One of the problems inherent in this system is that precoated membranes can be used to detect only one corresponding antigen. Two strategies can overcome this problem. One is to precoat membranes with a mixture of antibodies, thus enabling the system to detect a series of antigens. Another strategy is to precoat the membranes with antibodies against a common domain. This allows all of the antigens containing the common domain to be detected with enhanced sensitivity.

Fig. 6 shows that, indeed, membranes coated with a mixture of antibodies can be used to enhance detection sensitivity

Table 3 Classification and characteristics of normal and tumor subjects

	Normal (Female)	Endometrial cancer	Ovarian cancer	Cervical cancer	Vulvar cancer	Benign leiomyoma	Benign ovarian cyst	Benign endo-metriosis	Other benign
Total number	20	18	16	17	5	21	17	20	23
Age (median range)	50.7 (24–86)	65.1 (47–83)	62.8 (33–85)	41.4 (18–74)	58.4 (41–76)	47.5 (38–79)	47.1 (21–77)	39.6 (24–62)	50.5 (22–84)
Premenopausal	4	1	4	11	2	16	10	13	10
Postmenopausal	16	17	12	6	3	5	6	6	11
Stage									
I		7	2	2					
II		3	1	3					
III		4	8	0	1				
IV		2	3	2					
NA	2	2	10	4					
Histology		Adeno-carcinoma: 7	Serous papillary: 8	Squamous: 7	VAIN: 2				Fibroma: 5
		Serous papillary: 1	Mucinous: 2	Adeno-carcinoma: 1	Vagina: 1				Vulvtis: 3
		Clear cell: 3	Endo-metroid: 2	CIN: 5	CIS: 1				
		Sarcoma: 3	Granulosa cell: 1		Vagina: 1				
		MMMT: 2	Metastatic: 1						
			MMMT: 1						
			Borderline: 1						

[a] VAIN, vaginal intraepithelial neoplasia; CIN, cervical intraepithelial neoplasia; CIS, carcinoma *in situ*; MMMT, malignant mixed mullerian tumor.

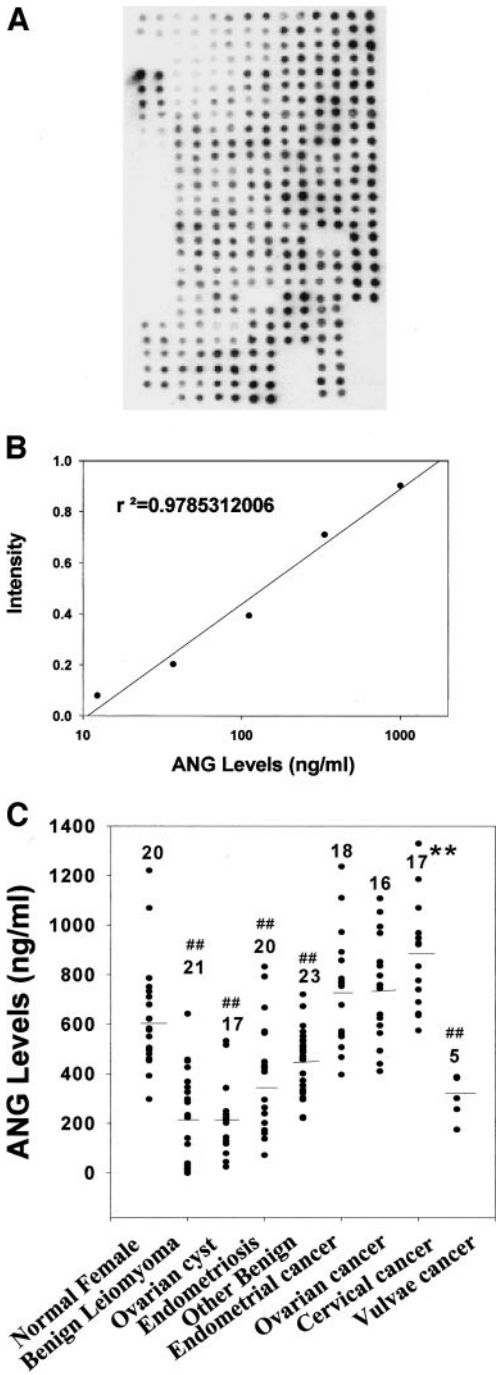


Fig. 2 The expression levels of angiogenin (*ANG Levels*) were assayed using enhanced protein profiling arrays. *A*, raw images of angiogenin expression levels detected by enhanced protein profiling arrays; 0.2 μl of plasma from patients and normal subjects, along with different amounts of recombinant angiogenin, were spotted onto anti-angiogenin antibody-coated membranes. Membranes were then incubated with biotinylated anti-angiogenin antibodies followed by horseradish peroxidase-conjugated streptavidin. Signals were visualized with enhanced chemiluminescence. *B*, the intensities of signals derived from standard angiogenin were determined by densitometry and were plotted against known concentrations of angiogenin. *C*, the expression levels of angiogenin (*ANG Levels*) in the control and patients' samples. Comparisons were made between normal subjects and patients by the Student *t* test. Significance is shown at the levels of $P \leq 0.05$ (*) and $P \leq 0.01$ (**) for high expression in patients compared with normal subjects, and at the levels of $P \leq 0.05$ (#) and $P \leq 0.01$ (##) for low expression in patients compared with normal subjects.

for all of the antigens corresponding to the coated antibodies. Furthermore, the results show specificity among the detection antigens. Similarly, precoated membranes using an antibody against three isoforms of growth-related oncogene (GRO; α, β, and γ) significantly enhanced the detection sensitivity for all three GRO isoforms.

To test whether such a system can be adapted to a protein chip format, we precoated nitrocellulose slides (Schleicher and Schuell, Keene, NH) with anti-EGF captured antibodies. Slides were then spotted with recombinant EGF. The slides were then probed with biotin-labeled antibodies against EGF. Finally, signals were detected by incubating the slides with cy^3-conjugated-streptavidin and creating an image with a laser scanner. As shown in Fig. 7, precoated slides significantly enhanced the detection sensitivity.

One of the advanced technologies in protein arrays is the three-dimensional substrate chip. To test whether our system can also be used to enhance detection sensitivity in three-dimensional substrate chips from Full Moon Biosystems Company (Sunnyvale, CA), chips were coated with or without antibody against angiogenin. Compared with noncoated slides, precoated slides again greatly enhanced the detection sensitivity (Fig. 7*B*).

To increase the binding of proteins to the surface of glass slides, one can covalently link proteins to glass sides through small chemical interactions. For instance, proteins are conjugated to phenyl(di)boronic acid [P(D)BA] and immobilized to the surface of glass slides through interaction with salicylhydroxamic acid (SHA; Prolinx, Bothell, WA). In theory, this may provide a better system to immobilize proteins to surfaces. We, therefore, tested whether our system has high detection sensitivity compared with the Prolinx system. As shown in Fig. 7*C*, treatment of surface according to the Prolinx complicated system did significantly increase the detection sensitivity. However, compared with our system, our system has a much higher sensitivity (maximal detection of EGF between 1 μg/ml and 0.1 μg/ml in Prolinx system *versus* 0.4 ng/ml in our system)

DISCUSSION

Previously, we developed several novel human cytokine protein array technologies for simultaneous detection of multiple cytokine expression levels from a variety of biological sources (33, 34). Such technologies are powerful tools in biomedical discovery (35, 36). Through antibody array techniques, we can simultaneously detect numerous cytokines and identify the key molecules important in specific cases. To further investigate the role of these key molecules, one usually needs to examine their expression in many samples. This is a costly and time-consuming process if protein arrays are repeated for all samples. At this stage of the investigation, limited protein molecules will be examined; therefore, it is much more efficient to screen as many samples as possible at one time. One of the difficulties of performing this type of experiment is decreased sensitivity when the sample volume is significantly scaled

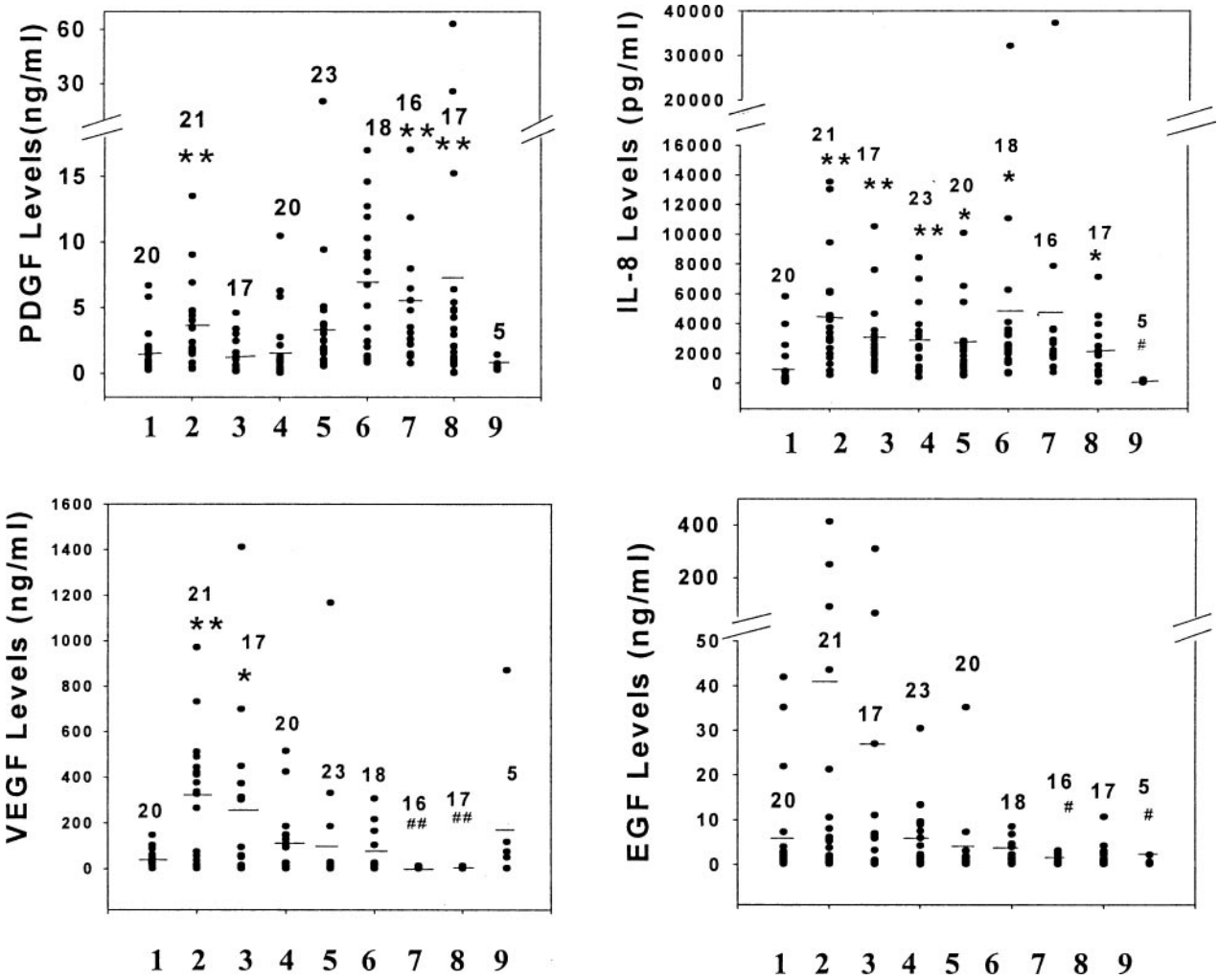


Fig. 3 Comparison of expression levels of interleukin 8 (*IL-8*), platelet-derived growth factor (*PDGF*), epidermal growth factor (*EGF*), and vascular endothelial growth factor (*VEGF*) between normal subjects and patients with gynecological diseases. The expression levels of IL-8, PDGF, EGF, and VEGF were measured quantitatively using enhanced protein profiling assays and were statistically analyzed using the Student *t* test. Significance is shown at the levels of $P \leq 0.05$ (*) and $P \leq 0.01$ (**) for high expression in patients compared with normal subjects, and at the levels of $P \leq 0.05$ (#) and $P \leq 0.01$ (##) for low expression in patients compared with normal subjects. 1, normal female; 2, benign leiomyoma; 3, ovarian cyst; 4, endometriosis; 5, other benign; 6, endometrial cancer; 7, ovarian cancer; 8, cervical cancer; and 9, vulvae cancer.

down. Another potential problem is the variation of antigens. Different antigens have considerably distinct binding abilities. To overcome both problems, we precoated membranes with specific antibodies before spotting analytes onto membranes or slides. This simple step significantly increased the detection sensitivity up to 50,000-fold.

Importantly, the increased detection sensitivity we found is a general phenomenon. Membranes also can be precoated with a mixture of antibodies or antibodies against a common domain. This allows multiple antigens to be detected using the same membranes or glass slides. This procedure can be extended to protein chip format. Thus, the methodology we describe in this article should have broad applications in the detection of a single to a few proteins in array format. To detect multiple proteins simultaneously, a cocktail of detection antibodies labeled with different fluorescent dyes can be applied to probe proteins in analytes. Because, unlike with cell or tissue lysate, it is almost impossible to concentrate serum or plasma, a highly sensitive approach is the key to detecting protein expression levels in serum and plasma. One of the immediate and extremely important applications is to detect tumor markers, hormones, and other clinically relevant proteins in patients' serum and plasma. In addition to detecting secreted proteins in serum and plasma, our approach can be used to detect any protein from any source. Profoundly enhanced detection sensitivity is also observed in cell lysate and tissue lysate.[1]

Proteins, particularly secreted proteins such as angiogenic factors, can be detected by many methods such as ELISA,

[1] R. Huang, Y. Lin, and R. P. Huang, unpublished data.

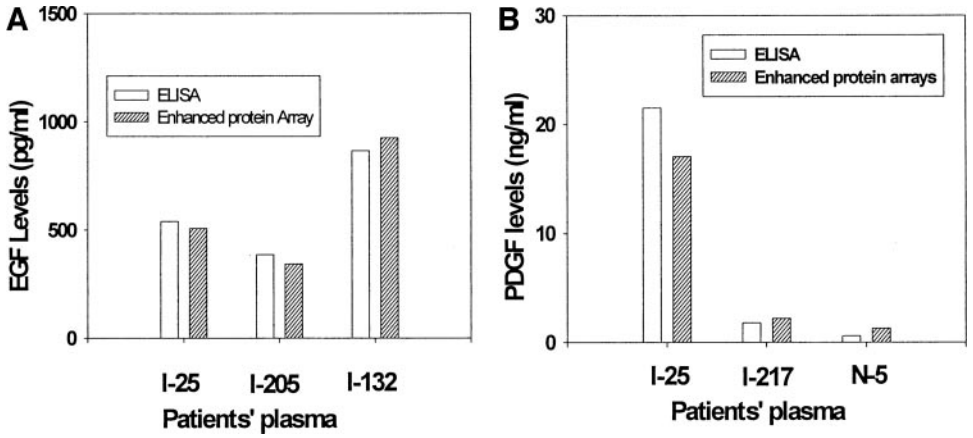


Fig. 4 Comparison of expression levels of platelet-derived growth factor (*PDFG*) and epidermal growth factor (*EGF*) by ELISA and enhanced protein profiling arrays. Expression levels of PDFG and EGF from several samples were determined by ELISA (*A*) and by enhanced protein profiling arrays (*B*). In general, the expression levels are similar between the two approaches.

Table 4 Summary of expression levels of interleukin 8 (IL-8), angiogenin (ANG), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF)

	Normal female	Benign leiomyoma	Benign ovarian cyst	Benign endometriosis	Other benign	Endometrial cancer	Ovarian cancer	Cervical cancer	Vulvae cancer
IL-8 (pg/ml)	899.2	4272[a]	3132[a]	2790[a]	2439[b]	4668[b]	4637	2060[b]	107.2[c]
ANG (ng/ml)	633.8	228.2[d]	227.6[d]	387.2[d]	452.7[d]	733.1	743.0	868.4[a]	332.5[d]
PDGF (ng/ml)	1.563	3.614[a]	1.283	1.884	3.220	6.528[a]	4.851[a]	6.669	0.661
EGF (ng/ml)	6.284	39.20	7.891	5.678	17.40	3.865	0.743[c]	1.947	0.668[c]
VEGF (ng/ml)	38.893	311.1[a]	250.2[b]	98.47	78.53	47.51	1.117[d]	2.087[d]	222.6

[a] $P < 0.01$, higher expression levels in patients than in normal subjects.
[b] $P < 0.05$, higher expression levels in patients than in normal subjects.
[c] $P < 0.05$, lower expression levels in patients than in normal subjects.
[d] $P < 0.01$, lower expression levels in patients than in normal subjects.

Western blotting, flow cytometry, enzyme-linked immunospot assay (ELISPOT), immunostaining, and immunoprecipitation. Among them, ELISA is the most common way to detect secreted proteins such as angiogenic factors. However, our approach has several advantages over ELISA. Because hundreds and thousands of samples can be detected simultaneously with high sensitivity in our system, our method offers high throughput, low variation, low cost, and high detection range as opposed to ELISA. Only minute amounts of sample are needed for the assays, and thousands of assays can be performed from just one blood drawing. Taking IL-8 as an example, our approach, with high sensitivity and specificity, can detect levels of IL-8 lower than 0.2 ng/ml in a 0.2-μl sample. Thus, less than 40 fg of IL-8 can be detected. This detection sensitivity exceeds that with ELISA in most cases. By using an arrayer to print samples onto membranes or slides, the detection limit should be ~1 fg if 1 nl of sample with concentration of 1 ng/ml is used. The detection sensitivity may be further enhanced by rolling circle amplification (RCA; Refs. 38–41) or catalyzed signal amplification (CSA; Dako, Carpinteria, CA) or 3-DNA technology (Genisphere, Hatfield, PA). The signals can be detected using either chemiluminescence or fluorescence. In general, chemilumines-

Table 5 Comparison of angiogenic factors between benign and malignant tumors

	Benign ovarian cyst	Ovarian cancer	Benign endometriosis	Endometrial cancer
IL-8[a] (pg/ml)	3132	4637 ↑	2790	4668 ↑
ANG (ng/ml)	227.6	743 ↑ ↑	387.2	733.1 ↑ ↑
PDGF (ng/ml)	1.283	4.851 ↑	1.884	6.528 ↑ ↑
EGF (ng/ml)	7.891	0.743 ↓	5.678	3.865 ↓
VEGF (ng/ml)	250.2	1.117 ↓ ↓	98.47	47.51 ↓

[a] IL-8, interleukin 8; ANG, angiogenin; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; ↑, the expression levels of cancer patients more than benign patients; ↑ ↑, $P < 0.05$ and the expression levels of cancer patients more than benign patients; ↓, the expression levels of cancer patients less than benign patients; ↓ ↓, $P < 0.05$ and the expression levels of cancer patients less than benign patients.

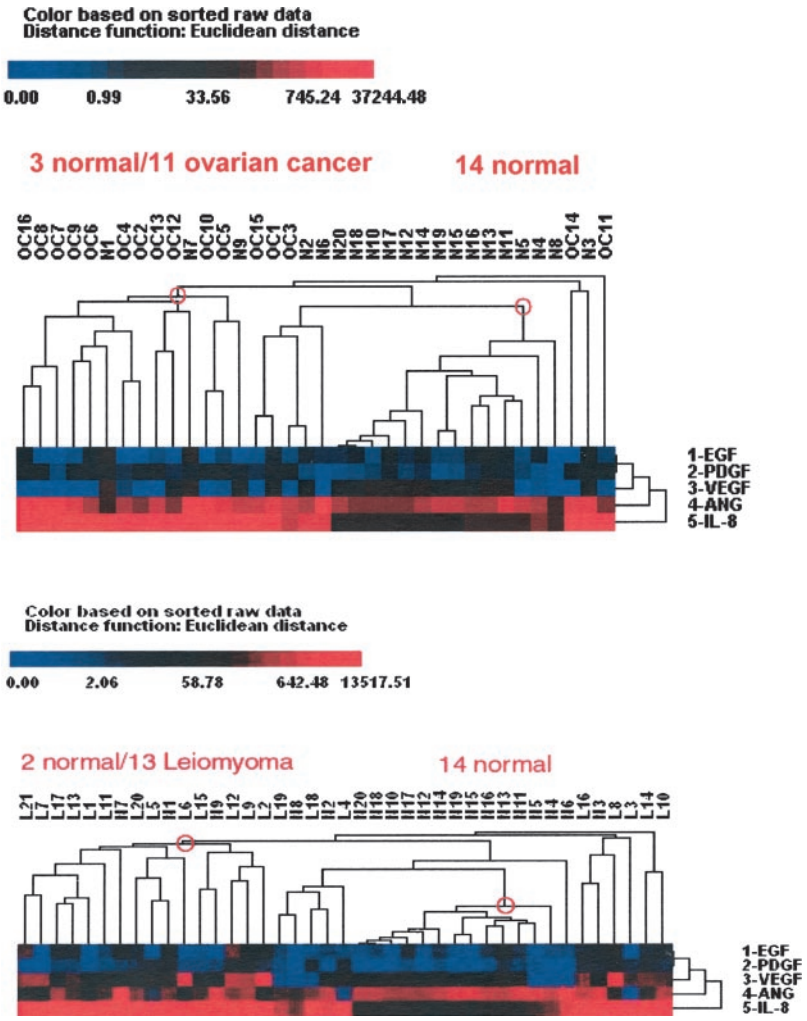


Fig. 5 Clustering analysis of angiogenic factors between normal subjects and gynecological diseases. Hierarchical clustering based on the five angiogenic factors was performed on the 20 normal subjects and 16 ovarian cancer patients or 21 leiomyoma patients. *EGF*, epidermal growth factor; *PDGF*, platelet-derived growth factor; *VEGF*, vascular endothelial growth factor; *ANG*, angiogenin; *IL*, interleukin.

cence is more highly sensitive than fluorescence and should be usable as a routine approach for signal detection.

To demonstrate the practical application of our approach, we measured the angiogenic factor levels in patients' plasma. Measurement of plasma levels of angiogenic factors and antiangiogenic factors, which reflect the balance of angiogenesis activity, may have practical implications in patient care management. Classification of tumor according to their angiogenic factor expression levels may help to identify subgroups of patients who might benefit from antiangiogenic therapy. To monitor the individual therapeutic response to those target-specific inhibitors of angiogenesis, angiogenic factors may be used as predictive markers. It has been reported that the expression patterns of angiogenic factors have been used to study the chemotherapeutic response in cancers such as leukemia (42). We found that all tumor patients exhibited altered expression levels of certain angiogenic factors. This is the first time that multiple angiogenic factor expression levels in gynecological malignancies have been measured using protein array technologies. Because tumors would grow very slowly without angiogenesis, it is an extensive investigation to measure the angiogenic factors in cancer patients' tissue and blood, including gynecological tumors. Our data are consistent with most of the data available in the literature as shown in Table 6.

Several important points can be derived from this study. First, enhanced protein profiling arrays are a powerful tool to measure protein expression levels from hundreds and thousands of samples simultaneously. Secondly, different gynecological malignancies express different amounts of angiogenic factors. Such variation may reflect the different origins of tumors. Thirdly, multiple expression levels of angiogenic factors may be used to classify normal cells and tumor cells. Recently, cDNA microarrays have been used to classify tumors according to gene expression patterns. Our approach, if successful, will have an even more profound effect and practical significance than the cDNA microarray approach because analyses using protein arrays can be performed using plasma or other fluids. Although at this moment it is too early to confirm that our approach can be used to classify tumors, by increasing the number of angiogenic factors in the test, we believe that it should be able to identify a pattern as a biomarker for diagnosis or prognosis.

Bioinformatics is the key to handling mass data. In this

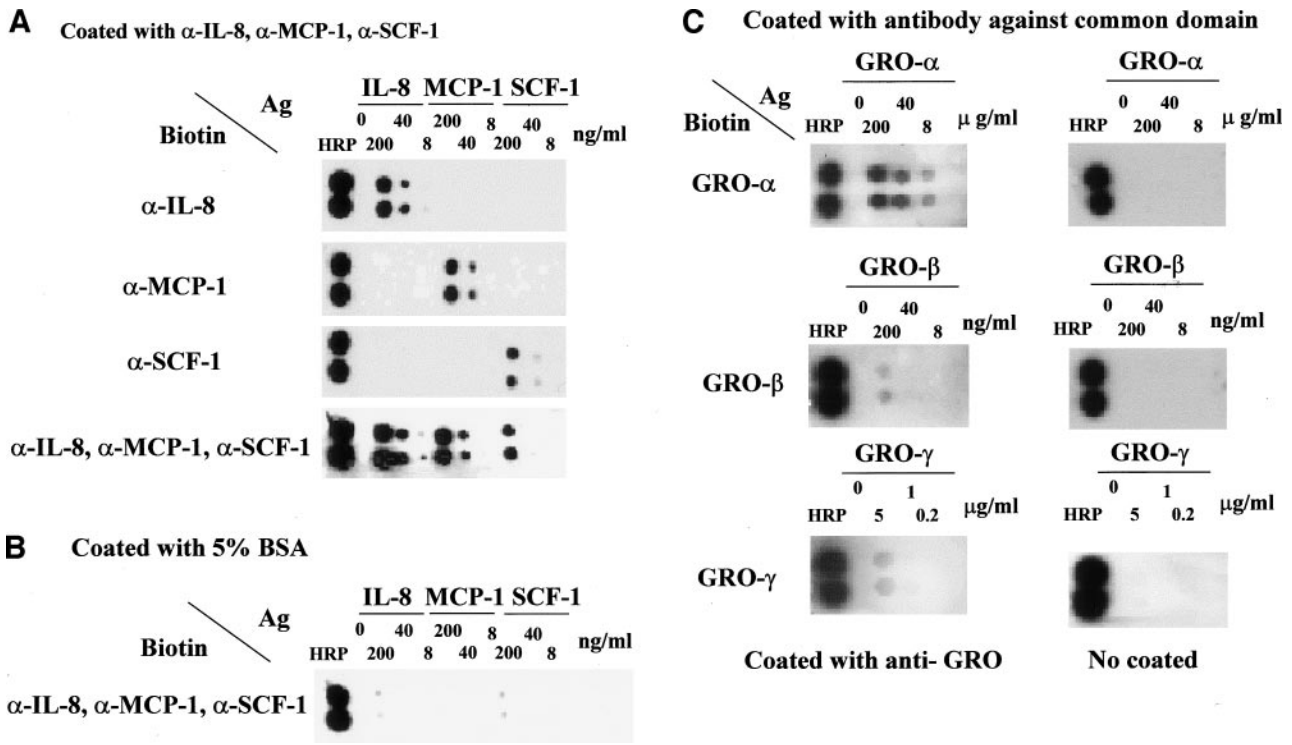
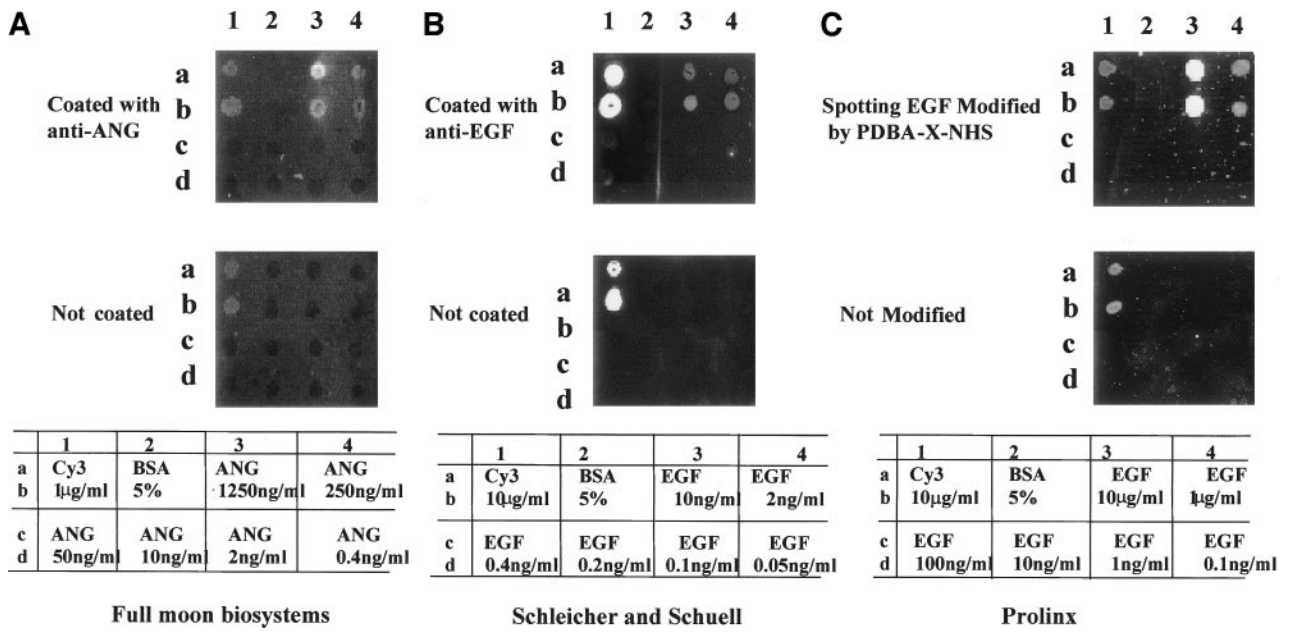


Fig. 6 Multiple proteins can be detected using the same precoated membranes. Membranes were coated with a mixture of antibodies against interleukin 8 (α-*IL-8*), monocyte chemotactic protein 1 (α-*MCP-1*), and stem cell factor (α-SCF-1) (*A*); BSA as a control (*B*); an antibody against a common domain of growth-related oncogene (GRO)α, GROβ and GROγ (*C, left panel*), and BSA as control (*C, right panel*). Recombinant proteins (IL-8, MCP-1, and SCF as in *A* and *B*; GROα, GROβ, and GROγ as in *C*) were spotted onto the antibody-coated membranes (*A* and *C*, left panel) and uncoated membranes (*B* and *C*, right panel) as indicated in the figure. Signals were then detected with corresponding biotin-labeled antibodies as described in the figure.



	1	2	3	4
a	Cy3 1μg/ml	BSA 5%	ANG 1250ng/ml	ANG 250ng/ml
b				
c	ANG 50ng/ml	ANG 10ng/ml	ANG 2ng/ml	ANG 0.4ng/ml
d				

Full moon biosystems

	1	2	3	4
a	Cy3 10μg/ml	BSA 5%	EGF 10ng/ml	EGF 2ng/ml
b				
c	EGF 0.4ng/ml	EGF 0.2ng/ml	EGF 0.1ng/ml	EGF 0.05ng/ml
d				

Schleicher and Schuell

	1	2	3	4
a	Cy3 10μg/ml	BSA 5%	EGF 10μg/ml	EGF 1μg/ml
b				
c	EGF 100ng/ml	EGF 10ng/ml	EGF 1ng/ml	EGF 0.1ng/ml
d				

Prolinx

Fig. 7 Enhanced protein profiling arrays can be adapted in protein chip formats. Different glass slides were coated with certain antibodies. Recombinant proteins and conditioned media were spotted onto the precoated glass slides. Slides were then incubated with biotinylated antibodies and 3-conjugated streptavidin. Signals were imaged using laser scanner. *ANG*, angiogenin; *EGF*, epidermal growth factor; *PDBA-X-NHS*, phenyl(di)-boronic acid-X-NHS.

Table 6 Change of angiogenic factor expression in gynecological tumors

	Endometrial cancer	Ovarian cancer	Cervical cancer	Vulvae cancer	Benign leiomyoma	Ovarian cyst	Endometriosis	Other benign
Interleukin 8								
Our assay	↑	↑	↑	↓	↑	↑	↑	↑
Literature	↑ (30)	↑ (47, 48)	↑ (48, 49)	N/A	N/A	N/A	↑ (30, 31, 50)	N/A
Angiogenin								
Our assay	↑	↑	↑	↓	↓	↓	↓	↓
Literature	↑ (30)	— (51)	↑ (49)	N/A	N/A	N/A	N/A	N/A
Platelet-derived growth factor								
Our assay	↑	↑	↑	↓	↑	—	—	↑
Literature	N/A	↑ (52)	N/A	N/A	↑ (53)	N/A	N/A	N/A
Epidermal growth factor								
Our assay	↓	↓	↓	↓	↑	—	—	↑
Literature	N/A	N/A	N/A	↑ N/A	(54)	N/A	N/A	N/A
Vascular endothelial growth factor								
Our assay	↑	↓	↓	↑	↑	↑	↑	↑
Literature	↑ (55, 56)	— (51) ↑ (32)	↑ (57)	↑	↑ (58, 59)	N/A	N/A	N/A

↑, increased expression in patient; ↓, decreased expression in patient; N/A, no data available; —, no significant change of expression level in patient.

article, we applied conventional unsupervised cluster analysis in which the experimental objective is to identify a group of proteins whose expression levels or patterns can be used to distinguish different types of diseases, different responses, or different outcome (43). This unsupervised learning method attempts to define groups within the data. It has been widely used as an analysis tool in cDNA microarrays and can be adapted for protein arrays. More sophisticated and power approaches are to use a well-defined learning set to develop a test that will be applied to additional samples of unknown types. These supervised learning approaches (43, 44) have been successfully applied in molecular classifications (45) and diagnoses (46) of cancer. Combining these approaches with enhanced protein array technology will greatly increase our ability to discover potential biomarkers for cancer diagnosis.

In summary, we have described a simple approach referred to as enhanced protein profiling arrays to detect one or a few proteins from hundreds and thousands of samples. In addition, we have demonstrated that the system can be used to profile angiogenic factor expression levels from patients' plasma with high sensitivity and high throughput. Such an approach should have broad application in the discovery of new mechanisms involved in detecting the development of diseases, of novel targets for drug development, and of biomarkers for diagnosis and prognosis.

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Enhanced Protein Profiling Arrays with ELISA-Based Amplification for High-Throughput Molecular Changes of Tumor Patients' Plasma

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