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A Biotin Label-based Antibody Array for High-content Profiling of Protein Expression

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Abstract. *Background/Aim: Profiling protein expression on a global scale will have significant impact on biomedical research, particularly in the discovery and development of drugs and biomarkers. Through the years, several antibody array systems have been invented and developed for multiple protein detection. However, a reliable and high-content system for protein profiling from many biological samples has yet been developed. This study aimed to develop a reliable, easy to use and cost effective method to profile protein expression levels in high-content manner with sufficient sensitivity and specificity. Materials and Methods: To address this problem, a high density antibody array was developed and used this technology to uncover the potential biomarkers of ovarian cancer. In this system, biological samples are labeled with biotin. The biotinylated proteins are then incubated with antibody chips. The presence of proteins captured by the antibody chip is detected using streptavidin-conjugated fluorescent dye (Cy3 equivalent) as a reporter. The signals, which are visualized by laser scanning, are normalized using positive, negative, and internal controls. Results: Using this biotin label-based antibody array technology, the expression levels of 507 human, 308 mouse and 90 rat target proteins can be simultaneously detected, including of cytokines, chemokines, adipokines, growth factors, angiogenic factors, proteases, soluble receptors, soluble adhesion molecules, and other proteins in a variety of samples. Most proteins can be detected at pg/ml and ng/ml levels, with a coefficient of variation of less than 20%. Using human biotin-based antibody arrays, we screened the serum expression profiles of 507 proteins in ovarian cancer patients and healthy individuals. A panel of protein expression showed significant difference between normal and cancer samples ($p<0.05$). By classification analysis and split-point score analysis of these two groups, a small group of proteins were found to be useful in distinguishing ovarian cancer patients from normal subjects. Conclusion: Our results suggest the biotin label-based antibody arrays that we have developed have great potential in applications for biomarker discovery.*

Antibody microarrays have emerged as a promising technology for multiplexed, quantitative, fast and cost-effective protein expression profiling, functional determination and biomarker discovery, using a tiny amount of sample (1, 2). Among different approaches for protein detection using antibody arrays, sandwich-based antibody arrays are the most common. Hundreds of publications have documented the suitability of sandwich-based antibody arrays to detect differential protein expression patterns using various sample types, including serum (3), plasma (4), cell-cultured media (5, 6), cell co-cultures (7), cell and tissue lysates (8, 9), cerebrospinal fluid (10), urine (11), abscess fluid (12), platelet releasates (13), bronchoalveolar lavage (14), sputum (15), breath condensates (16), saliva (6), tears (17), prostatic fluid (18) and milk and colostrum (19).

Sandwich-based arrays use the same method of detection as a standard ELISA, meaning that these multiplex arrays feature high detection sensitivity, specificity, reproducibility and the potential for quantitative measurement. However, the requirement of a pair of antibodies to detect each protein (analyte) hampers the development of higher density antibody arrays. Because antibodies can have unintended interactions with other antibodies, the higher the number of antibody pairs in the array, the greater the amount of developmental work needed to eliminate false signals in the multiplexed array. However, in most biomedical research and biomarker discovery programs, high density antibody arrays are more desirable, since they can reveal much more

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information and provide a more global view of protein expression patterns. Therefore, great efforts have been made to develop higher density antibody arrays.

One way to overcome this obstacle is to directly label samples with fluorescent dyes such as Cy3 and Cy5. The labeled samples are then applied on the antibody array chip. The bound proteins are then visualized by laser scanner. Since only capture antibodies are required, this approach can be used to detect hundreds of target proteins simultaneously. The main problems of this approach include low detection sensitivity, a complicated procedure and, in most reports, the limitation of comparison of two samples. Furthermore, this approach also suffers from limited sample compatibility and the requirement for laser scanner detection. Previously, we briefly reported the development of a biotin-label-based antibody arrays (20). In this report, we further improve the technology and demonstrate the reliability of this approach. In addition, the technology is used to screen and identify potential cancer biomarkers.

Materials and Methods

Antibody and protein. All antibodies were purchased either from commercial sources or were available from our own production. Some recombinant cytokine proteins were obtained from PeproTech (Rocky Hill, NJ, USA), BD Pharmingen (San Diego, CA, USA) and R&D (Minneapolis, MN, USA), while others were available from our own production.

Sample preparation and biotinylation of protein. To prepare U251 cell-conditioned media, 1×10^6 cells were seeded in a 100 mm plate with complete media. After two days, complete medium was replaced with low serum medium containing 0.2% bovine serum in the presence or absence of 50 ng/ml of recombinant human tumor necrosis factor alpha (TNF-alpha). Supernatants and cells were collected separately 48 h later. To prepare cell lysate, cells were lysed with RIPA buffer (20 mM Tris, pH 7.5, 0.15 M NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate). Protein concentrations were determined using bichinchonic acid (BCA) protein detection assay (Thermo Scientific Pierce, Rockford, IL, USA) and used to normalize the amount of conditioned media for the protein arrays. Serum samples were provided by Gynecologic Oncology Group and the Department of Gynecology of the Second Affiliated Hospital, Sun Yat-sen University. To prepare the cell lysate, cells growing in complete media were harvested immediately upon reaching confluence and lysed in RIPA buffer then homogenized. Prior to biotin-labeling, samples were extensively dialyzed against phosphate-buffered solution (PBS). The labeling process was carried out according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

Study populations. The serum samples from 47 patients diagnosed with early-stage (I and II) and late-stage (III and IV) ovarian cancer, 33 patients with benign tumor, and 39 healthy controls were tested as shown in Table I. Information about ovarian cancer diagnosis, staging, histology, grade and age was available to us, but the identity of patients such as name, address, day of birth was not provided. All serum samples were aliquoted and stored at –80˚C until use.

Table I. *Study population characteristics*.

	Normal	Benign	Cancer
Total samples	39	33	47
Age (years)			
Mean	44.64	48.09	46.70
Median	45	51	48
Range	35-58	17-62	31-54
Cancer histology			
Serous adenocarcinoma			20
Mucious adenocarcinoma			5
Endometrioid carcinoma			12
Clear cell carcinoma 1			0
Cancer stage			
I			8
II			1
III and IV			34
NA			4

NA, Not available.

Antibody chip technology. The array used was the RayBio® Human Biotin-labeled Antibody Array, glass slide format (AAH-BLG-1, RayBiotech, Inc., Norcross, GA, USA), detecting 507 different human proteins, including cytokines, chemokines, growth and differentiation factors, angiogenic factors, adipokines, adhesion molecules and matrix metalloproteases, as well as binding proteins, inhibitors and soluble receptors to these proteins. Antibodies were selected using the following criterion: i) The antibody has been extensive characterized and used in our sandwich-based antibody arrays; ii) The antibody recognizes a single band in Western blot analysis; iii) Proteins detected by the antibody are secreted or can be detected in body fluid. These antibodies (approx. 200 μg/ml) were printed onto Corning slides (Corning Incorporated, Corning, NY, USA) using a contact arrayer. A series of diluted anti-streptavidin, and biotin-conjugated IgG (BIgG) were included in the printed array as positive controls, while capture antibody diluent was used as a negative control. After blocking, the chips were incubated with 400 μl of biotin-labeled serum or cell-cultured media samples at room temperature for 2 h. The chips were then washed to remove unbound components. Streptavidin-conjugated fluorescent dye, HiLytePlus™ 555 (Cy3 equivalent) from AnaSpec (Freemont, CA, USA), was incubated with protein chips at room temperature for 1 h. The excess streptavidin was removed and the signals were scanned by a GenePix™ 4000B laser scanner (Axon Instruments, Sunnyvale, CA 94089, USA).

Enzyme-linked immunoassay (ELISA). Conventional ELISA was performed according to the RayBio® ELISA manual (RayBiotech, Inc., Norcross, GA, USA). Essentially, pre-coated 96-well ELISA plates for different captured antibodies were first blocked using a blocking buffer. Patient sera were diluted to a final volume of 100 μl. Aliquots of diluted sera and different concentrations of standard protein were loaded onto the ELISA plate in duplicate, 100 μl per well. The plates were incubated for 2 h at room temperature. Unbound materials were washed out, and biotinylated anti-cytokine detection antibody was added to each well. The plates were incubated for 1 h at room temperature. After washing, 100 μl of

streptavidin-HRP conjugated antibodies were added to the wells and incubation was continued for an 30 additional minutes at room temperature. After extensive washing, color development was performed by incubation with substrate solution in the kits. After adding stop solution, the optical densitiy (O.D.) at 450 nm was determined for each well by a microplate reader. Standard curves were generated by SigmaPlot v10.0 (Systat Software, San Diego, CA, USA) and the concentrations of the samples were determined by comparison to the standard concentration curves.

Data analysis. The array data of ovarian cancer patients and healthy controls were then normalized based on the positive control signal, consisting of biotin-labeled antibodies printed on each array, compared to a common reference array. After subtraction of local background signals, the fluorescent signal intensity for each spot was multiplied by a normalization factor, calculated as the average signal intensity of the positive control spots on the reference array divided by the average signal intensity of the positive control spots located on the same array as the data being normalized. Positive control normalization compensates for differences in the relative fluorescent signal responses to standardized amounts of biotin-labeled proteins bound on each array. Subsequently, a background threshold value was determined as the mean signal intensity +2×SE of 10 control samples where the slide arrays were assayed without patient's serum samples. The background threshold value was then subtracted from the signal intensities for each spot. After background subtraction, negative signal intensities were assigned a value of 1. Where signal intensities for a particular analyte were less than the background threshold in all samples tested, those cytokines were removed from further analysis.

Statistical analysis. ANOVA statistical methods were used to test the significance of the protein expression differences between ovarian cancer patients and healthy controls by using SSPS statistical software (SPSS, Inc., Chicago, IL, USA). For inclusion in further classification studies, the cut-off for statistical significance for each analyte was $p<0.05$.

Classification analysis. The classification tree method was used to discriminate between ovarian cancer and normal controls by first searching the range of each potential cytokine marker and finding the split that maximized the homogeneity of the two data classes. Within each resulting subset, the algorithm again sought the range of each variable to choose the optimal split. This process continued until all observations were perfectly discriminated, or the sample size within a given subset was too small to divide further.

Split-point score analysis. The split point divides the sample space into two intervals, one for ovarian cancer and one for normal controls. The best split score of each marker was chosen to ensure the minimization of misclassified samples. A score of 0 was assigned to a sample if it fell in the normal control interval; a score of 1 was assigned to a sample if it fell in the ovarian cancer interval. Overall, an individual was assigned a score as the sum of these assigned scores from N different markers. Therefore, the range of such score was between 0 to N. A given threshold T was chosen to optimally separate ovarian cancer from healthy controls *i.e.* a given individual with a total score of <T is predicted to have normal status, whereas an individual with a total score of >T was diagnosed as ovarian cancer.

Receiver operating characteristics (ROC) curve and assay performance characteristics. ROC curve was plotted by sensitivity (true positive) against 1–specificity (false positive) values. According to ROC, the following parameters were calculated to assess the assay's performance for discriminating ovarian cancer from normal controls: Specificity (true negative); sensitivity (true positive); positive predicted value (PPV); negative predicted value (NPV); and accuracy (both true positive and true negative).

Results

Performance of biotin labeled-based antibody arrays. Previously, we reported the development of sandwich-based cytokine antibody arrays to simultaneously detect multiple cytokine expression levels (21-23). Substantial obstacles must be overcome in order to develop higher content sandwich-based antibody arrays. Another approach to developing high-density antibody arrays is to label sample proteins with biotin. We have briefly described this method in our earlier publication on biotin label-based arrays (20). We have since expanded the content to include detection of 507 human proteins in a single array, as described in the materials and methods.

First we tested the overall sensitivity of the RayBio® Human Label-based Arrays, detecting 507 human protiens. As shown in Table II, most proteins were detected at pg/ml levels. The detection dynamics ranged from 5 pg/ml to 1,000 pg/ml. The minimal amount of proteins detected was 5 pg/ml. The detection sensitivity for individual proteins varied and depends mainly on the binding affinity for each antigen–antibody interaction, as well as the binding characteristics of the specific antibody to the solid support. Nevertheless, a linear increase in spot intensity was observed with concentration for all proteins that we tested.

To test the specificity of the arrays, we individually labeled one dozen (12) recombinant proteins. Labeled proteins were then incubated with the arrays at a final concentration of 100 ng/ml. As shown in Figure 1, individual biotin-labeled protein mainly bound to the spot where its corresponding antibody was printed, even at high concentrations, suggesting high specificity of the arrays. No signal was detected when biotin-labeled solvent was used. These results demonstrated the specificity of our system.

The variability was determined by comparing the signals from 3 different spots replicated in the same chip (spot to spot), from three distinct subarrays printed on the same chip (well to well) or from three different arrays on three separate chips (slide to slide), as shown in Figure 2. The coefficient of variation (CV; defined as the standard deviation divided by the average) was generally less than 20%, suggesting that the reliability of the system is quite good. The reproducibility was also examined by scatter plot analysis in the arrays using same serum samples in two different experiments as shown in Figure 2.

Table II. *Detection sensitivity of biotin label-based antibody arrays. Purified antigens were diluted into 1×PBS at 1,000 ng/ml. After biotin-labeling, biotinylated antigens were diluted with blocking buffer and incubated with array slide. Signals were detected by fluorescence conjugated streptavidin.*

	Target protein (pg/ml)	Array sensitivity (pg/ml)		Target protein	Array sensitivity
1	Activin A	10	56	IL-18 R alpha /IL-1 R5	20
2	Adiponectin/Acrp30	20	57	IL-18 R beta /AcPL	15
3	AgRP	10	58	IL-2 R alpha	20
4	ALCAM	20	59	IL-2R alpha	200
5	Angiogenin	20	60	IL-3	30
6	AR (Amphiregulin)	20	61	IL-4	50
7	Axl	10	62	I-TAC/CXCL11	10
8	B7-1 /CD80	10	63	Leptin (OB)	>1000
9	BCMA/TNFRSF17	10	64	LIF	100
10	BDNF	10	65	LIGHT/TNFSF14	80
11	beta-NGF	50	66	LIGHT/TNFSF14	100
12	BLC/BCA-1/CXCL13	100	67	MCP-2	100
13	BMP-5	100	68	MCP-3	10
14	BTC	10	69	MCP-4/CCL13	10
15	Cardiotrophin-1/CT-1	10	70	M-CSF	10
16	CTLA-4 /CD152	30	71	MMP-10	10
17	CXCL16	50	72	MMP-13	50
18	Dtk	15	73	MMP-9	30
19	EGF	10	74	MSP alpha chain	10
20	EGF R/ErbB1	15	75	MSP beta chain	100
21	Endoglin/CD105	20	76	NAP-2	100
22	Eotaxin/CCL11	10	77	NGF R	20
23	Eotaxin-2/MPIF-2	20	78	NT-4	10
24	Eotaxin-3/CCL26	10	79	OSM	15
25	ErbB3	10	80	Osteoprotegerin	50
26	Fas/TNFRSF6	30	81	PDGF R beta	15
27	Fas Ligand	10	82	PDGF-AA	10
28	FGF Basic	10	83	PDGF-AB	200
29	FGF-4	1000	84	PDGF-BB	5
30	FGF-6	100	85	PlGF	10
31	FGF-7/KGF	8	86	P-Selectin	10
32	FGF-9	8	87	RAGE	10
33	Follistatin	60	88	RANTES	20
34	GITR/TNFRF18	20	89	SCF	10
35	HB-EGF	5	90	SCF R /CD117	10
36	HCC-4/CCL16	8	91	sgp130	30
37	HGF	20	92	Siglec-9	20
38	I-309	30	93	Siglec-5/CD170	40
39	IGFBP-1	20	94	TARC	300
40	IGFBP-2	10	95	TGF-alpha	10
41	IGFBP-3	10	96	TNF RI/TNFRSF1A	10
42	IGF-I	1000	97	TNF RII/TNFRSF1B	30
43	IGF-I SR	10	98	TNF-beta	>1000
44	IGF-II	1000	99	TRAIL R1/DR4/TNFRSF10A	>1000
45	IL-1 alpha	>1000	100	TRAIL R3/TNFRSF10C	10
46	IL-1 beta	10	101	TRAIL R4/TNFRSF10D	10
47	IL-1 R4 /ST2	20	102	TRANCE	1000
48	IL-1 sRI	20	103	TREM-1	10
49	IL-1 sRI	20	104	TROY/TNFRSF19	10
50	IL-10	10	105	uPAR	1000
51	IL-10 R beta	8	106	VCAM-1 (CD106)	10
52	IL-13 R alpha 1	20	107	VE-Cadherin	10
53	IL-13 R alpha 2	1000	108	VEGF	10
54	IL-17	20	109	VEGF R2 (KDR)	10
55	IL-18 BPa	10	110	VEGF R3	10

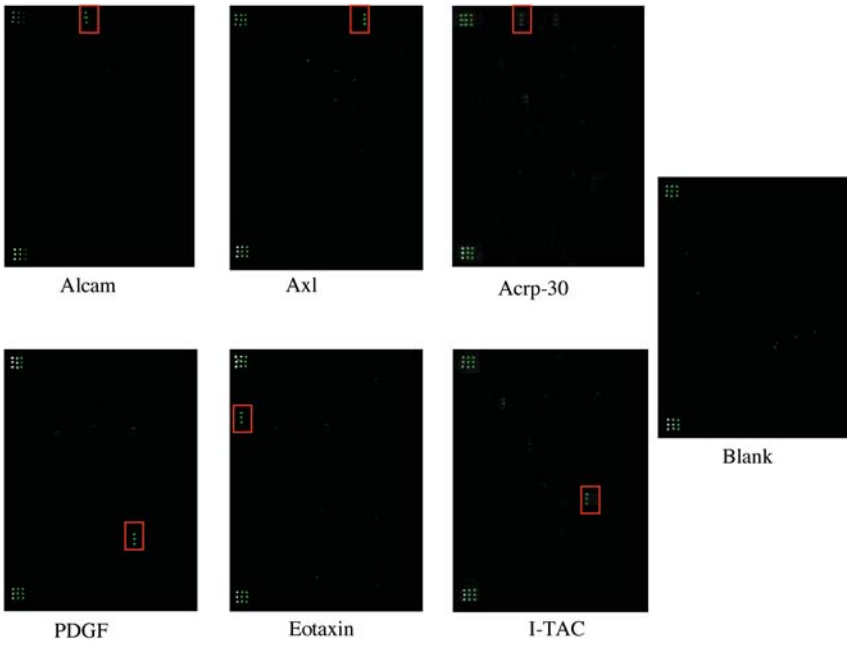


Figure 1. *Specificity testing. Each purified antigen was biotinylated at 1,000 ng/ml in 1X PBS. Biotinylated antigen was diluted 10-fold with blocking buffer and incubated with array slide. After extensive wash, fluorescence conjugated streptavidin was added to reveal signals.*

To test the detection linearity, we diluted the same conditioned medium sample by 2-fold, 10-fold and 20-fold. The diluted samples were labeled with biotin and subjected to the antibody array assay. As shown in Figure 3, linearity was observed for all analytes tested.

Validation of biotin label-based antibody arrays. After establishing the specificity, sensitivity and variability, we further validated the arrays in several different ways. First we performed spike-in experiment to assure that the arrays can detect the corresponding protein. As shown in Figure 4, individual proteins were spiked into the serum sample, labeled with biotin and probed with human 507 arrays. In general, a linear response was observed for all proteins we have tested.

The data obtained from the arrays were validated using conventional ELISA. It has been shown that TNF-alpha can induce interleukin (IL)-6 expression in human glioblastoma cells U251 (24). The conditioned media from U251 cells treated with or without TNF-alpha were assayed using biotin-labeled-based antibody arrays. Several proteins, such as IL-1 alpha, IL-6 and IL-8 were strongly induced by TNF-alpha as shown in Figure 5. The data were further validated using ELISA as shown in the Figure 5. The expression pattern was similar between the two data sets, with the ELISA data confirming the relative changes in protein expression seen in the semi-quantitative arrays. Similar results were obtained using TNF–alpha-treated human breast cancer cells. These results were further validated using

A

Reproducibility		
Spot to spot n=3 CV=10.8%	Well to well n=3 CV%=12.16%	Slide to slide n=3 CV=13.62%

B

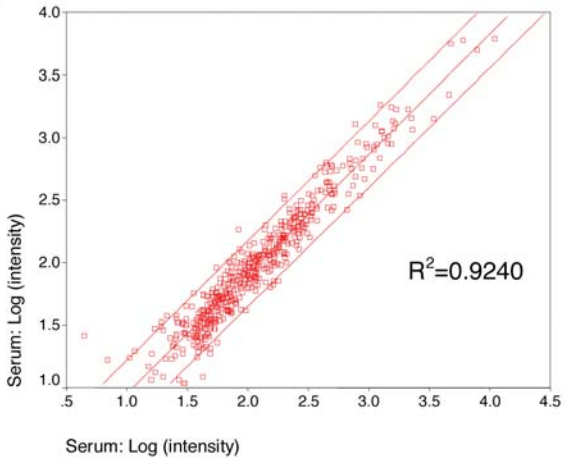


Figure 2. *The reproducibility assay was tested using serum samples. Scatter plot of normalized intensity from serum sample intra-slide on log scale. The log base 2 values of the signal intensities for duplicates experiments are plotted. R^2 is equal to 0.9240, suggesting a good reproducibility of two repeated experiments.*

human serum samples as shown in Figure 6. Brain-derived neurotrophic factor (BDNF) and adiponectin (ACRP30) were measured by both biotin label-based antibody arrays and ELISA for 7 human serum samples. Good correlation between array and ELISA data sets were obtained (Figure 6). Thus, the data obtained from arrays were well correlated with ELISA detection, widely considered the gold standard for measurement of protein concentration.

Ovarian cancer biomarker discovery using biotin label-based antibody arrays. To exploit the potential application of biotin label-based antibody arrays, we analyzed the expression levels of 507 protein markers in serum samples from 47 patients with ovarian cancer, 33 patients with benign ovarian masses and 39 healthy, age-matched controls using the RayBio® Human Biotin Label-based Antibody Array (Table I). There was no statistically significant difference in age among the three groups ($p>0.05$).

Protein levels larger than background +2×SE were subjected to ANOVA tests. A total of 84 proteins were differentially expressed between healthy women and those with ovarian cancer, with *p*-values of less than 0.05 (Table III).

To differentiate between ovarian cancer and normal healthy controls after sample decoding, statistical cluster analysis was performed. First, we used classification tree analysis.

As shown in Figure 7, the model used all observations in both normal and cancer groups to fit the model. Five markers were selected from the protein panels with significant differential expression between healthy women and those with ovarian cancer ($p<0.05$), including IL-2 receptor alpha, endothelin, osteprotegerin, vascular endothelial growth factor D (VEGF-D) and betacellulin (BTC). Overall, 90% of individuals were correctly classified when using these five markers to differentiate.

To develop a rapid assessment method for further testing, we used data for both ovarian cancer and normal healthy controls to develop split scores for each of the five markers plus osteoactivin.

The Table shown in Figure 8A gives the split-point signal level criterion for each marker. Individual marker classifications using split-point score method were inadequate to discriminate ovarian cancer from normal samples. However, by using split-point score analysis of six markers in which cancer is predicted to have a score of three or more, 98% of ovarian cancer cases (46 out of 47) were correctly diagnosed. In the healthy control group, 90% were correctly identified (35 out of 39). The overall accuracy rate for both cancer cases and normal healthy controls were 94% (81 out of 87) (Figure 8D).

The ROC was plotted using six-marker panel obtained from split-point score analysis, with sensitivity (true positive) as Y-axis and 1–specificity (false positives) as X-axis. According to the ROC, the overall performance of our 39 normal healthy controls and 47 cancer samples is shown in Figure 8C. The area under the curve (AUC) was 99%. Our six-marker panel clearly demonstrates the enhanced performance of the combination of six protein markers for prediction of ovarian cancer compared to single-biomarker discrimination (Figure 8C).

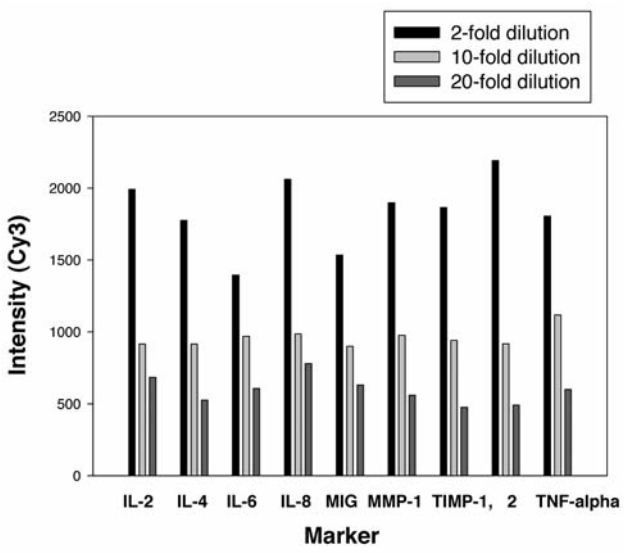


Figure 3. *Cell culture supernatant was diluted 2-, 10- and 20-fold with blocking buffer and then incubated with human label-based antibody arrays.*

A panel of 11 protein markers was also developed to distinguish benign ovarian tumors from ovarian carcinoma using split-point score analysis (Data not shown). These 11 markers are: CXCR2/IL-8 RB, Frizzled-1, IFN-alpha, IL-2 R alpha, IL-2 R beta/CD122, IL-3, IL-3 R alpha, IL-4, IL-1 R6/IL-1 Rrp2, IL-18 BPa, VEGF-D. Individual marker classifications using split-point score method were inadequate to discriminate ovarian cancer cases from those with benign ovarian tumors. However, by using split–point score analysis of 11 markers in which cancer is predicted to have a score of six or more, *i.e.* six or more markers for the sample falling in the ovarian cancer interval, 89% of ovarian cancer cases (41 out of 46) were correctly diagnosed. In the benign ovarian tumor group, 85% were correctly identified (28 out of 33). The overall accuracy rate for both cancer and normal healthy controls were 87% (69 out of 79). The ROC curve was also plotted using the 11-marker panel obtained from split-point score analysis. According to the ROC, the AUC was 90%.

Discussion

In the last decade, researchers, both in academia and in industry have developed several different formats of antibody arrays. The common principle of this technology is based on the interaction between capture antibodies printed on the solid surface in predetermined positions and the corresponding

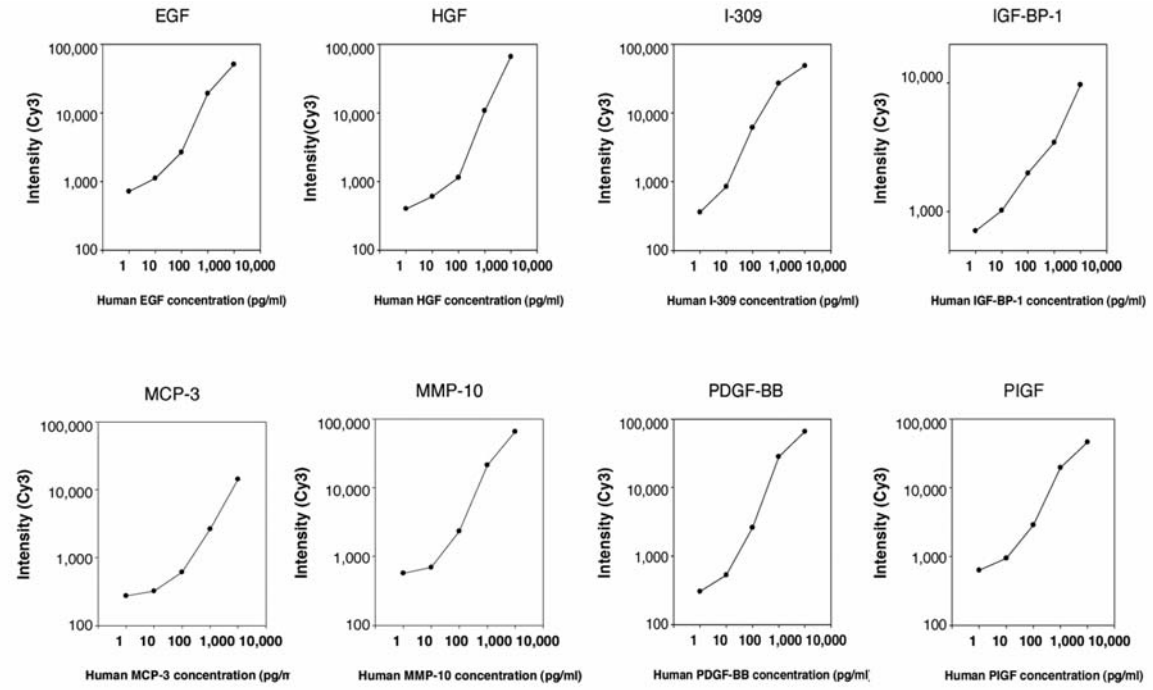


Figure 4. *Spiking test. A corresponding recombinant antigen was serially diluted into a human serum solution at 50,000, 5,000, 500, 50 and 5 pg/ml. The serum containing different concentrations of spiking antigen was then labeled with biotin. The biotinylated samples were diluted 5-fold with blocking buffer and incubated with each array slide.*

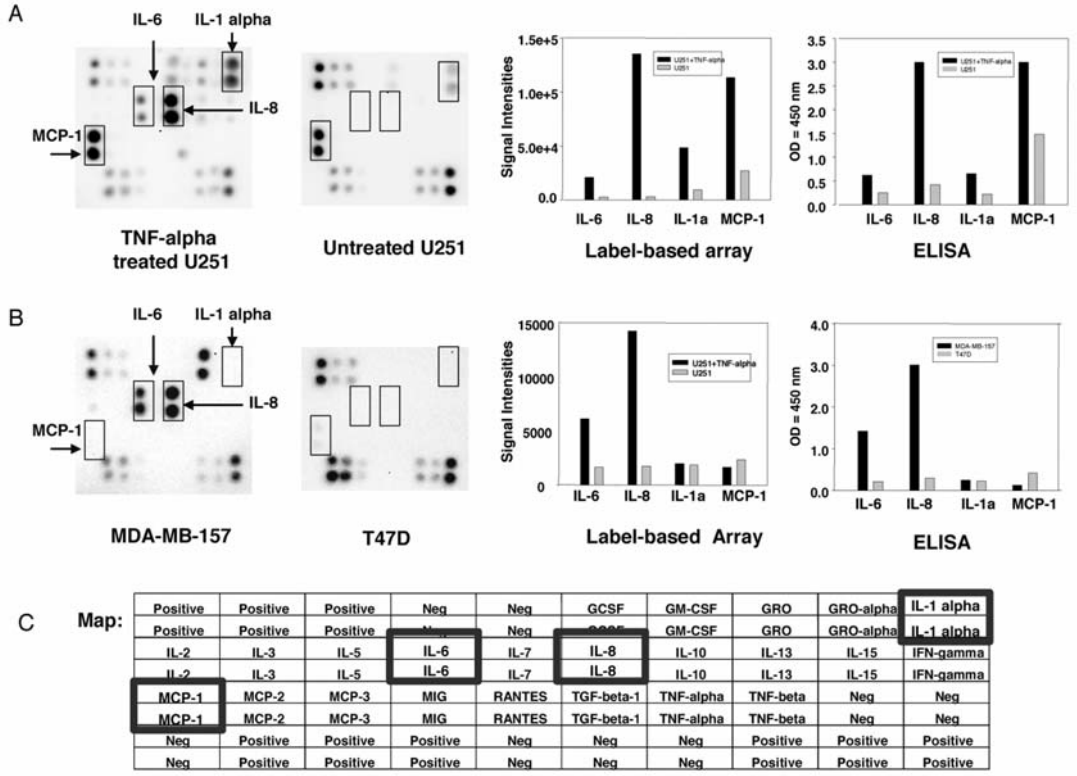


Figure 5. *Validation assay. A: The conditioned medium was prepared from human glioblastoma cells (U251) stimulated with or without TNF-alpha. Both biotin label-based antibody arrays and ELISA were performed and the result were compared. B: The conditioned medium prepared from human breast cancer cells (MDA-MB-157 and T47D) were assayed with both biotin label-based antibody arrays and ELISA. C: The mini map of antibody arrays.*

Table III. *Markers with significant differences (p<0.05 and SE>background+2×SE) expression using label-based antibody arrays.*

Marker	Healthy controls		Benign tumor		Ovarian cancer		*P*-value
	Mean	SE	Mean	SE	Mean	SE	
6Ckine	237.7	40.9	140.2	26.2	218.6	39.1	0.048
Angiopoietin-4	81.9	14.3	217.2	45.9	115.3	36.7	0.009
BDNF 5	29385.7	3126.8	21399.8	2423.2	10574.2	1353.2	0.049
BMP-8	4558.9	1117.4	1954.6	367.4	679.6	90.0	0.032
BTC	48.6	12.0	167.3	54.6	70.8	23.3	0.049
Cardiotrophin-1 / CT-1	48.2	18.8	106.2	20.2	17.7	4.8	0.042
CCR3	341.3	116.7	66.3	20.1	17.8	4.6	0.025
CCR4	1201.5	250.1	500.5	84.5	372.5	87.4	0.011
Chem R23	3209.6	758.0	1171.1	267.8	208.1	43.1	0.015
CLC	65.2	8.9	543.4	189.7	84.6	22.7	0.021
CXCR2/IL-8	49519.1	3254.1	39350.1	3607.8	32408.4	2909.2	0.042
DAN	38.1	7.0	117.5	25.6	172.2	80.1	0.006
EDG-1	2327.2	337.2	1488.5	219.8	1031.4	159.9	0.042
Endothelin	53.2	4.9	159.0	24.4	127.8	26.5	0.000
Eotaxin-3/CCL26	13.4	3.3	117.3	38.0	17.3	9.5	0.013
ErbB	22.2	4.1	99.4	23.2	34.0	7.1	0.003
FGF Basic	3538.7	665.3	1934.5	362.4	693.2	107.1	0.040
FGF R4	16959.7	3590.9	8506.7	1937.3	2803.2	1121.4	0.044
FGF-10/KGF-2	65.1	6.9	133.5	22.1	92.0	12.4	0.006
FGF-16	5309.5	853.7	2804.6	488.2	1401.3	270.3	0.014
FGF-6	33.9	6.0	129.3	24.3	46.0	9.0	0.000
GDF1	30.4	5.9	69.4	11.3	41.0	5.3	0.004
GDF-15	4432.7	656.2	1910.3	327.6	1800.5	494.7	0.001
GFR alpha-2	149.73	27.9	277.0	45.8	119.9	21.3	0.023
GITR ligand/TNFSF18	92.4	18.5	242.1	51.9	84.9	13.1	0.011
Glypican 5	11965.1	1542.9	7078.1	978.5	7066.4	879.4	0.010
Granzyme A	435.72	62.5	274.9	30.0	338.0	82.6	0.022
ICAM-1	116.78	19.4	212.9	36.9	80.1	9.0	0.029
IFN-alpha/beta R2	161.46	34.7	386.2	64.6	123.5	18.7	0.003
IFN-gamma	71.2	7.3	137.5	19.3	102.7	26.0	0.003
IGFBP-2	117.6	19.4	377.4	120.9	97.3	13.9	0.050
IGFBP-6	10504.0	1060.1	6856.8	938.7	6471.0	850.4	0.013
IGF-II	38.6	5.6	72.5	12.7	81.2	17.6	0.023
IL-1 R6/IL-1 Rrp2	81.3	16.5	144.9	22.7	70.0	9.0	0.031
IL-1 ra	29305.1	2757.9	21658.5	2420.7	16398.5	2300.9	0.040
IL-13 R alpha	398.8	58.4	1044.6	221.3	350.0	71.5	0.008
IL-17B	3981.7	673.5	1788.62	267.0	1377.2	189.7	0.004
IL-17C	6620.0	851.3	3631.5	535.0	3039.0	435.1	0.004
IL-17F	35221.4	3029.1	25044.9	3719.1	22451.1	3818.6	0.037
IL-18 BPa	121.54	12.9	180.2	17.1	136.2	26.4	0.009
IL-19	3499.4	551.0	1778.1	337.7	2347.4	477.5	0.010
IL-2 R alpha	218.97	65.8	859.1	164.0	251.7	42.5	0.000
IL-2 R	799.28	164.9	1519.6	263.8	370.5	61.0	0.027
IL-2 R gamma	171.24	25.6	322.1	64.5	140.9	18.9	0.042
IL-20 R	153.00	30.6	378.8	83.4	130.4	38.0	0.017
IL-22	2244.6	488.1	1060.4	169.9	890.5	206.9	0.027
IL-22	266.04	42.2	153.7	22.2	96.9	21.0	0.022
IL-3	149.3	20.4	246.9	29.7	115.7	18.9	0.010
IL-3 R alpha	103.7	18.6	237.4	39.9	55.1	10.5	0.004
IL-4	328.3	40.1	775.4	125.6	309.7	128.9	0.001
IL-4 R	56380.0	2622.1	66649.9	3717.6	57709.3	4362.9	0.032
IL-5	207.6	28.1	480.6	105.5	208.4	33.6	0.021
IL-9	74.3	23.2	191.1	39.2	56.7	11.8	0.015
Kremen-2	1443.6	285.7	3170.6	721.9	5595.0	1226.1	0.038
Lck	99.2	20.9	203.7	35.0	149.4	24.3	0.016
Leptin receptor	1260.8	195.0	580.6	61.7	1297.9	373.8	0.001
Lipocalin-8	11923.1	1841.3	6047.1	781.5	6768.2	1479.6	0.005

Table III. *continued*

Table III. *continued*

Marker	Healthy controls		Benign tumor		Ovarian cancer		*P*-value
	Mean	SE	Mean	SE	Mean	SE	
Lymphotoxin beta/TNFSF3	2010.6	431.3	955.5	195.9	481.3	89.3	0.031
MIP-1 alpha	42593.8	3562.6	31035.6	3634.0	26158.4	2677.7	0.027
MMP-10	2107.3	410.6	998.2	205.5	485.3	73.6	0.020
MMP-13	425.2	62.7	640.7	81.4	235.7	26.6	0.044
MMP-15	43.9	12.2	152.0	46.2	41.9	8.5	0.036
MMP-7	1214.0	248.0	632.3	119.7	567.2	81.3	0.041
MSP beta-chain	2490.8	710.8	933.9	213.4	959.8	238.7	0.042
Neuritin	1524.5	220.5	949.0	125.9	1168.5	165.9	0.028
Neuropilin-2	29.3	4.3	154.3	45.9	49.6	11.4	0.013
Neurturin 5	2128.4	397.4	1031.0	198.9	1397.5	285.5	0.017
NRG	54.0	16.8	189.5	41.2	133.5	39.1	0.004
Orexin B	54.9	13.7	280.0	104.8	60.3	17.2	0.049
Osteoactivin/GPNMB	12845.7	1630.5	7077.4	1141.6	8519.2	1588.7	0.005
Osteoprotegerin/TNFRSF11B	22163.9	3374.5	10674.6	1765.9	10954.8	2076.4	0.004
Pref-1	26.1	4.8	116.3	22.2	36.7	5.6	0.000
Prolactin	37.9	11.3	86.5	19.4	43.7	12.8	0.041
SIGIR	13455.5	2238.3	40335.2	8401.7	11353.5	3634.1	0.004
TGF-beta RI/ALK-5	73.7	10.3	108.8	11.2	71.0	10.3	0.027
Thrombospondin-4	55.1	13.0	99.1	17.8	99.8	45.8	0.045
TIMP-2	67.8	11.0	147.1	35.6	73.8	15.6	0.048
TSG-6	21.9	3.5	103.0	22.3	33.0	4.7	0.001
VE-Cadherin	27.0	7.5	259.9	87.2	25.5	3.9	0.015
VEGF R2 (KDR)	134.6	17.6	204.3	24.3	153.0	28.5	0.025
VEGF-D	41.2	7.3	79.0	10.1	37.4	4.2	0.004
WIF-1	49.4	9.7	112.2	23.2	44.3	7.0	0.019

target antigens. To visualize this interaction, a variety of detection methods can be used. The most common way is to apply detection antibodies which can recognize the different epitopes of same target protein. In practical application, this is the most common format since the whole procedure can be easily adapted to automation. This approach also can semi-quantitatively and quantitatively measure protein levels with high specificity, sensitivity and reproducibility. However, the limited source of pair antibodies and the cross-reactivity among capture and detection antibodies significantly hinders the potential for the development of high density antibody arrays.

The obvious solution for this is to avoid the use of detection antibodies in the array design. Surface plasmon resonance (SPR) detection provides a label-free and single-antibody approach (29). In this approach, proteins in the sample are captured by antibodies printed on the arrays and detected by light scatter reflection due to the interaction between capture antibody and the target protein. The major advantage of the SPR method is its ability to measure the relative protein levels, to monitor the affinity constant in real time, and no label is required. However, this approach suffers from low detection sensitivity and still awaits the improvement of instrumentation for high density detection.

Combinations of antibody arrays and mass spectrophotometry may provide a better solution for detection of protein levels with high content, high throughput and specificity, but the technology is not mature enough for routine application and lacks the sensitivity sufficient for biomarker discovery (4, 30).

Currently, one of the practical approaches for high density antibody arrays is to label protein with fluorescent dye. Most fluorescent dyes used in antibody arrays are Cy3 and Cy5 as in DNA microarrays (31, 32). The potential problem for fluorescent dye labeling is low detection sensitivity. To increase the detection sensitivity, we developed biotin label-based antibody arrays. The biotin label has several advantages over fluorescent label. First, it can be used as signal amplification. Second, biotin is the most common method for labeling protein, and the labeling process can be highly efficient. Furthermore, biotin can be detected either using fluorescence-streptavidin, visualizing signals using laser scanner, or using HRP-streptavidin, imaging signals using chemiluminescence. Indeed, in this paper, we showed that using the biotin label-based antibody arrays we developed, most proteins can be detected at pg/ml levels. The detection sensitivity may be further enhanced by using 3-DNA detection technology (unpublished observation) or

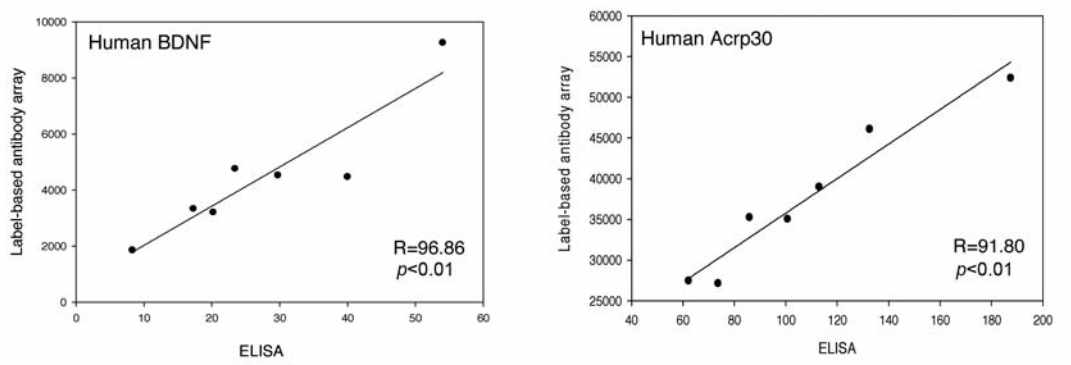


Figure 6. *ELISA confirmation. The correlation of BDNF (A) and Acrp-30 (B) between biotin label-based antibody arrays and ELISA were compared. The overall R value is larger than 0.9, suggesting a good correlation between the two assays.*

rolling circle amplification (33, 34). Furthermore, several detection methods can be used in this biotin label approach. If fluorescence detection is used, the whole experiment can be designed using glass slides and then a very tiny amount of samples is needed for the whole experiment. If chemiluminescence detection is used, the signal can be visualized using a chemiluminescence imager or x-ray processor. If colorimetric detection is used, no other equipment is needed for the entire experiment. This provides multiple choices in different settings. If sample volume is a major concern, fluorescence detection can be used. If high detection sensitivity is needed, chemiluminescence detection is the preferred choice. If no major equipment is available, colorimetric detection is the best method.

Since only one antibody is used, no interaction between capture antibody and detection antibody is a concern in the label-based approach. In general, the detection specificity of the single-antibody approach is lower compared with sandwich-based (paired antibody) format. But it can also avoid the cross-reactivity problem caused by detection antibody. As demonstrated in this paper, careful selection of highly specific antibodies, the gap in specificity between these two approaches can be minimized.

By comparison with ELISA-based antibody arrays, in some cases, the detection sensitivity is lower in the biotin label-based approach, but in others, it is higher.

The content of biotin label-based arrays can be easily expanded and changed. For instance, using the same technology, we have developed biotin label-based mouse antibody arrays to detect the expression levels of 308 mouse proteins, biotin label-based rat antibody arrays to detect the expression levels of 90 rat proteins and biotin label-based human adipokine antibody arrays to detect the expression levels of 182 adipokines.

High density antibody arrays are particularly useful in biomarker screening and expression profiling. To demonstrate the potential application, we screened 507

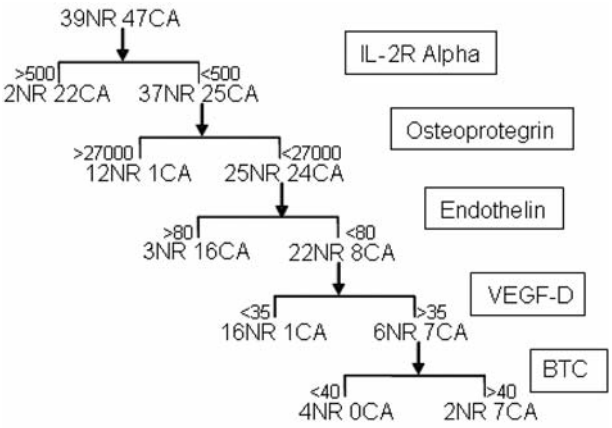


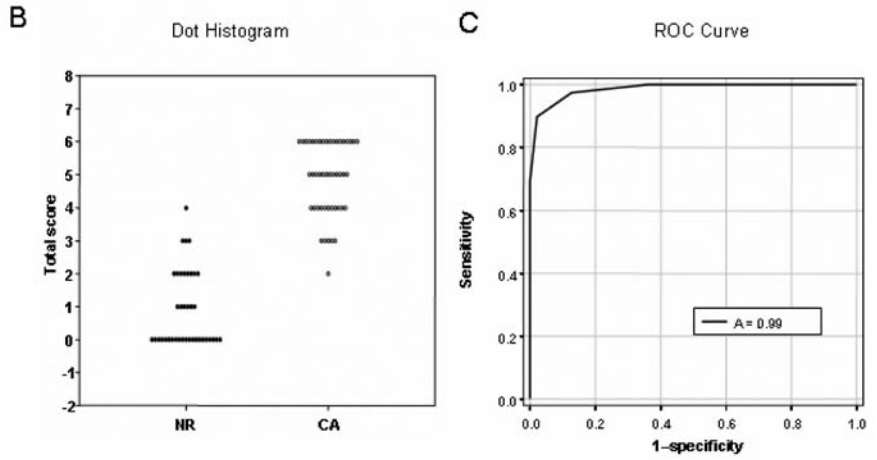
Figure 7. *Classification tree analysis. Proteins used in the classification tree analysis and their cut-off signal are listed on the left. The range of data specified at each split represents the subset of data which is further subdivided by branches to the right.*

human protein levels in ovarian cancer specimens and normal subjects. We found that a panel of serum proteins were differentially expressed differently between ovarian cancer patients and healthy controls ($p<0.05$), suggesting that this approach is a powerful approach in biomarker discovery. To validate the finding, quantitative antibody arrays can be developed and applied in more samples and different laboratories. If the results are validated, the quantitative antibody arrays can be used in the clinical setting. This is promising biomarker discovery platform.

Interestingly enough, the six biomarkers identified here are all involved in angiogenesis. Indeed, angiogenesis has been well-documented in the role of ovarian cancer development (35). The expression of many angiogenic factors has been found to be changed in ovarian cancer patients (36). They have also been reported to play role in solid tumor development and/or progression. ET-1 has been implicated in the

A

Marker	Split-Point	Left Region	Right Region
Endothelin	65	NR44(8)	CA42(7)
IL-2 R alpha	240	NR43(9)	CA43(5)
Osteoactivin	11200	CA50(9)	NR36(6)
VEGF-D	36	NR38(6)	CA48(12)
BTC	29.2	NR38(3)	CA48(14)
Osteoprotegerin	7000	CA29(0)	NR57(18)



D

	CANCER	NORMAL
Score>2	46	4
Score<2	1	35
Total samples	47	39

Specificity	90%
Sensitivity	98%
Accuracy	94%
PPV	92%
NPV	97%

Figure 8. *Split-point score analysis. A: The six markers used in split-point score classification analysis. Misidentified samples were in the bracket using individual markers. B: Dot histogram plot with six analyte split-point score classification of serum samples from healthy controls (N) and individuals with ovarian cancer (CA). Correctly classified normal serum samples should have a score of 0 to 2, whereas samples from ovarian cancer patients should have a score of 3-6. False-negative sample and false positive samples can easily be detected. C: The ROC curve for 5 marker panel of split-score analysis of ovarian cancer vs. healthy controls. The ROC is the curve plotted of sensitivity (true positive) against 1–specificity (false positive) values. D: Table using six-marker split-point score to diagnose ovarian cancer patients. A cut-off score of 3 was used.*

pathophysiology of a wide range of human tumors, including ovarian carcinoma (37). Recently, Salani *et al*. reported the role of ET-1 in the neovascularization of ovarian carcinoma, postulating that ET-1 could modulate tumor angiogenesis, acting directly and in part through VEGF (38). ET-1 has also been reported to play an important role in ovarian cancer progression. Rosano *et al*. reported that ETAR activation by ET-1 contributes to tumor progression by acting as a crucial mediator of epithelial-to-mesenchymal transition (EMT) in human ovarian carcinoma cells (39). sIL-2R have been found in elevated levels of sera from patients with several types of solid tumor, including ovarian cancer. Sedlaczek and Gebauer et al both reported that sIL-2R was highly expressed in ascites and sera of ovarian cancer patients compared with benign tumors (40, 41). Osteoactivin has been reported to play a role in some solid tumors including hepatocellular carcinoma, breast cancer, melanoma and glioma. Onaga et al have reported that overexpression of osteoactivin may be involved in the progression of hepatocellular carcinoma cells via stimulation of tumor invasiveness and metastatic potential (42). Rose et al has reported that osteoactivin plays a role in promoting breast cancer metastisis to bone. Our study is the first report to show that osteoactivin, in combination with other 5 markers, can be used in screening ovarian cancer patients (43). Interestingly enough, the 6 biomarkers identified here are all involved in angiogenesis.

In summary, we have developed a sensitive and reliable biotin-label-based antibody arrays for high content screening. By addition of new antibodies, the density of arrays can be expanded.

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