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Antibody Detection Using Tumor-Associated Antigen Mini-Array in Immunodiagnosing Human Hepatocellular Carcinoma

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Abstract

Background/Aims—Previous studies have demonstrated that during transition from chronic liver disease to hepatocellular carcinoma (HCC), autoantibodies can appear which are not detected in the prior pre-malignant conditions. These antibody responses may be stimulated by cellular proteins involved in carcinogenesis. This study determines the prevalence of antibodies to a selected panel of eight tumor-associated antigens (TAAs) in sera from patients with chronic hepatitis, liver cirrhosis and HCC, and considers the possibility and usefulness of antibodies to such a panel of TAAs in differentiating between these conditions. The panel of eight TAAs includes Imp1, p62, Koc, p53, c-myc, cyclin B1, survivin and p16 full-length recombinant proteins.

Methods—Enzyme-linked immunosorbent assay (ELISA) was used to detect antibodies against eight selected TAAs in 30 sera from chronic hepatitis, 30 from liver cirrhosis, and 142 from HCC. Positive results were also confirmed by slot blot, Western blotting and immunoprecipitation assay.

Results—Antibody frequency to any individual TAA in HCC varied from 9.9%–21.8%. With the successive addition of TAAs to a final total of eight antigens, there was a stepwise increase of positive antibody reactions reaching a frequency of 59.8% with whole cohort of HCC patients. This was significantly higher than the frequency of antibodies in chronic hepatitis (20%), liver cirrhosis (30%) and normal individuals (12.2%).

Conclusions—This study demonstrates that malignant transition to HCC is associated with increased autoantibody responses to certain cellular proteins which might have a role in tumorigenesis, and shows that a mini-array of eight TAAs enhanced antibody detection for diagnosis of HCC. More studies in patients with HCC and precursor conditions such as chronic hepatitis, alcoholic hepatitis and liver cirrhosis using enlarged TAA mini-array panels might further improve the sensitivity and specificity of this mode of cancer immunodiagnosis. Its additional usefulness might be in the early detection of cancer in some patients with predisposing conditions.

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Keywords

Autoantibodies; tumor-associated antigen (TAA); Cancer immunodiagnosis; hepatocellular Carcinoma (HCC)

1. Introduction

Many studies demonstrated that cancer sera contain antibodies which react with a unique group of autologous cellular antigens generally known as tumor-associated antigens (TAAs) [1–3]. Such autoantibodies from patients with hepatocellular carcinoma (HCC) have been used to immunoscreen cDNA expression libraries, and several novel TAAs such as HCC1 [4], p62 [5] and p90 [6] have been isolated and characterized. The hypothesis is that in sera from HCC patients who show autoantibody changes during progression from chronic liver disease to HCC, the novel autoantibodies appearing with malignant transformation will be more likely to be related to events associated with tumorigenesis, and that these autoantibodies can be used as reporters identifying aberrant cellular mechanisms in tumorigenesis, and also serve as immunodiagnostic markers for cancer detection [1]. One of the most extensively studied TAAs is p53, the tumor suppressor protein. Autoantibodies to p53 in cancer were first reported in 1982 [7] and since then there have been numerous reports confirming and extending this finding [reviewed in 8]. The mechanism underlying the production of such autoantibodies are not completely understood but the available data show that many of the target antigens are cellular proteins whose aberrant regulation could lead to tumorigenesis, such as p53 [8], HER-2/neu and ras [9,10], or are proteins whose dysregulation could have tumorigenic potential including mRNA binding proteins such as p62 [5] and cell-cycle control proteins such as cyclin B1 [11,12]. In the case of p62 which is primarily expressed in fetal tissues and is absent in adult tissues, immunogenicity appears to be related to abnormal expression of p62 in tumor cells [13]. In previous studies, we have observed changes in autoantibody profiles predating or coincident with clinical detection of liver cancer in chronic liver disease patients [14,15]. The results indicated that these were some features of tumorigenesis which induced immune responses in patients at the verge of developing cancer [14–17].

HCC is particularly prevalent in Africa and Asia. Most of the previous studies from China and other countries demonstrated that hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, dietary exposure to aflatoxin and excessive alcohol consumption were the principal etiological factors for HCC [18–23]. The majority of people with HCC will die within 1 year of its detection. This high case-fatality rate can in part be attributed to lack of diagnostic methods that allow early detection. Although alpha fetoprotein (AFP) is the most effective serological marker available to detect HCC, the sensitivity and specificity is not optimal. Therefore, there is a need for the development of more sensitive and specific methods that supplement AFP in the early detection of this cancer. This study determines the prevalence of antibodies to a selected panel of eight TAAs in sera from patients with chronic hepatitis, liver cirrhosis and HCC, and examines the possibility and usefulness of such a panel of TAAs in HCC immunodiagnosis.

2. Materials and Methods

2.1. Serum samples

Sera from 142 patients with HCC, 30 patients with chronic hepatitis (CH), 30 patients with liver cirrhosis (LC), and 82 normal human sera (NHS) were obtained from the serum bank of the Tumor Cell Engineering Laboratory of Xia'men University (Fujian Province, P.R. China). All HCC patients were diagnosed according to the criteria described in a previous study [24]. Of 142 HCC patients, 132 (93.0%) were histologically confirmed. General information

regarding HCC patients was shown in Table 1. Of 142 HCC patients, 116 (81.7%) were male, and 26 (18.3%) were female. Mean age was 56.8 ± 13.2 years (range, 24–79 years). One hundred and eight (76.1%) patients were positive for HBV, 16 (11.3%) patients for HCV, and 7 (4.9%) patients for both HBV and HCV. Ninety five (66.9%) patients had previous history with chronic hepatitis, 33 (23.2%) patients with liver cirrhosis, and 14 (9.9%) patients had no previous history with either chronic hepatitis or liver cirrhosis. Based on the Chinese general rules for liver cancer, 51 (35.9%) patients were in clinical stage I, 32 (22.5%) in stage II, 32 (22.5%) in stage III, 14 (9.9%) in stage IV, respectively and 13 (9.2%) patients had no available data on clinical stages. All HCC sera were collected at the time of initial cancer diagnosis, when the patients had not yet received treatment with any chemotherapy or radiation therapy. Patients with CH and LC had been followed for at least 18 months since the date of blood collection to exclude individuals with primary biliary cirrhosis and asymptomatic HCC. Normal human sera were collected from adults during annual health examinations in people who had no obvious evidence of malignancy. Due to regulations concerning studies of human subjects, the patient's name and identification number were blinded to investigators, and some clinical information for sera used in this study was not currently available. This study was approved by the Institutional Review Board of Xia'men University and collaborating institutions.

2.2. Expression and purification of recombinant proteins

Eight antigens, Imp1, p62, Koc, p53, c-myc, cyclin B1, survivin and p16 were selected for expression of recombinant proteins. Recombinant p62 has been expressed from a clone isolated from cDNA expression library by immunoscreening with antibody from a patient with HCC [5]. p62 cDNA was subcloned into pET28a vector producing a fusion protein with NH-terminal 6x histidine and T7 epitope tags. The recombinant protein expressed in *E. coli* BL21 (DE3) was purified using nickel column chromatography. Koc cDNA cloned in the pcDNA3 vector [25] was similarly subcloned to pET28a vector and recombinant protein expressed as above. Imp1 construct pCMV5-Imp1 was kindly provided by F.C. Nielsen [26] and p53 clone (p53SN3) by Yuxin Yin of Columbia University, New York, N.Y. and subcloned into pET28a for protein expression. cDNA from c-myc was amplified by polymerase chain reaction from human fetal liver tissue and survivin cDNA from human survivin EST clone (BG258433) before subcloning in pET28a vector. Recombinant cyclin B1 had been prepared and used previously [11] and was isolated from a pGEX construct expressing cyclin B1 with glutathione S transferase (GST) fusion partner. p16 cDNA was amplified by RT-PCR from human HeLa cells, and was subcloned into the pGEX vector. The GST gene fusion system was used for the expression and purification of p16 recombinant protein. Expression of adequate amounts of recombinant protein was examined in SDS-PAGE and Coomassie Blue staining was used to determine that expression products of expected molecular sizes were produced. In addition, Western blot analysis was used to confirm that the bands seen in SDS-PAGE were reactive with reference antibodies. The antibodies used were rabbit polyclonal anti-Imp1 against a C-terminal peptide and kindly supplied by F.C. Nielsen [26]. Anti-Koc/Imp3 was raised against specific C-terminal peptides of the respective proteins [25] and anti-p62 raised against the full-length protein [5]. Reactivities of p53, c-myc, cyclin B1 and p16 were determined with monoclonal antibodies obtained from Oncogene Research Products, Boston, MA. Rabbit polyclonal anti-survivin antibody raised against the C-terminal peptide was obtained from ProSci Inc., Poway, CA.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Purified recombinant proteins (Imp1, p62, Koc, p53, c-myc, cyclin B1, survivin and p16) were individually diluted in PBS to a final concentration of 0.5 µg/mL for coating Immunolon2 microtiter plates (Fisher Scientific, Houston, TX) overnight at 4°C. The human serum samples diluted 1:200 were incubated with the antigen-coated wells for 90 minutes, then incubated with

horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Caltag Laboratories, Burlingame, CA) as a secondary antibody diluted 1:3,000 for 90 minutes followed by washing with PBS containing 0.05% Tween 20. The substrate 2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma, St. Louis, MO) was used as the detecting agent. The O.D. of each well was read at 405nm, and the cut-off value for determining a positive reaction was designated as the mean absorbance of the 82 normal Chinese sera plus 3 standard deviations ($M + 3SD$). Each sample was tested in duplicate. As described in previous studies, 10 normal human sera representing a range of 3SD above and below the mean of the 82 Chinese normal sera, was always used in each experiment and the average value of the 10 normal sera was used in each run to normalize all absorbance values to the standard mean of the entire 82 normal samples. In addition, all positive sera were confirmed with repeat testing, as were some negative sera. The detailed protocol of ELISA was used as described by Rubin [27].

2.4. Western blotting and Slot blot

Serum samples that were determined to contain autoantibodies by the ELISA method were further tested by immunoblotting to confirm the immunoreactivity of the sera. Western blotting was performed essentially as described by Chan and Pollard [28]. In brief, the purified recombinant proteins (Imp1, p62, Koc, p53, c-myc, cyclin B1, survivin and p16) were electrophoresed by SDS-PAGE and subsequently transferred to a nitrocellulose membrane. Nitrocellulose membranes were cut in strips and the individual strips were pre-blocked in PBS containing 0.05% Tween 20 (PBST) with 5 % nonfat milk for 30 minutes at room temperature, then incubated for 90 minutes with patient sera diluted 1:100, and finally incubated with HRP-conjugated goat anti-human IgG diluted 1:3,000 for 90 minutes followed by washing with PBST solution. Positive signals were captured by autoradiography using chemiluminescence (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. The method for Slot blot was identical to Western blotting with the exception that the purified recombinant protein (100 ng/well) was applied directly to the nitrocellulose membrane using a vacuum source. Membranes were not cut into strips, and therefore, the detection of autoantibodies in an individual patient's serum to all eight TAAs could be performed in one blot simultaneously.

2.5. In vitro translation and immunoprecipitation

The full-length cDNA from individual TAAs such as Imp1, p62 and koc was transcribed and translated in vitro using the TnT coupled reticulocyte lysate system from Promega Biotec (Madison, WI) in the presence of [^{35}S]-methionine (ICN, Irvine, CA). Typically, 100 μl 10% protein A Sepharose, 500 μl buffer C (50 mM Tris-HCl, pH 7.4; 150mM NaCl; 5mM EDTA; 0.5% NP-40; 0.5% deoxycholic acid; 0.1% SDS; and 0.02% sodium azide), 10 μl BSA (10mg/ml), 5 μl in vitro translated products, 10 μl serum, and 10 μl proteinase inhibitors (Boehringer Mannheim) were added to a standard immunoprecipitation reaction. After incubation for 1 hour at 4°C, the immunoprecipitated beads were washed five times with 1 ml buffer C. Finally, beads were eluted in Laemmli's sample buffer and analyzed in SDS-PAGE, followed by autoradiography.

2.6. Statistical analysis

To determine whether the frequency of autoantibodies to eight TAAs in each cohort of patients' sera was significantly higher than that in sera from normal individuals, the data were analyzed using the χ^2 test with Yates' correction. Two significant levels (0.05 and 0.01) were used. Methods for calculating the sensitivity, specificity and positive predictive value was based on the methodology provided in *Epidemiology* [2nd edition, edited by Dr. Leon Gordis, W.B. Saunders Company, Philadelphia, 2000, reference 29].

3. Results

3.1. Frequency and titers of antibodies to a panel of eight TAAs in HCC

In this study, a panel of eight TAAs was used as coating antigens, and sera from patients with chronic hepatitis, liver cirrhosis, HCC and normal individuals were examined for the presence of antibodies to the individual TAA and cumulatively to the entire panel. The panel of eight TAAs includes IMP1, p62, Koc, p53, c-myc, cyclin B1, survivin and p16 full-length recombinant proteins. Seven TAAs except p16 were used in the previous study (30). p16, a cyclin-dependent kinase inhibitor, has been implicated as an important tumor suppressor (31). Table 2 show the frequency of autoantibodies to the eight TAAs in sera from patients with different conditions using ELISA. The sera that were tested included the 142 patients from China with HCC, 30 patients with chronic hepatitis, and 30 patients with liver cirrhosis. Eighty-two normal human sera from the same region in China as the patients with HCC and chronic liver diseases were also examined as controls. A positive test for antibodies was taken as an absorbance reading above the mean + 3S.D. of the 82 normal human sera. Of a total of 142 HCC sera analyzed, 59.8% (85/142) were shown to have antibodies cumulatively to any of these eight TAAs. Higher frequency ($p < 0.01$) of antibodies with individual TAA in HCC was found in p16 (21.8%), c-myc (20.4%), Imp1 (16.9%) and p62 (14.1%). In chronic hepatitis, antibody frequency to any individual TAA ranged from 0 to 13.3%, and in liver cirrhosis, it ranged from 0 to 10%. When the sera from patients with chronic hepatitis and liver cirrhosis were tested against the panel of these eight TAAs, the cumulative frequencies were 20% and 30%, respectively. The reactivity of normal human sera was low, ranging from 0 to 2.4% to any individual TAA, with a combined frequency of reactivity of 12.2% against the complete panel of eight antigens. The association between clinical stage and TAA positivity in HCC sera were also analyzed. The results showed that 29 (56.9%), 23 (71.9%), 18 (56.3%) and 8 (57.1%) sera were positive with any of the eight TAAs in clinical stage I, II, III and IV, respectively. There were no statistical differences for TAA frequencies among clinical stages.

The ranges of antibody titers to eight TAAs in different conditions are shown in Figure 1. The high titer reactivity of many HCC sera and the distinct difference between HCC and other conditions (chronic hepatitis, liver cirrhosis and normal individuals) were also demonstrated in this figure. Many HCC sera showed OD values several fold above the cut-off (mean + 3S.D. of normal sera), indicating that antibody responses to TAAs in some HCC patients were quite robust and not just mildly elevated. Positive results were also confirmed by slot blot and Western blotting as well as with immunoprecipitation assays. Slot blot analysis of four representative HCC sera is shown on Figure 2. Results from Western blotting and immunoprecipitation assay were confirmatory and are not shown.

3.2. Stepwise increase in rate of antibody positivity with successive addition of antigens

As described above, antibody frequency to any individual TAA in HCC was variable from 9.9%–21.8% but rarely exceeded 20%. The results from Table 3 demonstrated that with the successive addition of TAAs to a final total of eight antigens, there was a stepwise increase of positive antibody reactions up to 59.8%, which was significantly higher than the frequency of antibodies in chronic hepatitis (20%), liver cirrhosis (30%) and normal individuals (12.2%). The sensitivity in diagnosing HCC was 59.9%, and the specificity compared to normal individuals, chronic hepatitis and liver cirrhosis cases was 87.8%, 80.0% and 70.0%, respectively.

4. Discussion

HCC is one of the most common malignant cancers in the world. The unique feature of HCC is that antecedent HBV- or HCV-related chronic hepatitis and liver cirrhosis are common

precursor conditions and during transition to malignancy some patients develop autoantibodies which were not present during the preceding chronic liver disease phase [14–17]. In countries and regions where HBV or HCV infections are widely prevalent, patients with resultant chronic liver disease are followed regularly in outpatient clinics for treatment and for early detection of liver malignancy. In some of these patients, serial serum samples over a period of several years have been collected and in previously reported studies, it was shown that novel antibodies appeared during conversion to malignancy which were not present in the pre-malignant chronic liver disease phase. In HCC, where novel autoantibody responses are detected during conversion to malignancy, it has been proposed that characterization of the autoantigens driving the novel immune responses might provide insights into intracellular proteins participating in mechanisms leading to malignant transformation [1].

Many investigators have been interested in the use of autoantibodies as serological markers for cancer diagnosis, especially because of the general absence or a significantly lower frequency of these autoantibodies in normal individuals and in non-cancer conditions [30, 32]. Enthusiasm for this approach has been tempered by low sensitivity when individual antigen-antibody reactions were studied. We have observed that this drawback can be overcome by using a panel of carefully selected TAAs and that different types of cancer may require different panels of TAAs to achieve the sensitivity and specificity required to make immunodiagnosis a feasible adjunct to tumor diagnosis. This feature is one of the innovative notions in this study. Previous observations show that antibodies to any individual antigen such as p53 [8,30], c-myc [30,33], p62 [30] or NY-Eso-1 [34] do not reach levels of sensitivity which could become routinely useful in diagnosis. Recent studies indicate that combinations of multiple antigen-antibody systems may acquire higher sensitivity for diagnosis of cancer [30,32]. Wang et al. developed a phage-display library derived from prostate-cancer tissue, and a phage protein microarray, to analyze autoantibodies in serum samples from patients with prostate cancer and controls [32]. In this study, a 22-phage-peptide detector was constructed for prostate cancer screening, with 81.6% sensitivity and 88.2% specificity. Compared to our study [35], we constructed a decision tree for classifying prostate cancer, using five TAAs, with 79% sensitivity and 86% specificity. With a smaller panel, these rates are equivalent to those of Wang et al. and might be improved by the selection of other TAAs. Approaches from Wang et al. as well as our panel of TAAs do not as yet justify adoption in a screening program, and could be improved by the inclusion of additional tumor antigens. In a recent study, we added three selected TAAs (p90, cyclin D1 and cyclin A) into our previously used mini-array system for detection of antibodies in prostate cancer. The results showed that the cumulative positive reactions in prostate cancer sera reached 92.5%, significantly higher than in benign prostatic hyperplasia (BPH) and other controls [36]. All these results strongly support the hypothesis and rationale which we proposed in this study. In searching the literature, we found that the notion of using panels of TAAs for detecting cancer autoantibodies is rarely discussed and is a fairly new approach. We stress the notion that panels of “customized” TAAs should be used for different types of tumor and that these customized panels should be rigorously tested for sensitivity and specificity not only against other tumors but also against other disease conditions. In the case of HCC, the natural conditions would be chronic hepatitis and liver cirrhosis. The basis for the notion of the necessity of customized panels of TAAs is based not only on empirical observations but also on retrospective analysis of our own data accumulated in previously published work [4–6,14–17,30]. The main purpose of this study is to identify a specific panel of TAAs for HCC and compare and contrast this with chronic hepatitis and liver cirrhosis.

In a recent publication [37], we have studied another group of HCC patients, and found that 7 of 8 HCC patients with autoantibodies against multiple TAAs had a normal range of serum AFP levels. We have also demonstrated that 6 of 8 HCC patients with anti-TAAs had comparatively small nodules of HCC (<30 mm). In addition, all 5 HCC patients who had anti-

TAA and were confirmed histologically had well- to moderately-differentiated HCC. This finding indicated that anti-TAAs seem to be supplementary serological markers for the diagnosis of HCC, especially for the AFP-negative cases. As mentioned in the section on *Materials and Methods*, all serum samples used in this study were derived from the serum bank of our collaborating institution. In compliance with regulations concerning studies of human subjects, the patient's name and identification number was blinded to investigators. At the current time, we only have the general information about the diagnosis of each sample, and we are not able to get the detailed clinical information such as tumor size, AFP titer and so on. This is a limitation of this study. Additional cohort studies in patients with chronic liver diseases such as chronic hepatitis and liver cirrhosis would still be needed to support the notion that TAA mini-array for the detection of autoantibodies might be a useful non-invasive approach for the early diagnosis of patients during transformation from chronic liver disease to malignancy.

Although there have been a steadily increasing number of studies describing and characterizing autoantibodies to "cancer-related antigens" in recent years, many of these antigen-antibody systems are not found to be useful in differentiating cancer and normal. For example, we clearly demonstrate that antibodies to three so-called "cancer antigens" α -enolase, B23 and elongation factor-1 γ (EF-1 γ) are not unique to HCC, and suggest that its detection in HCC might be attributed to antibody being already present in the precursor liver diseases such as chronic hepatitis, liver cirrhosis or primary biliary cirrhosis (PBC) [38]. The data also emphasizes the importance of a comprehensive analysis of antibody responses to putative cancer antigens in different pre-cancer conditions such as chronic hepatitis and liver cirrhosis before conclusions can be made regarding their contribution to HCC. In the present study, p16 was a new TAA added to seven other TAAs used in a previous study [30]. P16 protein is a cyclin-dependent kinase (CDK) inhibitor, which plays an important role in the regulation of the cell cycle by inactivating the cyclin-dependent kinase (CDK) that phosphorylates retinoblastoma (Rb) protein [31]. Overexpression of p16 protein has been found in many types of human malignancy, such as gastric [39], prostate [40] and colorectal cancer [41]. Autoantibody response to p16 in cancer was recently observed in our laboratory. The frequency of antibodies to p16 appeared relatively higher in nasopharyngeal cancer (28.6%), breast cancer (17.1%) and HCC (21.4%) than in the other cancers examined (unpublished data).

In conclusion, this study further demonstrates that malignant transition in HCC can be associated with autoantibody responses to certain cellular proteins which might have some role in tumorigenesis, and suggests that a mini-array of eight TAAs selected on the basis of an association with transformation processes may enhance antibody detection for diagnosis of HCC. As noted in this study, our efforts was aimed at increasing both the sensitivity and specificity of antibodies as markers in HCC by expanding the TAA array to include antigens which might be more selectively associated with HCC and not with others. According to the data in this study, we thought that our mini-array of multiple TAAs might be used as a novel non-invasive approach to identify HCC in normal population and also in high-risk individuals such as in patients with chronic hepatitis and liver cirrhosis. Our concern is that the approach may be not suitable to distinguish HCC (liver cancer) with other types of cancer such as lung, breast and prostate cancer. The reason is that certain TAAs which we have used in our mini-array approach (for example p62, p53 and c-myc) may be also associated with other types of cancer such as lung, breast and prostate cancer. We propose that some antibody-antigen systems may be unique to HCC, and some may not. A comprehensive analysis and evaluation of various combinations of selected antibody-antigen systems will be useful for the development of autoantibody profiles involving different panels or arrays of TAAs in the future, and the results could be useful for diagnosis of specific types of cancer. Important additional studies would be to determine whether mini-arrays of antibody markers might be

useful in identifying the early stage of certain types of cancers such as lung and breast in high-risk individuals.

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Fig. 1.

Titer of autoantibodies to the panel of eight TAAs in HCC, chronic liver diseases (chronic hepatitis, liver cirrhosis), and normal individuals. The range of antibody titers to each of eight TAAs is expressed as optical density (OD) obtained from enzyme-linked immunosorbent assay (ELISA). The high titer reactivity of many HCC sera and the distinct difference between cancer and chronic liver diseases and normal individuals are demonstrated in this figure. The Y-axis represents the OD values. The X-axis represents the panel of eight TAAs. 1, Imp1; 2, p62; 3, Koc; 4, p53; 5, c-myc; 6, cyclin B1; 7, survivin; 8, p16.

Fig. 2.

Slot blot analysis of four representative HCC sera. Each blot represents a duplicate test for autoantibodies against a panel of eight recombinant cancer antigens, with phosphate buffered saline (PBS) as a negative control. Lane 1: Imp1; Lane 2: p62; Lane 3: Koc; Lane 4: p53; Lane 5: c-myc; Lane 6: cyclin-B1; Lane 7: survivin; Lane 8: p16; Lanes 9 and 10: PBS. **(A)** Normal human serum shows no reactivity to any of the eight TAAs. **(B)** One HCC serum shows reactivity to only p53. **(C)** Another HCC serum shows a strong reactivity to Imp1, and also a weak reactivity to p62. **(D)** A third HCC serum shows reactivity against Imp1, p62, and Koc. **(E)** A fourth HCC serum shows reactivity against Koc, survivin, and p16.

Table 1

Characteristics of 142 patients with HCC

Variables	Results
Mean age (mean $\pm$ SD, year)	56.8 $\pm$ 13.2
Age range (year)	24–79
Gender:	
Male (%)	116 (81.7%)
Female (%)	26 (18.3%)
Hepatitis virus infection:	
HBV (%)	108 (76.1%)
HCV (%)	16 (11.3%)
HBV + HCV (%)	7 (4.9%)
Background:	
Chronic hepatitis (%)	95 (66.9%)
Liver cirrhosis (%)	33 (23.2%)
Clinical Stages:	
Stage I (%)	51 (35.9%)
Stage II (%)	32 (22.5%)
Stage III (%)	32 (22.5%)
Stage IV (%)	14 (9.9%)
Histological diagnosis (%)	132 (93.0%)

HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 2
Frequency of antibodies to eight tumor-associated antigens^a

No. and (percentage) of autoantibodies to:										
Sera	No. Tested	Imp1	p62	Koc	p53	c-myc	Cyclin B1	survivin	p16	Cumulative to eight Ags
HCC	142	24(16.9)**	20(14.1)**	14(9.9)*	20(14.1)**	29(20.4)**	19(13.4)*	16(11.3)*	31(21.8)**	85(59.8)**
LC	30	0(0)	1(3.33)	4(13.3)*	2(6.7)	1(3.33)	0(0)	0(0)	4(13.3)*	9(30)*
CH	30	3(10)	0(0)	2(6.67)	1(3.3)	0(0)	0(0)	0(0)	2(6.7)	6(20)
NHS	82	2(2.4)	1(1.2)	1(1.2)	2(2.4)	0(0)	2(2.4)	2(2.4)	1(1.2)	10(12.2)

LC, liver cirrhosis; CH, chronic hepatitis; NHS, normal human sera.

^a *P* Values relative to NHS:

* *p* < 0.05,
** *p* < 0.01.

Table 3
Sequential addition of antigens to the panel eight TAAs

Antigen	No. and (percentage) of autoantibodies in:			
	HCC (142)	LC (30)	CH (30)	NHS (82)
Imp1	24(16.9)	0(0)	3(10)	2(2.4)
Imp1 and p62	33(23.2)	1(3.3)	3(10)	3(3.6)
Imp1, p62 and Koc	43(30.3)	4(13.3)	4(13.3)	3(3.6)
Imp1, p62, Koc and p53	53(37.3)	6(20)	4(13.3)	5(6.1)
Imp1, p62, Koc, p53 and c-myc	67(47.2)	6(20)	4(13.3)	5(6.1)
Imp1, p62, Koc, p53, c-myc and cyclin B1	72(50.7)	6(20)	4(13.3)	7(8.5)
Imp1, p62, Koc, p53, c-myc, cyclin B1 and survivin	75(52.8)	6(20)	4(13.3)	9(11.0)
Imp1, p62, Koc, p53, c-myc, cyclin B1, survivin and p16	85(59.8)	9(30)	6(20)	10(12.2)

LC, liver cirrhosis; CH, chronic hepatitis; NHS, normal human sera.