Original Article

Protein Microarrays for Quantitative Detection of PAI-1 in Serum

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ABSTRACT

Objective: Plasminogen activator inhibitor-1 (PAI-1), one crucial component of the plasminogen activator system, is a major player in the pathogenesis of many vascular diseases as well as in cancer. High levels of PAI-1 in breast cancer tissue are associated with poor prognosis. The aim of this study is to evaluate rigorously the potential of serum PAI-1 concentration functioning as a general screening test in diagnostic or prognostic assays.

Methods: A protein-microarray-based sandwich fluorescence immunoassay (FIA) was developed to detect PAI-1 in serum. Several conditions of this microarray-based FIA were optimized to establish an efficacious method. Serum specimens of 84 healthy women and 285 women with breast cancer were analyzed using the optimized FIA microarray.

Results: The median serum PAI-1 level of breast cancer patients was higher than that of healthy women (109.7 ng/ml vs. 63.4 ng/ml). Analysis of covariance revealed that PAI-1 levels of the two groups were significantly different (P<0.001) when controlling for an age effect on PAI-1 levels. However, PAI-1 values in TNM stage I–IV patients respectively were not significantly different from each other.

Conclusion: This microarray-based sandwich FIA holds potential for quantitative analysis of tumor markers such as PAI-1.

Key words: Breast cancer; Plasminogen activator inhibitor-1; Protein microarray; Serum

INTRODUCTION

Plasminogen activator inhibitor-1 (PAI-1), a member of the plasminogen activator system, is of the serine protease inhibitor superfamily. It protects the extracellular matrix from excessive degradation and also interacts with the extracellular matrix component vitronectin. Given these biological properties involved in cell adhesion and migration, it is hypothesized that PAI-1 may play an important role in cancer invasion and metastasis[1]. Indeed, high tissue levels of PAI-1 have been consistently reported to predict poor prognosis in several types of human cancers. In breast cancer, tumor tissue levels of urokinase plasminogen activator (uPA) and PAI-1 were more predictive of both disease-free and overall survival than estrogen receptor (ER) status and tumor size in a pooled analysis of over 8,000 patients[2].

A few studies examined serum or plasma PAI-1 levels in cancer patients, but unfortunately, the results were frequently conflicting. Grebenchtchikov, et al. demonstrated that determination of plasma PAI-1 did not reflect its concentration in tumor tissue. Therefore, they did not recommend measurement of PAI-1 in blood for assessing prognosis in breast cancer[3]. Conversely, Palmirotta's team found that the 5-year relapse-free survival rate of PAI-1 positive patients was statistically lower than that of PAI-1 negative patients. They concluded that plasma PAI-1 levels in breast cancer patients could represent a useful prognostic variable for relapse[4].

Protein microarray is a widely used new technique, but there are still some intractable problems strangling its advance[5]. Protein microarray technique has several unique features beyond traditional methods in term of assessment of biomarkers. First, protein microarray technique has the potential to analyze a multiple set of biomarkers simultaneously in biological samples[6,7]. Second, its miniaturized assay format allows for the

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profiling of biomarkers using only minute amounts of biological materials, such as antibodies or tumor biopsies[8]. Third, protein microarrays are suitable for automation[9] and could be adapted to large-scale applications by hospitals or independent laboratories.

In this report, we described the development and validation of a sandwich fluorescence-immunoassay (FIA) microarray for measuring PAI-1 in serum using the monoclonal PAI-1 antibodies prepared by our laboratory. Moreover, we analyzed the PAI-1 data of breast cancer patients and healthy women obtained by this newly established method.

MATERIALS AND METHODS

Preparation of Recombinant Fusion Protein

A prokaryotic plasmid expressing fusion protein MS2-PAI-1 was constructed by our laboratory previously. It was transformed into *Escherichia coli* strain POP2136. The positive colony was first cultured at 30℃ for 4.5 h, and then cultured at 42℃ for 4.5 h to induce large quantities of the fusion protein. We then employed alkaline denaturation and ultrasonic schizolysis to extract insoluble fusion protein from inclusion bodies. The extract was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The target protein band was electroeluted (40 V overnight, the next day 80 V for 1 h), followed by dialysis in 1×PBS buffer and concentration embedded in sucrose[10]. In this way, we eventually got the recombinant protein which would be used as standard protein in protein microarrays.

Purification of PAI-1's Antibodies

PAI-1 monoclonal antibodies were generated according to standard hybridoma technique by our laboratory before. BALB/c mice were immunized with MS2-PAI-1 and their spleen cells were isolated to fuse with mouse myeloma cells. The strategies for monoclonal antibody selection are described in the "Results". Isotyping was performed using a mouse Monoclonal Antibody Isotyping Test Kit (Sigma, Missouri, USA) according to the manufacturer's instruction.

Two hybridoma cell strains that can secrete PAI-1 monoclonal antibody F6 or D11 were obtained. The two different monoclonal antibodies are both of the IgG1 subclass. First, the antibodies in mouse ascites were precipitated by saturated ammonium sulfate. Then, we conducted affinity chromatography using a Protein G-Sepharose 4B antibody purification column (GE Healthcare, Shanghai, China) according to antibody subclass. The antibody eluate was dialyzed in 1× PBS buffer and its concentration was determined by a bicinchoninic acid (BCA) protein assay kit (Applygen, Beijing, China).

Biotinylation of PAI-1 Monoclonal Antibody

In order to develop sandwich FIA microarrays of two monoclonal antibodies, one of two antibodies must be alternatively labeled (with biotin etc.). (+)-Biotin N-hydroxysuccinimide ester (NHS-D-Biotin) (Sigma, Missouri, USA) was used to biotinylate antibodies according to the manufacturer's protocol. NHS-D-Biotin was dissolved in dimethyl sulfoxide (DMSO) to 40 mg/ml while the concentration of the antibody solution to be labeled must be above 1 mg/ml. NHS-D-Biotin and the antibodies were mixed at 1:2 with gentle stirring at room temperature for 4 h, followed by dialysis against several changes of 1× PBS buffer at 4℃. After dialysis, the biotinylated antibody was stored at –20℃ until use.

Manufacturing Protein Microarrays

Protein microarrays were produced by SmartArrayer™48 Microarray Spotter (CapitalBio, Beijing, China) as the manufacturer's recommended procedure. Briefly, the capture antibody was diluted in spotting buffer (0.05% PBS-Tween 20) at a certain concentration. A hydrophobic paper containing 18 holes was stuck on the surface of aldehyde-coated slides (VSS-25C, CEL Associates, Inc. Pearland, Texas) to form 18 reaction wells. Microarrays of 3×3=9 spots with a spot-to-spot distance of 1,300 μm were printed using non-contact spray technology. In every well, 6 spots of the capture antibody and 3 spots of negative control (mouse IgG) were printed. Freshly printed microarrays were incubated in a moist chamber at 4℃ until use.

Processing Protein Microarrays

The protocol for processing protein microarrays has been described in the literature[11]. In the first step, the printed chips were blocked with 30 μl blocking solution (PBS containing 5% *W*/*V* non-fat milk)/well for 1 h at room temperature. After that, standard protein, blank control, and serum samples were added in different wells for 45 min at room temperature followed by 1-h incubation with detection antibody. Finally, diluted streptavidin-Cy3 (Sigma, Missouri, USA) was added for 1-h incubation at room temperature. Extensive washing with 0.05% PBS-Tween 20 followed each incubation step to reduce nonspecific binding. The chip images were obtained by LuxScanTM 10 K Microarray Scanner (CapitalBio, Beijing, China) using Cy3 settings. The scanner's accompanying software, LuxScan 3.0.0720, was used to quantify the spot fluorescence intensity from the scanned images.

Optimization of Experiment Conditions

Several experimental conditions of protein microarrays were optimized so as to maximize the detection sensitivity. The time to fix the capture

antibodies to the slide was either overnight at 4°C or 4 h at room temperature. The blocking efficiencies of 10 mg/ml bovine serum albumin (BSA) in PBS and 5% non-fat milk in PBS were compared. In addition, the blocking time was tested at 0.5, 1.0, 1.5, and 2.0 h. The washing times between each incubation step were also optimized.

Establishment of Standard Curve

After optimizing the experimental conditions, a series of diluted standard antigens (MS2-PAI-1) over a larger range were measured to create a standard curve. The concentrations of the standards were 2-fold diluted and ranged from 62.5–5,000 ng/ml. The standard antigens were diluted in blocking solution and the blocking solution was used as blank control.

Patient and Control Samples

The human samples for this study were obtained from Beijing Cancer Hospital, Health Science Centre of Peking University between 2009 and 2010. A cohort of 285 breast cancer patients were pathologically confirmed and staged according to the TNM classification. Criteria for exclusion from the study included male patients, benign breast tumor, and patients experiencing more than one cancer. Patient clinical and pathological characteristics are summarized in Table 1. The control group was recruited from healthy women receiving physical examinations and comprised 84 unrelated healthy women (mean age 45.5±5.92 years). Samples and clinical information were obtained under Institutional Review Board approval. Written informed consent was obtained from each participant.

Serum Collection

Blood samples for serum analyses were left to clot for 1 h; thereafter, the samples were centrifuged at 3,400 r/min for 8 min, and serum aliquots were stored frozen.

Clinical Sample Test

Samples were tested by using the sandwich FIA microarrays as described above. Generally, samples were analyzed directly without dilution, whereas a few samples required dilution because of too high fluorescent signal. A six-point standard curve was generated in each chip to quantify PAI-1 concentrations. Three printed spots in the last row of each well were 1 mg/ml mouse IgG. The precise fluorescent signal of a certain sample was the signal of specific capture antibody (PAI-1 antibody) minus the signal of mouse IgG. This correction was performed because the PAI-1 monoclonal antibodies originated from mouse.

Table 1. Patient clinicopathological characteristics

Tumor characteristic	*N*	%
Primary site*		
Left side	138	48%
Right side	129	45%
Bilateral	17	6%
Pathological type*	213	75%
Ductal	9	3%
Lobular	37	13%
Others**		
TNM Stage*	64	22%
I	76	27%
II	15	5%
III	81	28%
IV		
Lymph node status	230	81%
Negative	55	19%
Positive		
ER status*	68	24%
Negative	28	10%
<25%	10	4%
25%–50%	21	7%
50%–75%	53	19%
>75%		
HER2 status*	70	25%
Negative	40	14%
1+	30	11%
2+	45	16%
3+		
Age, mean (range)		
51.2 (17–79) years	285	

*Unknown data: primary site (*n*=1); pathological type (*n*=26); TNM stage (*n*=49); ER status (*n*=105); HER2 status (*n*=100).
**Other histotypes included medullary, tubular, mucinous, and mixed histotypes.

Statistical Analysis

All analyses were performed using the SPSS version 16.0 statistical package (SPSS Inc., Chicago, IL, USA). Considering the age effect on serum PAI-1 levels, analysis of covariance was applied to compare PAI-1 levels of the disease and control group after transforming PAI-1 values for normal distribution. The associations between serum PAI-1 and tumor size, carcinoembryonic antigen (CEA), cancer antigen 15−3 (CA15−3), CA125, estradiol (E2), and follicle stimulating hormone (FSH) were calculated by Spearman's correlation. Absolute levels of PAI-1 were compared by nonparametric tests. The following variables were studied: primary site, pathotype, TNM stage, lymph node status, ER status, human epidermal growth factor receptor 2 (HER2) status, menopausal state, hypertension or non-hypertension, and family history. All tests were two-tailed and only *P* values lower than 0.05 were regarded as statistically significant.

RESULTS

Recombinant PAI-1 and Antibodies

The specificity of PAI-1 monoclonal antibodies was

rigorously identified by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, the hybridoma cells were first screened against both MS2 and MS2-PAI-1. Those hybridoma cells only reacting with MS2-PAI-1 but not with MS2 were maintained. They then were screened against MS2-PAI-1, asparagine synthetase (ASNS), Survivin, neuron-specific enolase (NSE), Midkine, α6-integrin and BSA. The reaction between hybridoma cells' supernatant and MS2-PAI-1 was far strong than other reactions[10].

Recombinant PAI-1 and its antibodies were purified. Their purity was identified by SDS-PAGE and their concentration was determined by a BCA protein assay kit (data not shown).

Optimum Conditions for Microarrays

We chose F6 as the capture antibody because it had the highest specificity[10]. The antibody D11 was biotinylated and dialyzed in a tube filter against 1×PBS buffer. The concentration of capture antibody F6 sprayed onto the surface of aldehyde-coated slides was 1 mg/ml based on a previous study[11]. At first, 10 mg/ml BSA-PBS was used as blocking solution[12]. Unfortunately, the background signal was always high despite testing several other conditions. We substituted PBS containing 5% (*W*/*V*) non-fat milk for 10 mg/ml BSA-PBS and the background signal decreased to an acceptable level. Other processing conditions, including fixing time, blocking time and wash times, were also optimized. Whether incubated overnight at 4℃ or 4 h at room temperature made no difference to the final results, so either manner could be used to fix the capture antibodies. A blocking time of 1 h was enough to greatly reduce non-specific binding, and longer time did not yield further improvement. Results were not significantly different whether the slides were washed three times or twice between incubation steps. In sum, blocking solution was the most important factor determining test specificity and signal to noise ratio.

Standard Curve

The fluorescent signals increased over a larger range (62.5–5,000 ng/ml) of PAI-1 concentrations, but the linear relationship was only maintained below 2,000 ng/ml. As a result, we measured standard antigen PAI-1 over a smaller range (62.5–1,000 ng/ml). Meanwhile, some serum samples were detected. The linear relationship of standard curves was similar in repeated trials, and the fluorescence signals of randomly selected samples were not beyond the range of PAI-1's fluorescence signals (Figure 1).

Repeated Trials

We performed repeated trials with standard protein to verify the method's reliability and calculate within-run and between-run variation (Table 2).

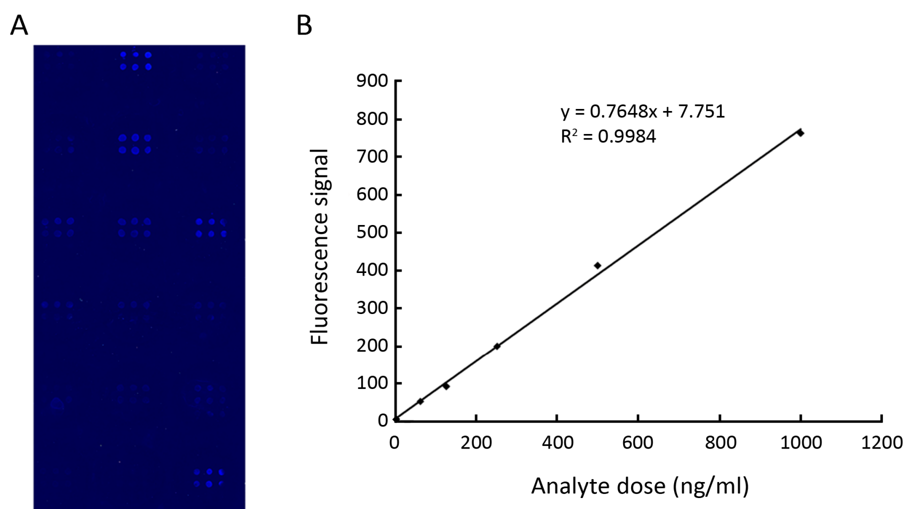

Figure 1. Image of a protein chip and standard curve. **A:** Image obtained by scanning a processed glass slide; standard protein reactions were set in column 2. **B:** The standard curve was drawn from (A). Individual data points represent the mean signals of identical replica spots (*N*=6).

Table 2. Within-run and between-run variation of standard protein detection

PAI-1 (ng/ml)	Within-run			Between-run		
	Average	*s*	CV (%)	Average	*s*	CV (%)
1,000	984	107	11%	1094	128	12%
500	462	72	16%	494	25	5%
250	257	47	18%	265	39	15%
125	166	27	16%	150	20	13%
62.5	61	16	25%	62	7.7	12%
0	7.4	1.3	17%	7.0	2.8	40%

s: standard deviation.

Analysis of Serum PAI-1 Level in Women with Breast Cancer

The median PAI-1 level of breast cancer patients was higher than that of control group (109.7 ng/ml vs. 63.4 ng/ml, Figure 2). Analysis of covariance showed that PAI-1 levels of the two groups were significantly different (P<0.001) after controlling age effect on serum PAI-1.

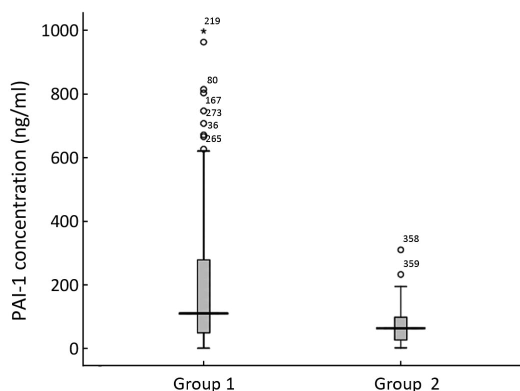

Figure 2. The concentrations of serum PAI-1 were significantly higher in the breast cancer group (group 1) compared to the control group (group 2). The upper bar, box and lower bar represent the 90%, 75%, 50%, 25% and 10% percentiles.

Using Spearman Correlation analysis, serum PAI-1 levels were not be found to correlate with levels of CEA (r=0.059, P=0.342, n=263), CA15-3 (r=0.037, P=0.554, n=265), CA125 (r=0.144, P=0.231, n=71), E2 (r=0.016, P=0.803, n=257), or FSH (r=−0.018, P=0.772, n=261). Serum PAI-1 level did not correlate with tumor size, either (r=−0.07, P=0.379, n=162).

Serum PAI-1 levels were increased weakly, but not significantly, associated with the fact that whether a breast cancer patient was simultaneously a hypertension patient (P=0.069). In addition, we did not find any association between PAI-1 level and other patient characteristics (Table 3).

Table 3. Associations between serum PAI-1 levels and other tumor characteristics

Characteristic	PAI-1, $\bar{x}$±s (median)	Mann-Whitney U/Kruskal-Wallis H test
Primary site		
Left	200.1±18.0 (108.3)	P=0.529
Right	175.3±15.5 (109.7)	
Bilateral	188.7±38.9 (128.8)	
Pathotype		
Ductal	179.5±12.9 (106.7)	P=0.831
Lobular	262.6±94.2 (124.0)	
Others	194.2±31.8 (111.7)	
TNM stage		
I	196.8±24.5 (110.3)	P=0.551
II	214.3±26.1 (125.5)	
III	112.6±27.1 (71.3)	
IV	202.8±21.6 (124.4)	
Lymph node status		
Negative	191.8±12.9 (111.2)	P=0.512
Positive	169.7±24.2 (98.3)	
ER status		
Negative	185.4±25.9 (105.0)	P=0.310
<25%	209.9±32.4 (160.8)	
25%–50%	125.8±47.9 (84.6)	
50%–75%	266.5±51.5 (145.4)	
>75%	181.9±26.4 (104.0)	
HER2 status		
negative	189.5±23.1 (114.5)	P=0.503
1+	155.8±28.3 (89.8)	
2+	222.7±37.4 (146.6)	
3+	220.2±33.1 (162.8)	
Menopausal state		
Pre-menopausal	180.3±17.4 (102.1)	P=0.535
Post-menopausal	193.7±15.5 (118.9)	
Hypertension		
No	176.7±11.9 (102.4)	P=0.069
Yes	241.1±32.9 (178.1)	
Family history		
No	182.4±11.6 (108.3)	P=0.552
Yes	231.2±45.0 (130.7)	

DISCUSSION

Serum PAI-1 levels show a diurnal variation, being higher in the early morning hours, due to the upregulation of its promoter by circadian genes[13]. The majority of patient samples were collected in the afternoon, so we surmised that the diurnal fluctuation of serum PAI-1 level would not affect the final statistical results. Under normal physiological state, serum PAI-1 concentration keeps in low level (10–30 ng/ml). In our study, the median of serum PAI-1 value of control group was approximately 60 ng/ml, which was higher than the values of some previous reports[4,14]. This is probably attributable to the following aspects. First, the standard protein we used was a recombinant fusing protein. There is a 9-kDa polypeptide, MS2, at the amino terminal of the fusing protein which consequentially makes PAI-1 quantification relatively higher. Second, the fusing protein was expressed by *Escherichia coli*, and so it could not be glycosylated after translation, while the native form of PAI-1 is a 52-kDa single-chain glycoprotein[13]. Thus, the recombinant protein was different from the native PAI-1 in structure and activity, thereby affecting the absolute values obtained. And third, there are several forms of PAI-1 in human blood, including functionally active PAI-1, latent PAI-1, and complexed PAI-1. We still do not know the specificity of our antibodies against these specific PAI-1 forms and this requires further investigation.

The current study demonstrated that serum PAI-1 levels were elevated in women with breast cancer, which is consistent with previous reports, not only concerned with breast cancer[4,15,16]. However, PAI-1

levels in serum were not positively correlated to TNM stage, in contrast to our initial expectations. The differences in serum levels possibly just reflect a difference between healthy persons and diseased patients such as cancer patients. A few perspective studies reported that preoperative plasma PAI-1 could be used as a prognostic indicator in breast cancer[4], rectal cancer[17,18], and colorectal cancer[19]. In sum, it is most possible that plasma or serum PAI-1 can take the place of tumor tissue PAI-1 as a prognostic factor for a variety of tumor types, including breast cancer.

The samples were collected randomly from patients at Beijing Cancer Hospital. The patient number in TNM I, II and IV stage was nearly the same, and only the patients in TNM III stage were dramatically fewer (only 15 cases). For these breast cancer patients who do not discovery themselves disease at an early stage, a specific marker or markers combination in blood is crucial to accomplish screening test.

PAI-1 is a major player in the pathogenesis of many vascular diseases as well as in cancer. Accordingly, plasma PAI-1 levels were elevated in obesity, metabolic syndrome, type 2 diabetes, inflammatory states, and cancer[20]. In this study, we found breast cancer patients with hypertension had a higher serum PAI-1 level than patients without hypertension, even though the difference was not statistically significant. PAI-1 levels also change due to some physiologic factors. The study indicated menopausal state did not affect serum PAI-1 level, which was discordant with previous results. We speculate that how serum PAI-1 concentration changes under different physiological and pathological conditions is still uncertain and requires further study.

To date, protein chip technology is far from fully developed. Potential pitfalls in microarray-related techniques mainly include sample preparation and handling, microarray platform, detection technologies, and data analysis[12]. We optimized most experimental conditions and finally set up the reproducible protein-microarray immunoassay. But the quantitative data still seem to vary greatly (Table 2) compared to conventional methods. Although there are many challenges to be overcome in the development of robust protein microarrays, the technology is worthy of continued support.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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