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Original Article

Application of high-throughput protein array in clinical screening for tumor markers

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Abstract: Objective: We evaluated the value of high-throughput protein array in clinical screening for tumor markers. Method: HuProt array, which contained over 19000 proteins, was used. The case group consisted of 160 patients with various tumors and the control group consisted of 160 healthy subjects. The serum was collected and detected using protein array. The positive criteria were determined and the protein array data were interpreted according to the criteria by searching the databases and sequence alignment. The differences between the two groups were compared and the validity of protein array detection for serum tumor markers was confirmed. Results: The criteria were based on signal-to-noise ratio (SNR) combined visual inspection. This method had high sensitivity and specificity for interpreting protein array data. For the positive result based on SNR≥10 and number of positive spots in visual inspection ≥6/20, the detection had 100% sensitivity and 100% specificity. The differences compared with other criteria reached significance level ($P<0.05$). For BLAST analysis, the amino acid sequences of 45 positive protein spots related to important tumor markers were selected. The protein array detection in the two groups indicated that the tumor markers in patients of the case group could be effectively detected; the detection results showed significant difference from the control group ($P<0.05$). Conclusion: Tumor markers were accurately detected using HuProt array with the criteria of SNR≥10 and number of positive spots in visual inspection ≥6/20. The high-throughput protein array was clinically valuable in tumor screening.

Keywords: Protein array, tumor markers

Introduction

Cancer is the disease that poses serious threat to human life due to high mortality and brings great burden to the patients and their family [1]. It is the second leading cause of death throughout the world, and cancer death rate only ranks second to cardiovascular diseases [2]. Every year the newly diagnosed cases of cancer reach up to 10 million, of which 60% of the cases are found in developing countries. Early diagnosis and treatment are the key factors for treating cancers. Tumor markers are specific indicators of tumors which can be reliably used to monitor and diagnose tumors [3]. They have already been applied to early diagnosis and prediction of sensitivity to treatment, metastasis, relapse and prognosis of tumors at present [4].

Protein array, or protein microarray, is a high-throughput proteomic technique with wide applications [5]. The microarray is formed by depositing a large number of proteins on a solid surface. The microarray hybridization and scanning are involved for the detection [6]. The specific interaction between the proteins and the antibodies can be utilized to analyze and compare the serum tumor antibodies in cancer patients and healthy controls, thus screening the specific antibodies of tumors [7]. We applied the HuProt array to the screening for tumor markers among tumor patients and healthy controls.

Materials and method

Subjects

One hundred and sixty patients with various types of tumors treated at our hospital from February 2013 to March 2015 were selected (98 males, 62 females, aged 53.2±9.5 years).

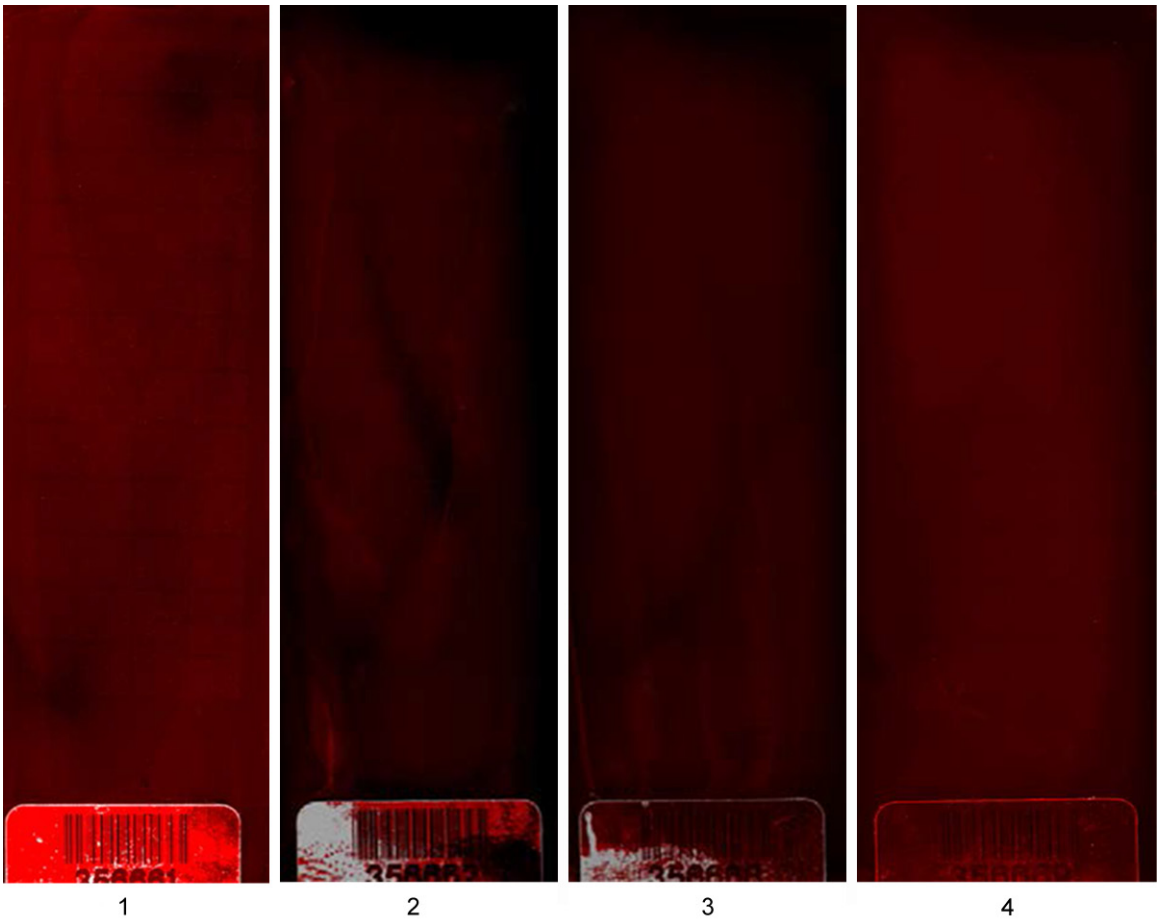

Figure 1. Chip scan results (1-4 represent sample number).

There were 63 cases with gastrointestinal tumors, including 36 cases with liver cancer, 21 cases with colorectal cancer and 6 cases with gastric cancer; 58 cases with respiratory tract tumors, including 39 cases with lung cancer and 19 cases with nasopharyngeal carcinoma; 30 cases with reproductive system tumors, including 28 cases with ovarian cancer and 2 cases with testicular cancer; 9 cases with other types of cancer, including 4 cases with melanoma, 3 cases with aortic aneurysm and 2 cases with mucinous adenocarcinoma. All cases were confirmed by clinicopathology, imaging examination or laboratory tests.

One hundred and sixty healthy subjects receiving physical examination at our hospital during the same period were chosen as controls (102 males, 58 females, aged 50.1±7.2 years). They had no diseases according to clinical examination and no significant differences in age and gender compared with the controls.

Detection method

The drug was disused one day before blood sampling and the patients were instructed to eat only light diet. The fasting blood samples were collected in the morning and the serum was preserved at -20°C. HuProt array was used for the detection. The serum was diluted in 5% BSA as the blocking solution at 1:500-1:5000 to reach the final volume of 3.0 ml. The blocking solution was removed from the 4-well plate, and the serum was added, with the array surface facing upwards. The serum was incubated at room temperature on a shaker for 1 h (60 rpm/min), and the washing was done three times after incubation. The secondary antibodies were diluted in the blocking solution according to the manufacturer's instruction. Into each well 3.0 ml of freshly diluted secondary antibodies were added, covered with aluminum foil and incubated at room temperature on a shaker for 1-1.5 h (60 rpm/min). Washing was done

Table 1. Interpretation results using different SNR thresholds

SNR	≥10	≥15	≥20	≥25	≥30	≥35
Number of real positive spots (cases)	45	45	45	45	45	45
Number of positive spots/real positive spots	486/45	325/45	182/45	121/45	78/45	59/45
Sensitivity	100%	100%	100%	100%	100%	100%
P value			>0.05			

Table 2. Interpretation results using SNR combined with visual inspection

	SNR≥2+ number of positive spots in visual inspection ≥5/20	SNR≥10+ number of positive spots in visual inspection ≥6/20
Number of real positive spots (cases)	45	45
Number of positive spots/real positive spots	112/45	45/45
Specificity	59.80%	100%
χ^2 value	50.31	
P value	P<0.05	

three times after incubation. The absorbent paper was placed at the bottom of the slide box to remove the water from the edge of the array. The array was vertically placed into the slide box and centrifuged at 800 rpm for 3 min. The array was scanned with LuxScan 10 K Microarray Scanner.

Data analysis

The raw images were processed with GenePix Pro software, and Z value was calculated for each spot using the following formula: $\alpha = Foreground_{sample\ channel}-Background_{sample\ channel}$, $z = (\alpha-\alpha_{avg})/\alpha_{std}$. α_{avg} is the average α value of all spots on an array, and α_{std} is the standard deviation of α value of all spots. If the Z values of two replicate spots of a protein were both above 3, this protein was considered positive. The array data interpretation was performed based on SNR and visual inspection. SNR was calculated by dividing the background subtracted signal by the background. Visual inspection (Flags): the greater the value of positive spots by visual inspection, the higher the reliability was.

The amino acid sequences of the selected positive proteins were searched against the databases. The sequencing results were analyzed with the GenBank using BLAST.

Statistical process

Statistical analysis was performed using SPSS 16.0 software. The qualitative variables were compared with chi-square test and quantitative variables by t-test or analysis of variance. P<0.05 indicated statistical significance.

Results

Positive criteria for array data interpretation

The Chip scan results were shown in **Figure 1**. When SNR was used as the sole criterion with the threshold set as SNR≥10, all 45 tumor markers were detected in 160 cancer patients and the sensitivity was 100%. There were no statistically different differences in the number of positive spots using this threshold (P>0.05) (**Table 1**). However, the use of SNR alone would lead to a reduction in specificity. For example, the positive spots on the array totaled 485 using the criterion SNR≥10, and the number was still as high as 59 when SNR≥35. These results all exceeded the allowable range of number of real positive spots.

SNR was combined with visual inspection for array data interpretation, as shown in **Table 2**. The number of positive spots was 112 when SNR≥2 and number of positive spots in visual inspection ≥5/20, which exceeded the allowable range of number of real positive spots. The number of positive spots was 45 when SNR≥10 and number of positive spots in visual inspection ≥6/20, with specificity of 100%. The interpretation results were statistically significant from those using SNR≥2 and number of posi-

Table 3. Database searching and alignment analysis of 45 positive protein spots

No.	Accession No.	Description	Related diseases
1	XP_939608	PREDICTED: hypothetical protein LOC55036 isoform 7 [Homo sapiens]	Ciliary dyskinesia
2	NM_001098413	Homo sapiens G antigen 10 (GAGE10), mRNA	Cancer-testis antigen
3	NM_001040663	Homo sapiens G antigen 1 (GAGE1), transcript variant 2, mRNA	Melanoma
4	NM_001009615	Homo sapiens SPANX family, member N2 (SPANXN2), mRNA	Cancer-testis antigen
5	NM_001009609	Homo sapiens SPANX-N3 protein (SPANX-N3), mRNA	Cancer-testis antigen
6	NM_001008708	Homo sapiens ChaC, cation transport regulator homolog 2 (E. coli) (CHAC2), mRNA	Cancer-testis antigen
7	NM_001002756	Homo sapiens NFU1 iron-sulfur cluster scaffold homolog (S. cerevisiae) (NFU1), nuclear gene encoding mitochondrial protein, transcript variant 3, mRNA	Mitochondrial DNA depletion syndrome
8	NM_183041	Homo sapiens dystrobrevin binding protein 1 (DTNBP1), transcript variant 3, mRNA	Muscular dystrophy
9	NM_182498	Homo sapiens zinc finger protein 428 (ZNF428), mRNA	-
10	NM_153757	Homo sapiens nucleosome assembly protein 1-like 5 (NAP1L5), mRNA	Malignant tumor of liver
11	NM_152298	Homo sapiens nuclear autoantigenic sperm protein (histone-binding) (NASP), transcript variant 3, mRNA	Ovarian cancer
12	NM_152296	Homo sapiens ATPase, Na+/K+ transporting, alpha 3 polypeptide (ATP1A3), mRNA	Medulloblastoma and hepatocellar carcinoma
13	NM_058163	Homo sapiens TSR2, 20S rRNA accumulation, homolog (S. cerevisiae) (TSR2), mRNA	-
14	NM_053031	Homo sapiens myosin light chain kinase (MYLK), transcript variant 7, mRNA	Aortic aneurysm
15	NM_052957	Homo sapiens acidic repeat containing (ACRC), mRNA	Mucinous adenocarcinoma
16	NM_052848	Homo sapiens coiled-coil domain containing 97 (CCDC97), mRNA	-
17	NM_032907	Homo sapiens ubiquitin-like 7 (bone marrow stromal cell-derived) (UBL7), transcript variant 1, mRNA	-
18	NM_031899	Homo sapiens golgi reassembly stacking protein 1, 65 kDa (GORASP1), mRNA	-
19	NM_024948	Homo sapiens family with sequence similarity 188, member A (FAM188A), mRNA	-
20	NM_024793	Homo sapiens clusterin associated protein 1 (CLUAP1), transcript variant 2, mRNA	Osteosarcoma, ovarian cancer, colon cancer, lung cancer
21	NM_019088	Homo sapiens Paf1, RNA polymerase II associated factor, homolog (S. cerevisiae) (PAF1), mRNA	Tumor-related genes
22	NM_018975	Homo sapiens telomeric repeat binding factor 2, interacting protein (TERF2IP), mRNA	Breast cancer
23	NM_016449	Homo sapiens chromosome 22 open reading frame 43 (C22 or f43), mRNA	-
24	NM_015952	Homo sapiens RWD domain containing 1 (RWDD1), transcript variant 1, mRNA	-
25	NM_015874	Homo sapiens recombination signal binding protein for immunoglobulin kappa J region (RBPJ), transcript variant 2, mRNA	-
26	NM_012196	Homo sapiens G antigen 8 (GAGE8), mRNA	Cancer-testis antigen
27	NM_005594	Homo sapiens nascent-polypeptide-associated complex alpha polypeptide (NACA), mRNA	Monocytic leukemia
28	NM_004343	Homo sapiens calreticulin (CALR), mRNA	Systemic lupus erythematosus
29	NM_003946	Homo sapiens nucleolar protein 3 (apoptosis repressor with CARD domain) (NOL3), mRNA	Markers of colon cancer and epicytoma
30	NM_003011	Homo sapiens SET translocation (myeloid leukemia-associated) (SET), mRNA	Myeloid leukemia
31	NM_002824	Homo sapiens parathymosin (PTMS), mRNA	-
32	NM_002823	Homo sapiens prothymosin, alpha (gene sequence 28) (PTMA), mRNA	Gastric cancer
33	NM_002482	Homo sapiens nuclear autoantigenic sperm protein (histone-binding) (NASP), transcript variant 2, mRNA	Ovarian cancer and autoantigen of other diseases
34	NM_002118	Homo sapiens major histocompatibility complex, class II, DM beta (HLA-DMB), mRNA	Ovarian cancer
35	NM_001087	Homo sapiens angio-associated, migratory cell protein (AAMP), mRNA	Breast cancer
36	BC098149	Homo sapiens variable charge, X-linked 3A, mRNA (cDNA clone MGC: 118976 IMAGE: 40002644), complete cds	-
37	BC090928	Homo sapiens SLAM family member 6, mRNA (cDNA clone IMAGE: 3066534), with apparent retained intron	-

38	BC070336	Homo sapiens immunoglobulin kappa constant, mRNA (cDNA clone IMAGE: 30330282)	-
39	BC062732	Homo sapiens immunoglobulin kappa constant, mRNA (cDNA clone IMAGE: 30351013)	-
40	BC036743	Homo sapiens ubiquilin 3, mRNA (cDNA clone MGC: 44847 IMAGE: 5167911), complete cds	-
41	BC032749	Homo sapiens SET nuclear oncogene, mRNA (cDNA clone MGC: 45315 IMAGE: 5587291), complete cds	-
42	BC014122	Homo sapiens angio-associated, migratory cell protein, mRNA (cDNA clone IMAGE: 4552582), containing frame-shift errors	Spinal degeneration
43	BC007200	Homo sapiens acidic (leucine-rich) nuclear phosphoprotein 32 family, member A, mRNA (cDNA clone MGC: 12667 IMAGE: 3677623), complete cds	-
44	BC000267	Homo sapiens GC-rich promoter binding protein 1, mRNA (cDNA clone IMAGE: 3357748), complete cds	-
45	AK314899 (NOL3)	Homo sapiens cDNA, FLJ95803, Homo sapiens nucleolar protein 3 (apoptosis repressor with CARD domain) (NOL3), mRNA	-

Table 4. Comparison of array detection results of case group and control group

	Array detection results	
	Positive	Negative
Case group	160	0
Control group	1	159
χ^2 value	316.02	
P value	P<0.05	

tive spots in visual inspection ≥5/20 (P<0.05). Thus, the criteria were finally selected as SNR≥10 and number of positive spots in visual inspection ≥6/20.

Database searching and sequence analysis

Forty-five positive protein spots obtained by hybridization were further analyzed. The amino acid sequences of these proteins were searched in the database and the alignment was performed against Genbank using BLAST. The sequences with identity ≥99% were obtained, as shown in **Table 3**. Thus these positive proteins were related to important tumor markers: cancer-testis antigen GAGE10, GAGE1 related to antigen of tumors, NASP and RCAS1 related to ovarian cancer, ATP1A3 related to medulloblastoma and liver cancer, MYLK related to aortic aneurysm, ACRC related to mucinous adenocarcinoma, and CLUAP1 related to ovarian cancer, colon cancer and lung cancer.

Comparison between case group and control group

Tumor markers were detected in all 160 patients, but in only 1 control subject. The difference between the two groups was of statistical significance (P<0.05) (**Table 4**).

Discussion

Protein array has the features of high throughput, miniaturization and fastness and it has been applied in clinical detection and monitoring [8]. Huprot array containing over 19000 proteins was used in the present study. This protein array was constructed using tumor-related antigens and specific antibodies of antigens that had been identified, including GAGE10 [9] (cancer-testis antigen), GAGE1 [10, 11] (tumor-related antigen), NASP and RCAS1 [12] (ovarian cancer), ATP1A3 [13] (medulloblastoma and hepatocellar carcinoma, MYLK [14] (aortic aneurysm), ACRC (mucinous adenocarcinoma), CLUAP1 [15] (ovarian cancer, colon cancer, lung cancer) and NOL3 [16, 17] (colorectal cancer). ID of all these tumor markers can be found in Genbank. The protein array can be constructed with tumor antigens synthesized by mRNA or amino acid sequences from the database. Thus the protein array can rapidly screen the tumor markers with high throughput.

Although tumor markers can be detected in different samples, serum is the most ideal with easy availability and convenience of detection [18]. By immobilization of thousands of antigens on the array, a global, fast, high-throughput sequencing can be done without bias. The healthy subjects and the cancer patients can be easily differentiated using a very small amount of sample [19, 20], as shown by the present study. The protein array technique has high application value in the screening for tumor markers.

The protein array detection is based on fluorescence with high sensitivity, and therefore, the choice of positive criteria is highly important [21, 22]. In this study, the positive criteria were SNR combined with visual inspection. It was confirmed by the array data interpretation that the combined use of SNR and visual inspection could achieve high specificity, sensitivity and accuracy. However, different positive criteria should be formulated for different protein array based on experimental data in clinical applications.

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Disclosure of conflict of interest

None.

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