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J. Proteome Res., **2008**, 7 (2), 611-620 • DOI: 10.1021/pr070525r • Publication Date (Web): 28 December 2007

research articles Journal of proteome research

Identification of Hepatocellular-Carcinoma-Associated Antigens and Autoantibodies by Serological Proteome Analysis Combined with Protein Microarray

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Received August 11, 2007; Accepted October 31, 2007

To comprehensively study autoantibodies in patients with hepatocellular carcinoma (HCC), we used an approach-based serology and proteomics technologies. Total proteins extracted from HepG2 cells and HepG2.2.15 cells were separated by two-dimensional gel electrophoresis (2DE) and then transferred onto polyvinylidene difluoride (PVDF) membranes, which were subsequently incubated with sera from HCC patients or from normal controls. As a result, 13 HCC-associated antigens were identified. Antigenicity of eight proteins was further confirmed using recombinant proteins by Western blotting (WB) and protein microarray. The results of antigen microarray analysis showed strong signals of keratin 8 and lamin A/C in chronic hepatitis controls; therefore, the autoantibodies to keratin 8 and lamin A/C may not be HCC-specific. These two antigens were removed from subsequent analyses. The frequencies of positive reactions to DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, eukaryotic translation elongation factor 2 (eEF2), apoptosis-inducing factor (AIF), heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2), prostatic binding protein, and triose-phosphate isomerase (TIM) were significantly higher in HCC than in chronic hepatitis and normal individuals. Positive reactions to DEAD box polypeptide 3, eEF2, AIF, and prostatic binding protein were significantly more frequent in HCC than in any other cancer. The sensitivity of any individual antigen in HCC at stage I ranged from 50 to 85%. When the combinations of six antigens were analyzed, the sensitivity increased to 90%. We conclude that the detection of autoantibodies against the six antigens may have value on early diagnosis of HCC.

Keywords: Autoantibody • autoimmunity • hepatocellular carcinoma • serological proteome analysis

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent and lethal malignancies worldwide, and the 5 year survival rate after hepatectomy is 30–50%.[1] Given the high incidence and mortality of HCC, it is important to develop biomarkers for assessing onset or prediction of therapy outcome as well as to identify targets for the development of novel therapies. Although there are multiple promising diagnostic biomarkers of HCC in the development phase, there are at the present time no definitive antibody-based serologic markers for its early diagnosis in clinic.[2,3] Several experimental studies have led to the identification of autoantigens via recognition by autoantibodies present in HCC sera.[4] Antigens that have been shown to induce a humoral response in HCC include p53[5] and diverse other nuclear proteins.[6] Autoantibodies to cyclin B1[7] and to a novel cytoplasmic protein with RNA-binding motifs[8] have also been reported. However, efforts to consistently predict HCC based on autoimmunity to antigens have not resulted in serologic markers with definitive specificity and sensitivity.

Serological proteome analysis (SERPA) is a powerful tool for the identification of a large group of candidate cancer biomarkers recognized as autoantigens by the sera of cancer patients.[9] This approach permits the transfer and immobilization of proteins to a semirigid support, allowing for the subsequent immunodetection of relevant antigens among thousands of individual proteins separated by two-dimensional gel electrophoresis (2DE). To survey autoantibodies associated with HCC, we used this SERPA approach and identified 13 proteins that induced a humoral response in HCC patients but not in healthy individuals and subsequently investigated the prevalence of the autoantibodies in a large number of HCC patients and negative controls.

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10.1021/pr070525r CCC: $40.75

Published on Web 12/28/2007

Table 1. Clinical Characteristics of the Research Subjects

subjects	*n*	age (mean)	sex (male/female)	serum AFP ≤20/>20 (ng/mL)	$HBV^+/HCV^+/HBV^-HCV^-$	clincal stage I/II/III/IV[a]
HCC patients						
used for SERPA	28	40–77 (62.5)	18/10	7/21	18/0/10	6/9/7/6
used for WB and microarray	146	32–78 (58.2)	97/49	44/102	91/30/25	25/47/49/25
total	174	32–78 (58.9)	115/59	51/123	109/30/35	31/56/56/31
chronic hepatitis	63	30–73 (61.2)	39/24	26/37	43/20/0	
other cancers	66	32–75 (59.7)	38/28	60/6		12/21/20/13
normal controls	71	28–75 (56.6)	47/24	71/0		

[a] Assessment based on TNM classification by the International Union Against Cancer.

Materials and Methods

Serum Samples and Cell Lines. Serum samples for SERPA were obtained from 28 HCC patients during routine blood tests prior to any treatment, consisting of 18 hepatitis B virus (HBV)-related HCC patients and 10 virus-negative HCC patients. Sera from 18 healthy volunteers were used as controls. Healthy volunteers without a history of cancer or autoimmune disease were asymptomatic and had normal physical exams and normal routine blood tests of liver function.

Serum samples for blind validation by Western blotting (WB)/microarray were collected from 146 HCC patients at the time of initial cancer diagnose. Among 146 HCC patients, 91 patients were positive for HBV, 30 patients were positive for hepatitis C virus (HCV), and 25 patients were negative for hepatitis virus. Sera from 63 patients chronically infected with HBV or HCV without HCC, from 66 patients with other cancers, including 17 with lung cancer, 16 with esophageal cancer, 15 with breast cancer, and 18 with gastric cancer, and from 53 healthy volunteers were used as controls. Aliquots of sera were immediately frozen at −80 °C until used and were never refrozen. Clinical characteristics of the research subjects are shown in Table 1.

Patients were only included in the study if they had provided written consent to participate in the study after receiving oral and written information regarding its course and purpose. Approval for the study was received from the Ethics Committee of the host institution.

The human hepatoma cell lines HepG2 were cultured in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA), 100 units/mL penicillin, and 100 units/mL streptomycin. HepG2.2.15 cells were kindly provided by Beijing Medical University, who received them from Mount Sinai Medical Center in New York. The HepG2.2.15 cells were cultured in minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 380 μg/mL antibiotic G-418 sulfate (Promega, Madison, WI), 2 mM L-glutamine (Sigma, St. Louis, MO), and 200 units/L amikaxin sulfate (QiLu medicine company, Shan-Dong, China).

Two-Dimensional Gel Electrophoresis (2DE). All reagents for 2DE were from Amersham Biosciences, except mentioned specially. Cultured cells were solubilized in lysis buffer containing 40 mM Tris, 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate (CHAPS), 40 mM dithiothreitol (DTT), 2% immobilized pH gradient (IPG) buffer (pH 3–10), protease inhibitor mix, and nuclease mix. The protein concentration was measured with Bradford's method. The IPG strips (pH 3–10, 7 cm longer) were rehydrated overnight in 7 M urea, 2 M thiourea, 2% CHAPS, 18 mM DTT, 0.5% IPG buffer, and trace bromophenol blue. The sample (120 μg) was applied to gels by rehydration loading. Isoelectric focusing (IEF) was initiated at 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, and then gradually increased to 5000 V for 5–6 h. Focus was carried out for 20 000 V h. After IEF, IPG strips were equilibrated for 2 × 15 min. Equilibration buffers contained 75 mM Tris-HCl (pH 8.8), 6 M urea, 29.3% glycerol, 2% sodium dodecyl sulfate (SDS), and trace bromophenol blue, with 1% DTT for the first step and 2.5% iodoacetamide for the second step. For the second-dimension electrophoresis, 12.5% SDS−polyacrylamide gels were used. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Schleicher and Schuell Biosciences, Keene, NH) or visualized by Colloidal Coomassie staining of the gels.

Two-Dimensional Immunoblot Analysis. After transfer, membranes were incubated in Tris-buffered saline (TBS) containing 5% nonfat dry milk and 0.1% Tween 20 for 3 h at room temperature. The membranes were incubated overnight at 4 °C with a mixture of serum samples diluted at 1:100 with blocking buffer. After washing 6 times for 15 min each in TBS with 0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated mouse anti-human IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) for 1 h at room temperature. Immunodetection was performed with enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by autoradiography on Kodak film. Two-dimensional electrophoresis gels and immunobloting maps were scanned using ImageScanner II (Amersham Biosciences, Buckinghamshire, U.K.) and analyzed by ImageMaster 2D Platinum 5.00 software (Amersham Biosciences Buckinghamshire, U.K.). The proteomic profile of proteins from the hepatoma cell line was used as a reference map for spot analysis. Spots on immunoblotting maps were matched to the reference map, and those observed from HCC sera but not from normal sera were excised for protein identification.

In-Gel Digestion and Mass Spectrometry (MS) Identification. In-gel digestion of proteins from 2D gels was performed as described by Steiner et al.[10] Spots were excised and destained with 25 mM ammonium bicarbonate/50% acetonitrile and dried in a vacuum concentrator (Savant, Holbook, NY). The dried gel pieces were rehydrated with 5 μL of 10 mg/L trypsin (Roche Molecular Biochemicals, Mannheim, Germany) in 25 mM ammonium bicarbonate and digested at 37 °C for 18–20 h. Tryptic peptides were first extracted using 5% trifluoroacetic acid (TFA) at 40 °C for 1 h and then 2.5% TFA/50% acetonitrile at 30 °C for 1 h. The extracted solutions were mixed in an Eppendorf tube and dried in a vacuum concentrator. The peptides mixture was solubilized with 0.5% TFA for MS analysis. MS was performed on a matrix-assisted laser desorption/

ionization−time-of-flight (MALDI−TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany), with saturated α-cyano-4-hydroxycinnamic acid (CHCA) solution in 0.1% TFA/50% acetonitrile as the matrix. Mass spectra were externally calibrated with autodigest peaks of trypsin (MH^+: 906.505, 1020.504, 1153.574, 2163.057, and 2273.160 Da). Database searching (NCBI or Swiss-Prot) was performed using the Internet-available program Mascot (Matrix Science, London, U.K.). Search parameters were set up as follows: the taxonomy was selected as *Homo sapiens*; the mass tolerance was 0.3 Da; the missed cleavage sites were allowed up to 1; and the variable modifications were selected as carbamidomethyl (cysteine) and oxidation (methionine). The criteria for protein identifications included the extent of sequence coverage, the number of peptides matched, and the probability-based Mowse score, and also, the molecular weight and isoelectric point of identified proteins should match the estimated values obtained from image analysis.[11]

Preparation of Recombinant Proteins. According to the reported cDNA sequence of the protein identified, we prepared corresponding oligonucleotide primers to amplify DNA fragments by reverse transcription and polymerase chain reaction. The nucleotide sequences of the primers included the restriction enzyme recognition sequence for subcloning. The resulting cDNA fragments were subcloned separately into the plasmid expression vector pGEX-4T-1, pET-28a(+), and pET-43.1a(+). Using these constructs, recombinant proteins were produced in *Escherichia coli* BL21 (DE3) as fusion proteins with corresponding tags. After induction with isopropyl-β-D-thiogalactopyranoside, the recombinant proteins were separately purified on Glutathione Sepharose columns (Amersham Biosciences, Buckinghamshire, U.K.) or Ni−NTA columns (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Next, serum samples either from HCC patients or from normal controls were used for WB with recombinant proteins and the corresponding tags as negative controls.

Probing and Scanning of Antigen Microarrays. Antigens were diluted to 0.5 μg/μL in phosphate-buffered saline (PBS) with 0.02% SDS and 1% glycerol and robotically attached in ordered arrays on aldehyde-activated glass slides, using a computer-controlled microchip spotting instrument (Cartisan Pixsys 3000). Samples were transferred from 384 microtiter plates to glass slides using stainless-steel microspotting pins (400 μm in diameter). Each pin was estimated to transfer ~2 nL of sample to the slide. The size of the glass slide was 25.4 × 76.2 mm, including 10 matrixes (7.5 × 7.5 mm). Each antigen was printed in two replicates within a matrix. Human IgG (Sigma, St. Louis, MO) was used as a positive control, and purified glutathione-*S*-transferase (GST) protein and Nus protein were used as negative controls in microarrays. Protein microarrays were blocked in PBS containing 25% FCS and 0.05% Tween 20 for 3 h at 37 °C and probed with 1:10 dilutions of HCC serum or negative control serum for 30 min, followed by washing 5 times for 10 s each in PBS with 0.1% Tween 20 and incubation with a 1:400 dilution of Cy3-conjugated mouse antihuman IgG secondary antibody (Sigma, St. Louis, MO). Arrays were scanned using the GenePix 4000 scanner (Axon, Foster City, CA), and the signal intensities of the spots and background values were determined using GenePix Pro version 5.1 (Axon, Foster City, CA).

The technical steps of the WB/microassay in the blinded validation phase were performed by individuals who did not know the diagnoses of patients and who had not been directly involved in the initial SERPA.

Statistical Analysis. χ^2 tests were used to determine the difference of positive reactions to HCC-associated antigens between different classes and the correlation between antigens and the clinicopathological parameters. A value of $p < 0.05$ was considered statistically significant.

Results

Proteomic Analysis of Autoantigens in Patients with HCC. We separated total proteins extracted from the two cell lines (HepG2 and HepG2.2.15) by 2DE and transferred them onto PVDF membranes. Initially, mixed serum samples from eight HBV-related HCC patients were incubated with HepG2 proteins. Normal sera were used as controls. Then, mixed serum samples from 10 HBV-related HCC patients and 10 virus-negative HCC patients were separately reacted with HepG2.2.15 proteins. We detected six antigenic protein spots of HepG2 cells that reacted to HCC sera but did not reacted to normal controls. In the case of HepG2.2.15, we detected 10 antigenic protein spots that reacted to HCC sera but not to normal controls. Among the 10 proteins, 5 reacted to sera from all HCC patients with or without HBV infection, 2 reacted only to sera without HBV infection, and 3 reacted only to sera with HBV infection, reflecting a difference between the prevalence of autoantibodies in HBV-related HCC and virus-negative HCC.

Identification of the Autoantigens. We tried to identify the proteins detected by SERPA. The proteins of interest were excised from stained Colloidal Coomassie Brilliant G-250 gels and submitted to digest with trypsin. The peptide mixtures were analyzed by MALDI−TOF MS. The resulting peptide mass maps were used for protein database searches using Mascot software. Six protein spots from the HepG2 cell were identified as four distinct proteins, including keratin 8, lamin A/C, DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, and eukaryotic translation elongation factor 2 (eEF2). Of the 10 protein spots from the HepG2.2.15 cell, 2 reactive proteins with autoantibodies in virus-negative HCC sera were identified as triosephosphate isomerase (TIM) and prostatic binding protein, 3 reactive proteins with autoantibodies in HBV-related HCC sera were identified as pyruvate kinase, phosphoglycerate kinase 1, and phosphoglycerate mutase isozyme B, and the rest, which reacted to both of the HCC sera, were identified as programmed cell death 8 (apoptosis-inducing factor, AIF), heterogeneous nuclear ribonucleoprotein (hnRNP) A2, cyclophilin A, and aspartate aminotransferase 1. Locations of the identified protein spots on 2D gels are shown in Figures 1 and 2. The results of identification are summarized in Table 2.

Analysis of the Prevalence of Autoantibodies against the Identified Antigens by WB. Antigenicity of the identified protein was confirmed by WB. Four identified antigens from the HepG2 cell and three from the HepG2.2.15 cell were randomly selected for the expression of recombinant proteins. The protein TIM was purchased from Sigma-Aldrich Company. Keratin 8 cDNA was subcloned into pGEX-4T-1 vector, producing a fusion protein with a GST tag. cDNA from lamin A/C, eEF2, and prostatic binding protein was subcloned into pET28a(+) vector, respectively, and recombinant proteins were expressed as fusion proteins with His tags. cDNA from DEAD box polypeptide 3, hnRNP A2, and AIF was separately subcloned into pET43.1a(+) vector for protein expression as fusion proteins with Nus and His tags. The purified fusion proteins were separated on 10% SDS−PAGE and stained with Coomassie

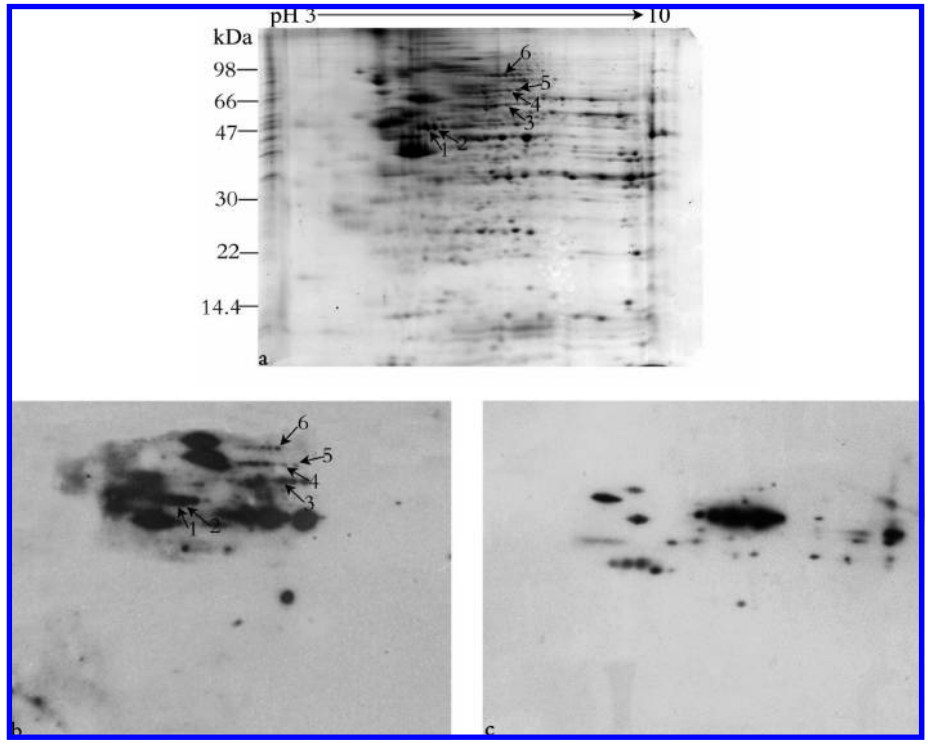

Figure 1. Two-dimensional electrophoresis map of proteins from the HepG2 cell line and the corresponding immunoblotting maps. The proteins from the hepatoma cell line HepG2 were separated by IEF (pH 3–10) and then 12.5% SDS−PAGE and subsequently stained with Colloidal Coomassie Brilliant Blue (a) or transferred on PVDF membranes for WB experiments using mixed sera from eight patients with HCC (b) or from eight healthy subjects (c) as a first antibody. The position of the six identified reactive protein spots are labeled on the 2D patterns.

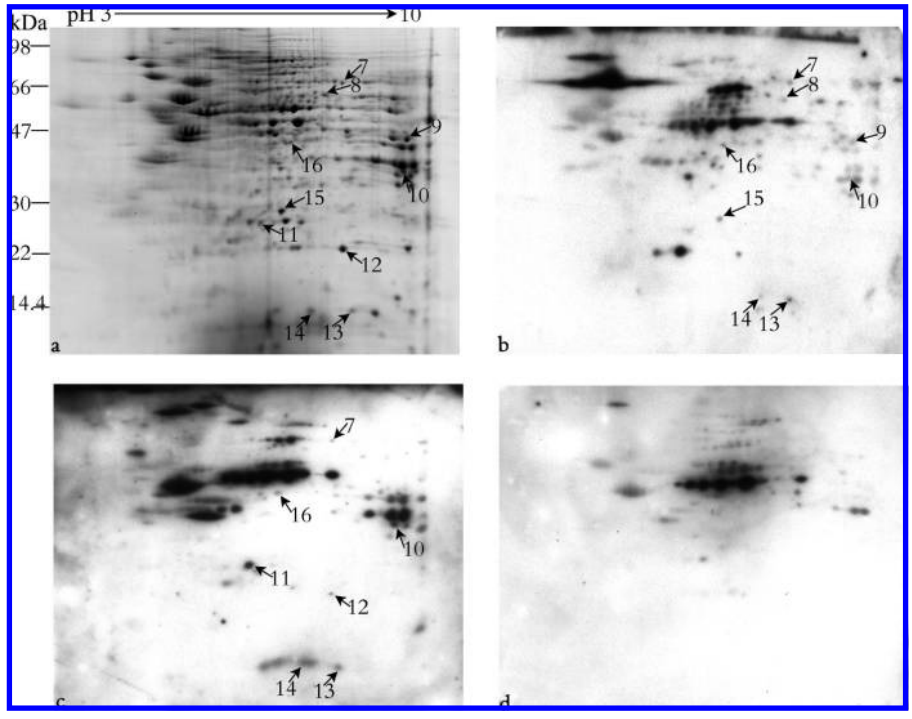

Figure 2. Two-dimensional electrophoresis map of proteins from the HepG2.2.15 cell line and the corresponding immunoblotting maps. The proteins from the hepatoma cell line HepG2.2.15 were separated by IEF (pH 3–10) and then 12.5% SDS−PAGE and subsequently stained with Colloidal Coomassie Brilliant Blue (a) or transferred on PVDF membranes for WB experiments using mixed sera from 10 HBV-related HCC patients (b), from 10 virus-negative HCC patients (c), or from 10 healthy subjects (d) as a first antibody. The position of the 10 identified reactive protein spots are labeled on the 2D patterns.

Blue. The purity of the recombinant proteins was over 90%, which was evaluated with the software Bandscan.

Using the eight proteins as antigens, sera from 28 HCC patients and 10 normal controls were screened individually by

Table 2. Proteins Identified by Mass Spectrometry

spot number	protein	NCBI accession number	theoretical pI/Mr	sequence coverage (%)	score	peptides matched[a]
1	keratin 8	gi|62913980	4.94/41 083	43	115	15/29
2	keratin 8	gi|62913980	4.94/41 083	34	96	12/21
3	lamin A/C	gi|13111979	6.40/65 096	22	71	11/24
4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3	gi|15080078	6.73/73 198	15	68	10/20
5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3	gi|15080078	6.73/73 198	28	129	14/21
6	eukaryotic translation elongation factor 2	gi|4503483	6.41/95 277	17	77	13/23
7	programmed cell death 8	gi|4757732	9.04/66 859	23	83	9/14
8	chain A, structure of human muscle pyruvate kinase	gi|67464392	8.22/59 707	24	70	10/25
9	phosphoglycerate kinase 1	gi|4505763	8.30/44 586	37	87	11/28
10	heterogeneous nuclear ribonucleoprotein A2	gi|4504447	8.67/35 984	37	68	9/29
11	chain A, triosephosphate isomerase (TIM)	gi|999892	6.51/26 522	77	212	20/48
12	prostatic binding protein	gi|4505621	7.01/21 044	79	119	14/45
13	cyclophilin A	gi|1633054	7.82/17 870	63	84	10/33
14	cyclophilin A	PPIA_HUMAN[b]	7.82/17 870	46	57	8/31
15	predicted: phosphoglycerate mutase 1	gi|89035672	6.67/28 832	63	177	18/46
16	aspartate aminotransferase 1	gi|4504067	6.52/46 219	47	144	14/27

[a] Represents the peptides matched among the peptide list searched. [b] Swiss-Prot ID in the Swiss-Prot database.

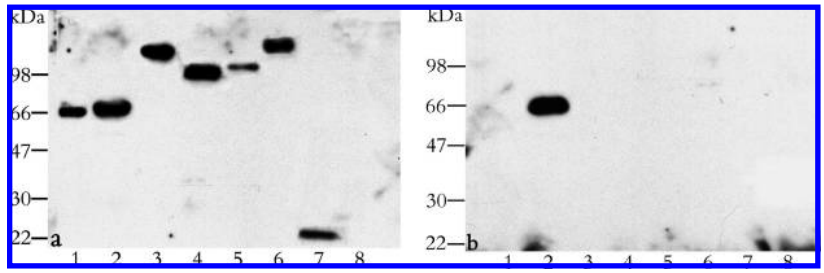

Figure 3. WB analysis of recombinant proteins. Recombinant proteins were reacted with serum from a patient with HCC (a) or from a normal control (b). Lane 1, keratin 8 with a GST tag; lane 2, lamin A/C with a His tag; lane 3, DEAD box polypeptide 3 with His and Nus tags; lane 4, eEF2 with a His tag; lane 5, hnRNP A2 with His and Nus tags; lane 6, AIF with His and Nus tags; lane 7, prostatic binding protein with a His tag; lane 8, TIM.

Table 3. Occurrence of Autoantibodies to Recombinant Proteins among HCC Patients and Negative Controls by WB Analysis

subjects	keratin 8	lamin A/C	DEAD box	eEF2	AIF	hnRNP A2	prostatic binding protein	TIM
HCC patients								
HBV^+ ($n = 14$)	5	11	11	5	7	6	4	1
HCV^+ ($n = 8$)	6	5	4	3	3	3	3	3
HBV^-/HCV^- ($n = 6$)	4	4	3	3	2	4	6	2
total ($n = 28$)	15	20	18	11	12	13	13	6
normal controls ($n = 10$)	1	2	0	0	0	0	0	0

WB analysis. Images of representative blottings are presented in Figure 3. The results are shown in Table 3. The frequency of autoantibodies to individual proteins ranged from 21.4 to 71.4% in HCC sera but from 0 to 20% in normal sera.

Analysis of the Prevalence of Autoantibodies against the Identified Antigens by Microarray. The antigen arrays contained seven recombinant proteins and TIM purchased from Sigma. These candidate antigens were robotically attached in ordered arrays to the aldehyde surface of slides, upon which the binding of serum autoantibodies was detected. Sera from 118 patients with HCC, 63 patients with CHB or CHC, 66 patients with other cancers, and 43 healthy individuals were analyzed by protein microarrays. Digital images of representative arrays are presented in parts b−e of Figure 4. After the removal of the local background, the mean signal intensity of 43 normal sera against each protein respectively plus 2 standard deviation was used to determine whether the target was considered to be positive or not. Two antigens, keratin 8 and lamin A/C, showed strong signals in chronic hepatitis with a high frequency, presumably because of the cross-reacting with the antibodies in both HCC sera and chronic hepatitis sera. These two antigens were removed from subsequent analyses.

The frequency of positive reactions to each antigen in HCC was significantly higher than that in normal controls ($p < 0.001$). When the frequency of autoantibodies was compared between HCC and chronic hepatitis, it was observed that HCC patients had a higher percentage of positive reactions to DEAD box polypeptide 3, eEF2, AIF, and TIM at $p < 0.001$ and to hnRNP A2 and prostatic binding protein at $p < 0.05$. For α-fetoprotein (AFP), no significant difference was found between HCC and chronic hepatitis (Table 4).

To determine whether the autoantibodies could distinguish HCC with other cancers, the prevalence of autoantibodies to the six antigens in sera from other cancers was further studied. For DEAD box polypeptide 3, AIF, and prostatic binding protein, antibody frequency in any other individual cancer was significantly lower than that in HCC ($p < 0.05$), ranging from 17.6 to 35.3%. In breast cancer, it was observed that high

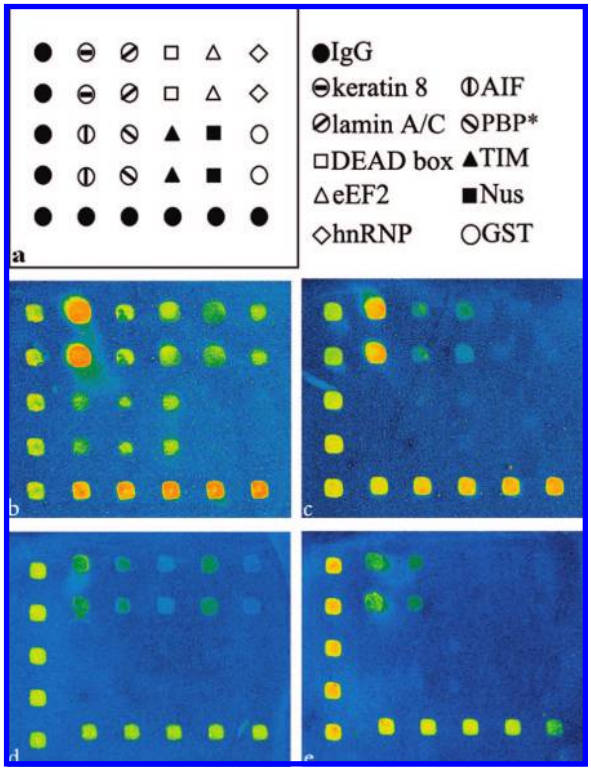

Figure 4. Scan images of antigen arrays. Schematic representation of protein microarrays (a). Individual arrays were incubated with HCC serum (b), chronic hepatitis serum (c), other cancers serum (d), or normal serum (e) at 532 nm detection wavelength. (∗) PBP, prostatic binding protein.

positive reactions to eEF2 and hnRNP A2 were 73.3 and 53.3%, respectively. In lung cancer, high positive reactions to hnRNP A2 and TIM were both 64.7%. Differences in the reactions of different cancers were observed for each of the six antigens (Table 4).

Using an AFP level of 20 ng/mL as a cutoff value for diagnosing HCC, the sensitivity and specificity were 72.9 and 75%, respectively. For the identified autoantibodies, the sensitivity of DEAD box polypeptide 3, eEF2, AIF, hnRNP A2, prostatic binding protein, and TIM was 85.6, 78.8, 55.9, 64.4, 48.3, and 64.4%, respectively, and the specificity versus all non-HCC subjects ranged from 69.8 to 82.6%. In the case of HCC at TNM stage I, the sensitivity for AFP was only 50% ($^{10}/_{20}$) and the sensitivity for DEAD box polypeptide 3, eEF2, AIF, hnRNP A2, prostatic binding protein, and TIM was 85% ($^{17}/_{20}$), 75% ($^{15}/_{20}$), 60% ($^{12}/_{20}$), 70% ($^{14}/_{20}$), 50% ($^{10}/_{20}$), and 60% ($^{12}/_{20}$), respectively (Table 5). In particular, we observed that 8 of 20 stage I HCC patients with autoantibodies against any of the six antigens had a normal range of serum AFP levels. With the addition of antigens to a total of six, it appeared that the sensitivity in diagnosing stage I HCC increased from 50 to 85% when one antigen was used and to 90% when six antigens were used.

Table 6 demonstrates the correlation between the frequencies of autoantibodies and clinicopathological parameters in HCC. There was no statistical difference among each autoantibody expression and sex, histological grade, or TNM classification. Tumors (>5 cm) were more frequently anti-eEF2 positive than small tumors (≤5 cm) ($p < 0.05$). The positive rates of AIF and hnRNP A2 in HCC without regional lymph node metastasis were significantly higher than those with regional lymph node metastasis ($p < 0.05$).

Discussion

Recent studies demonstrate that cancer sera contain antibodies that react with a group of autologous cellular antigens called tumor-associated antigens.[12,13] We have used the proteomic approach to identify a diverse set of tumor-associated antigens that might induce a humoral response in patients with HCC. We used protein extracted from the HepG2 cell line and HepG2.2.15 cell line as autoantigen sources because they would contain both hepatocyte-specific and ubiquitous proteins. A stably HBV-transfected HepG2.2.15 cell line was derived from hepatoblastoma HepG2 cells and has been a useful model for evaluation of HBV-related HCC. In some of these patients, transition from chronic liver disease to HCC was associated with the appearance of novel autoantibodies.[14]

In the present study, 13 proteins were identified from the 2DE. The first identified antigen was keratin 8. Keratin 8 is major component of the intermediate filaments of simple or single-layered epithelia, as found in the liver, gastrointestinal tract, and exocrine pancreas, from which many carcinomas arise.[15] Circulating antikeratin 8 antibody immune complexes were reported in sera of patients with pulmonary fibrosis[16] and autoimmune hepatitis.[17]

The second identified autoantigen was lamin A/C. Lamin A/C is a type-V intermediate filament protein that forms the nuclear lamina, a filamentous network underlying the inner nuclear membrane of eukaryotic cells. Although the autoantibodies to lamin A/C have been detected in autoimmune cytopenias[18] and uveal melanoma,[19] antibodies specific for lamin A/C have been rarely observed in sera from patients with HCC. This production of antilamin A/C antibodies might be under the idiotypic network regulation. It is possible that polyclonal activation of B lymphocytes and mutation of light chains of antibodies were correlated highly with the presence of antilamin A/C antibodies.[18]

The third identified protein was DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, which belongs to RNA helicase superfamilies. Consistent with their central roles in nucleic acid metabolic processes, an increasing number of helicases were found to be involved in severe diseases, often associated with significant cancer susceptibility. Autoantibodies to DEAD box protein have been reported in diabetes mellitus,[20] pancreatic cancer,[21] and connective tissue disease.[22] Our study demonstrated that autoantibodies to DEAD box polypeptide 3 were present in sera of patients with HCC.

eEF2 is an essential factor for protein synthesis. It promotes the GTP-dependent translocation of the nascent protein chain from the A site to the P site of the ribosome. Anti-eEF2 autoantibodies were found previously in sera of patients with breast cancer.[23] However, the significance of the anti-eEF2 autoantibodies in HCC was not described previously. Further studies are needed to elucidate this aspect.

hnRNP A2/B1 proteins are one of the most abundant and important nuclear RNA-binding proteins involved in pre-RNA processing. It is known that patients with autoimmune diseases frequently have blood autoantibody against hnRNP A2/B1.[24,25] Upregulation of hnRNP A2/B1 is thought to play an important role in early stages of carcinogenesis. Moreover, we have found anti-hnRNP A2 in the sera of HCC patients. There has been little understanding as to why hnRNP A2 becomes immunogenic. Recent studies using hnRNP A2 recombinant fragments

Table 4. Occurrence of Autoantibodies to Recombinant Proteins among HCC Patients and Negative Controls by Antigen Array Analysis

subjects	DEAD box	eEF2	AIF	hnRNP A2	prostatic binding protein	TIM	AFP
HCC patients							
HBV^+ ($n = 77$)	69	64	42	51	30	41	62
HCV^+ ($n = 22$)	17	15	12	11	12	17	13
HBV^-/HCV^- ($n = 19$)	15	14	12	14	15	18	11
total ($n = 118$)	101	93	66	76	57	76	86
hepatitis controls							
HBV^+ ($n = 43$)	22	10	10	17	13	11	28
HCV^+ ($n = 20$)	6	4	4	6	4	5	9
total ($n = 63$)	28	14	14	23	17	16	37
other cancers							
lung ($n = 17$)	6	4	4	11	3	11	1
esophageal ($n = 16$)	5	3	4	3	3	5	1
breast ($n = 15$)	5	11	3	8	3	5	1
gastric ($n = 18$)	6	4	4	4	4	6	3
normal controls ($n = 43$)	2	1	3	1	0	0	0
p(HCC versus hepatitis)	<0.001	<0.001	<0.001	0.001	0.007	<0.001	>0.05
p(HCC versus heathy)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 5. Sensitivity and Specificity of Autoantibodies by Antigen Array Analysis

characteristics	DEAD box	eEF2	AIF	hnRNP A2	prostatic binding protein	TIM	AFP
all HCC (TNM I, II, III, and IV)							
sensitivity (%)	85.6	78.8	55.9	64.4	48.3	64.4	72.9
specificity (%)	69.8	78.5	81.4	70.9	82.6	75	75
early stage HCC (TNM I)							
sensitivity (%)	85	75	60	70	50	60	50
specificity (%)	69.8	78.5	81.4	70.9	82.6	75	75

Table 6. Correlation between the Occurrence of Autoantibodies and Clinicopathological Parameters in HCC

parameters	DEAD box	eEF2	AIF	hnRNP A2	prostatic binding protein	TIM
sex						
male ($n = 78$)	65	59	43	49	37	50
female ($n = 40$)	36	34	23	27	20	26
size						
≤5 cm ($n = 51$)	42	35[a]	30	35	27	31
>5 cm ($n = 67$)	59	58	36	41	30	45
histology						
well and moderately differentiated ($n = 56$)	48	42	33	36	30	35
poorly differentiated ($n = 62$)	53	51	33	40	27	41
regional lymph nodes metastasis						
absence ($n = 71$)	60	52	46[b]	52[b]	39	44
presence ($n = 47$)	41	41	20	24	18	32
distant metastasis						
absence ($n = 100$)	85	77	58	67	50	63
presence ($n = 18$)	16	16	8	9	7	13
TNM classification						
I ($n = 20$)	17	15	12	14	10	12
II ($n = 38$)	33	30	23	26	21	25
III ($n = 40$)	35	32	21	24	18	26
IV ($n = 20$)	16	16	10	12	8	13

[a] $p < 0.05$, patients with tumor < 5 cm versus patients with tumor > 5 cm. [b] $p < 0.05$, patients without regional lymph node metastasis versus patients with regional lymph node metastasis.

have revealed that antibodies of patients preferentially reacted with domains located in RNA-binding domains.[26] Further study is required for the specific reactivities of the autoantibodies to hnRNP A2 in HCC.

AIF is a bifunctional NADH oxidase involved in mitochondrial respiration and caspase-independent apoptosis. Recently, it has been demonstrated that AIF plays a potential role in tumor formation/suppression.[27] In another paper, AIF determined the chemoresistance of nonsmall-cell lung carcinomas.[28] However, autoantibodies against AIF have not been reported as yet. Our results showed that autoantibodies against AIF were observed in sera of HCC, indicating that AIF might be associated with hepatic carcinogenesis.

A novel protein designated as Raf-1 kinase inhibitor protein (RKIP) is the major peptide fragment of prostatic binding protein. RKIP has been shown to disrupt the Ras/Raf/MEK/

ERK signaling cascade, thereby abrogating the survival and antiapoptotic properties of these signaling pathways. Schuierer et al.[29] detected that expression of RKIP mRNA and protein was downregulated in HCC cell lines and tissue as compared to primary human hepatocytes or nontumorous liver tissue, respectively. Overexpression of RKIP sensitized tumor cells to chemotherapeutic drug-induced apoptosis.[30] In our study, the presence of autoantibodies directed against prostatic binding protein was observed in sera from patients with HCC.

Cyclophilin A, also known as rotamase A or peptidyl-prolyl *cis–trans* isomerase A, is important for peptidyl-prolyl *cis–trans* isomerizations, protein folding/repair, maintaining mitochondrial functions, apoptosis, regulation of T-cell function and inflammation, vascular disease, HIV infection, and tumor biology.[31] Autoantibodies to cyclophilin A have been described in rheumatoid arthritis, systemic lupus erythematosus,[32] and uveal melanoma,[19] but it was not known to be associated with HCC before.

The other five identified proteins are all members of metabolic enzymes, consisting of TIM, pyruvate kinase, phosphoglycerate kinase 1, phosphoglycerate mutase 1, and aspartate aminotransferase 1. Some of the five antigenic proteins were associated previously with autoantibodies in various conditions. Kuramitsu et al.[33] reported that the antibody against TIM was found in the sera from HCC patients with HCV infection. The process by which TIM becomes immunogenic has not been understood yet. Previous studies suggested that a C to T mutation resulting in a Thr to Ile conversion in TIM might create a new epitope to increase immune response.[34] Furthermore, Yang et al.[12] found that overexpression and secretion of TIM into patient serum might account for one of the mechanisms of developing autoantibodies in cancer. Autoantibodies against phosphoglycerate kinase 1 have been observed in sera from patients with systemic lupus erythematosus and lupus nephritis. The presence of autoantibodies against phosphoglycerate mutase 1 has been reported in sera of patients with autoimmune encephalomyelitis[35] and systemic sclerosis,[36] as well as in HCC patients with HCV infection.[33]

The reasons that intracellular antigens result in the elicitation of autoimmune responses have not been completely elucidated. It has been postulated that necrosis of tumor tissue could be an important factor leading to the shedding of large quantities of cellular autoantigens.[37] Also a role of microorganisms in the induction of autoimmunity has been suggested because autoantibodies were detected at increased frequency during some infections. HBV−DNA integration could alter the transcription of host genes modifying the structure and function of encoding proteins, resulting in autoimmune reactions.[37] Other factors that influence the immunogenicity of autoantigens may include a wide variety of overexpressed or mutated proteins involved in repeated cycles of necrosis and regeneration during liver transformation or in tumor growth. In our study, the prevalence of autoantibodies in sera of HCC patients with hepatitis virus infection was different from that without hepatitis virus infection, suggesting a possible interaction between virus-induced carcinogenesis and humoral autoimmune response in HCC.

The pathophysiological role of autoantibodies is unclear. Whether they are protective or deleterious is due to a combination of their interaction with other immune or inflammatory factors.[38] A total of 13 HCC-associated antigens identified have profound implications beyond the quest for novel diagnostic biomarkers of cancer, because these autoantibodies target important molecules involved in biosynthesis, signal transduction, cell proliferation, and apoptosis, all of them key processes in carcinogenesis.[23] Moreover, we cannot rule out the possibility that such antibodies may enter into the cell and react with their targets, leading to cell damage.[39]

Many investigators have been interested in the detection of novel autoantibodies as serological markers for HCC diagnosis, but it is difficult to develop clinically valuable markers with definitive specificity and sensitivity for early diagnosis. In a recent study, Takashima et al.[40] reported that autoantibodies against heat-shock 70 kDa protein, peroxiredoxin, and manganese superoxide dismutase showed significantly higher frequency immunoreaction in HCC sera than in normal sera. They demonstrated that the three autoantibodies may be candidate diagnostic biomarkers for HCC. However, most autoantibodies can be associated with various diseases, and their relevance to the diagnosis of a particular disease should be treated with caution. To examine the specificity of the autoantibodies for HCC diagnosis, we investigated the occurrence of autoantibodies in sera from other cancers, chronic hepatitis, and healthy volunteers. An unexpected finding with strong signals of keratin 8 and lamin A/C was shown in chronic hepatitis with a high frequency, presumably because of the cross-reacting with the antibodies in both HCC sera and chronic hepatitis sera. After the removal of the two antigens, the results showed that the frequency of the positive reaction to each antigen in HCC classes was significantly higher than that in chronic hepatitis and normal classes. However, AFP was frequently elevated in patients with chronic hepatitis and without HCC. Positive reactions to DEAD box polypeptide 3, AIF, and prostatic binding protein were significantly more frequent in HCC than in any other cancer. However, it was observed that antibodies to eEF2 and hnRNP A2 were frequently detected in breast cancer, and anti-hnRNP A2 and anti-TIM were frequently detected in lung cancer. The results indicated that autoantibodies to DEAD box polypeptide 3, AIF, and prostatic binding protein might be HCC-specific, whereas eEF2, hnRNP A2, and TIM might elicit an immune response not only in HCC but also in other cancers. Certain antigens that we identified in SERPA may be also associated with the development of other cancers, such as breast and lung cancers. Our further studies would be aimed at analysis and evaluation of various combinations of antigens for early diagnosis of different cancers.

Although AFP is the effective marker available to detect HCC, the sensitivity and specificity is not optimal, especially in patients with small or well to moderately differentiated tumors. Our studies in HCC patients at TNM stage I might be helpful for early diagnosis of HCC. The sensitivity of a single antigen in diagnosing stage I HCC ranged from 50 to 85%. In particular, 8 of 20 stage I HCC patients with autoantibodies against any of the six antigens had a normal range of serum AFP levels. This finding was consistent with other reports[41] and suggested that HCC-associated antigens might have value in early diagnosis of HCC, especially for the AFP-negative cases. Moreover, recent studies indicate that combinations of multiple antigen–antibody systems may acquire higher sensitivity for diagnosis of cancer.[41,42] In our study, with the successive addition of antigens to a final total of six, there was a stepwise increase of the sensitivity up to 90% in diagnosing HCC on stage I. The combination of the six antigens might enhance antibody detection for early diagnosis of HCC.

To identify markers associated with clinicopathological characteristics of patients, the relationship between tumor size,

histological grade, metastasis, TNM classification, and antibody levels needs additional research. Previous studies rarely considered the clinical meaning of the presence of autoantibodies in HCC. In the present study, we found a relationship between the presence of antibodies to eEF2 and tumor size, and anti-eEF2 was more frequent in sera from HCC of bigger than 5 cm. Antibodies to AIF and hnRNP A2 occurred more often among patients without lymph node metastasis. It is suggested that anti-AIF and anti-hnRNP A2 are expected to be involved at an early stage of HCC, and antibodies to eEF2 are associated with HCC progression to advanced stage. However, there were no statistical differences for each autoantibody frequency among TNM classification.

Other markers, such as *Lens culinaris* agglutinin-reactive AFP (AFP-L3) and des-γ carboxyprothrombin (DCP), have also been proposed as markers for HCC diagnosis. AFP-L3, known as a fucosylated variant of AFP, appeared to be useful at improving the specificity for patients with AFP elevation between 20 and 200 ng/mL but suffered from a poor sensitivity in detecting early HCC. In HCC (<2 cm), AFP-L3 had a sensitivity of only 35–45%.[43–45] The efficacy of DCP in diagnosis of early HCC still remains unclear. It has been reported that the clinical utility of DPC might be dependent upon tumor size, and the sensitivity of DPC (41%) was worse than that of AFP (55%) for tumors less than 3 cm.[46] In comparison to these two biomarkers, the sensitivity of autoantibodies that we identified varied from 50 to 85%, enabling the detection of HCC at stage I. Further analysis with large randomized controlled trials is needed to compare HCC-associated antigens with AFP, AFP-L3, and DCP. Because AFP, AFP-L3, DPC, and HCC-associated antigens behave independently, they have the potential to complement each other for diagnosis of early HCC.

In conclusion, we have identified several novel autoantibodies present in the sera of HCC patients with SERPA combined with protein microarray. Some of the autoantibodies against HCC-associated antigens that we identified may have value on HCC early diagnosis. Additionally, the combination of proteomic technique and highly sensitive protein microarrays may provide a powerful diagnosis system in the near future.

Acknowledgment. The study was supported by a grant from the National Natural Sciences Foundation of China (30572128). We gratefully acknowledge the National Center of Biomedical Analysis for support in MS identification.

Supporting Information Available: MALDI−TOF MS analyses of identified proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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PR070525R