Original Article

Profiling of Serum Autoantibodies in Japanese Patients with Hepatocellular Carcinoma

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ABSTRACT

Background: Conventional serum markers frequently yield negative results in the early stages of hepatocellular carcinoma (HCC). Serum IgG autoantibodies to tumor-associated antigens have been reported even in the early stages. However, very little information is available for Japanese patients with HCC. This study aimed to profile serum autoantibodies in Japanese patients with HCC, using enzyme-linked immunosorbent assay (ELISA) systems based on tumor-associated antigens (TAAs) /antigenic fragments.

Methods: Sera were obtained from patients with HCC at stages I (n = 11), II (n = 30), and III (n = 9). In total, 18 serum autoantibodies were detected using ELISA. The TAAs used were RalA, Hsp70, p90, KM-HN-1, Ny-ESO-1, Galectin-1, Sui1, p53, Annexin A2, Prx6, VEGF, c-myc, HCC-22-5, p62, HCA25a, HER2, Hsp40, and Cyclin B1.

Results: The positive rates of autoantibodies against RalA (24%), Hsp70 (22%), and P90 (20%) were the highest. Among 50 patients who showed seropositivity, 40 (80%) showed seropositivity for at least one antibody and 27 (54%) showed seropositivity for two or more antibodies. The positive rates of serum autoantibodies at each stage were as follows: 91% for stage I, 80% for stage II, and 67% for stage III.

Conclusions: We developed ELISA systems to detect autoantibodies and successfully profiled 18 serum autoantibodies in Japanese patients with HCC. As pleural antibody responses were detected even in patients with stage I tumors, combination assay for these autoantibodies may be useful to detect early-stage HCC. Clinical trial registration number: UMIN 000014530.

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KEYWORDS: autoantibodies, hepatocellular carcinoma, enzyme-linked immunosorbent assay, RalA, Hsp70

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Introduction

Hepatocellular carcinoma (HCC) accounts for over 90% of all primary liver cancers and is a major global health concern.[1] Moreover, HCC is the sixth most common cancer and the third most common cause of cancer-related deaths worldwide.[2] Despite improvements in surgical techniques and other conditions, the prognosis of HCC remains poor because of late diagnosis; therefore, early detection holds utmost importance.

Alpha-fetoprotein (AFP) and prothrombin induced by the absence of vitamin K or antagonist II (PIVKA-II) are useful serum tumor markers for detecting HCC.[3] However, these conventional serum markers are frequently negative during the early stages of HCC. Although AFP is the only serum tumor marker available for HCC surveillance, AFP does not yield satisfactory results for the diagnosis of HCC in the early stages. Using a cutoff value of 20 ng/ml, the sensitivity and specificity of AFP are 41% − 65% and 80% − 90%, respectively, and the sensitivity is even lower when AFP is used to detect early-stage HCC.[4] Therefore, novel serum tumor markers that can detect HCC before the occurrence of symptoms in stage I or stage II are urgently required. Recently, some IgG autoantibodies were found to respond to tumor-associated antigens in the sera of patients, even at early stages. Using IgG autoantibodies, such as NY-ESO-1, p53, and RalA, HCC and healthy control sera could be satisfactorily differentiated.[2] However, very little information is available for Japanese patients with HCC.

The present study aimed to profile serum autoantibodies in Japanese patients with HCC, using enzyme-linked immunosorbent assay (ELISA) systems based on tumor-associated antigens (TAAs) /antigenic fragments.

Patients and Methods

Patients and healthy controls

The sera of 50 patients with HCC before treatment were obtained from BioBank, Japan. In total, 74 matched healthy control samples were also obtained. The samples obtained from BioBank were anonymous and had no information about patient sex, age, the type of viral infection, or prognosis. The TNM stage of HCC was classified on the basis of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (5th Edition) .[5] Among the 50 patients, 11 had stage I disease, 30 had stage II disease, and 9 had stage III disease. This study was approved by the institutional review board of the Toho University School of Medicine (#22-112 #22-047 #24-045). Written informed consent was obtained from all subjects under the management of BioBank. This clinical study was registered in the UMIN Clinical Trials Registry (UMIN 000014530). Optimized antigen cutoffs and a standard cutoff that corresponded to a value greater than the mean plus 3 standard deviations (SD) of the healthy control cohort were applied to each of the 18 antigens, and specificity was maintained at over 95%.

Purification of recombinant protein and enzyme-linked immunosorbent assay for the detection of serum autoantibodies

Among the 18 ELISA systems, three systems, including RalA, galectin-1, and NY-ESO-1, have been described previously.[6–9] As described previously, serum samples were analyzed using ELISA.[6]

HSP70

For HSP70 recombinant protein, full-length cDNA of HSP70 (GenBank accession number: NM 004134) was amplified using polymerase chain reaction (PCR). The amplified gene was inserted between the NcoI and XhoI sites of pET-28a (+) plasmid (Novagen, Darmstadt, Germany) for expression as a C-terminal 6 × histidine tag. The plasmid was introduced in *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA). The expressed recombinant protein was dissolved in 8 M urea-phosphate buffered saline (PBS). The extract was applied to a Ni Sepharose™ 6 Fast Flow column (GE Healthcare, Buckinghamshire, UK), and purified HSP70 recombinant protein was eluted with 50 mM − 200 mM imidazole in 8 M urea-PBS. The expression and purity of the recombinant protein was examined using 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). DNA sequencing analysis of the constructed plasmid confirmed that the inserted gene was accurate. HSP70 was diluted in a binding buffer to a final concentration of 5.0 μg/ml and was added to microtiter plates (100 μl/well). Only buffer was added to control wells. HSP70 signals were evaluated by calculating the difference in absorbance values between the wells containing HSP70 and the control wells.[10] The s-HSP70-Ab titers were divided as follows: negative optical density (OD) values (<0.248; mean + 3 SD of the values in the healthy controls) and positive values (>0.248).

Annexin A2

For Annexin A2 recombinant protein, full-length cDNA of Annexin A2 (GenBank accession number: NM

001002858) was amplified using PCR. The amplified gene was inserted between the NcoI and XhoI sites of pET-28a (+) plasmid (Novagen) and was expressed. The extract was applied to a Ni Sepharose™ 6 Fast Flow column (GE Healthcare), and the column was washed with 50 mM imidazole in PBS. Purified Annexin A2 recombinant protein was eluted with 200 mM imidazole in PBS. Annexin A2 was diluted in PBS to a final concentration of 4.0 μg/ml. Annexin A2 signals were evaluated by calculating the difference in absorbance.[11] The s-Annexin A2-Ab titers were divided as follows: negative OD values (<0.359) and positive values (>0.359).

HCC-22-5

For HCC-22-5 recombinant protein, full-length HCC-22-5 cDNA (GenBank accession number: NM 004683) obtained from human testis was amplified using PCR. The amplified gene was inserted between the EcoRI and XhoI sites of pGEX-4T-1 plasmid (GE Healthcare) for expression as an N-terminal GST fusion protein. The homogenized inclusion body was boiled in SDS-PAGE sample buffer and was fractionated using SDS-PAGE. The HCC-22-5 antigen was purified by eluting it from the gel. HCC-22-5 was diluted in PBS to a final concentration of 1.0 μg/ml. HCC-22-5 signals were evaluated by calculating the difference in absorbance.[12] The s-HCC-22-5-Ab titers were divided as follows: negative OD values (<0.290) and positive values (>0.290).

KM-HN-1

For KM-HN-1 recombinant protein, the cDNA region of 37-524 of KM-HN-1 (GenBank accession umber: NM152775) was amplified using PCR. The amplified gene was inserted between the NcoI and XhoI sites of pET-28a (+) plasmid (Novagen) for expression as a C-terminal 6 × histidine tag. The extract was applied to TALON Metal Affinity Resin column (Clontech, Palo Alto, CA), and the column was washed with 10 mM imidazole in 8 M urea-PBS. Purified KM-HN-1 recombinant protein was eluted with 200 mM imidazole in 8 M urea-PBS. KM-HN-1 was diluted in PBS to a final concentration of 0.8 μg/ml. KM-HN-1 signals were evaluated by calculating the difference in absorbance.[13] The s-KM-HN-1-Ab titers were divided as follows: negative OD values (<0.138) and positive values (>0.138).

Prx6

For Prx6 recombinant protein, full-length cDNA of Prx6 (GenBank accession number: NM 004905) was amplified using PCR. The amplified gene was inserted between the NdeI and XhoI sites of pET-28a (+) plasmid (Novagen), which had a modification of NcoI to NdeI, for expression as a C-terminal 6 × histidine tag. The extract was applied to a Ni Sepharose™ 6 Fast Flow column (GE Healthcare), and the column was washed with 50 mM imidazole in PBS. Purified Prx6 recombinant protein was eluted with 200 mM imidazole in PBS. Prx6 was diluted in PBS to a final concentration of 1.0 μg/ml. Prx6 signals were evaluated by calculating the difference in absorbance.[14] The s-Prx6-Ab titers were divided as follows: negative OD values (<0.223) and positive values (>0.223).

VEGF

For VEGF recombinant protein, the cDNA region of 27-191 aa of VEGF (GenBank accession number: AF486837) was amplified using PCR. The amplified gene was inserted between the NcoI and XhoI sites of pET-28a (+) plasmid (Novagen) for expression as a C-terminal 6 × histidine tag. The extract was applied to a Ni Sepharose™ 6 Fast Flow column (GE Healthcare), and the column was washed with 50 mM imidazole in 8 M urea-PBS. Purified VEGF recombinant protein was eluted with 200 mM imidazole in 8 M urea-PBS. VEGF was diluted in binding buffer (10 mM Tris, 6 M urea, 0.05% octyl-glucoside) to a final concentration of 1.0 μg/ml. VEGF signals were evaluated by calculating the difference in absorbance.[15] The s-VEGF-Ab titers were divided as follows: negative OD values (<0.182) and positive values (>0.182).

p53

For p53 recombinant protein, full-length cDNA of p53 (GenBank accession number: AB082923) was amplified using PCR. The amplified gene was inserted between the BamHI and HindIII sites of pFastBac Dual vector (Invitrogen, Carlsbad, CA). The p53 recombinant protein was expressed in Sf21 cells using the Bac-to-Bac® Baculovirus Expression System (Invitrogen). The cells were dissolved in RIPA buffer, and the target protein was fractionated using SDS-PAGE. p53 was diluted in PBS to a final concentration of 0.15 μg/ml. p53 signals were evaluated by calculating the difference in absorbance.[16] The s-p53-Ab titers were divided as follows: negative OD values (<0.118) and positive values (>0.118).

Sui1

A Sui1 construct inserted in a pET28 plasmid for expression as an N-terminal His-tagged protein was provided by Dr. Jian-Ying Zhang (University of Texas, El Paso, TX). Additional details of the procedure have been described previously.[17] The Sui1 extract was applied to a TALON Metal Affinity Resin column (Clontech), and the column was washed with 10 mM imidazole in 8 M urea-PBS. Puri-

fied Sui1 recombinant protein was eluted with 200 mM imidazole in 8 M urea-PBS. The s-Sui1-Ab titers were divided as follows: negative OD values (<0.176) and positive values (>0.176).

p90

For p90 recombinant protein, the cDNA region of 618－905 aa of p90 (GenBank accession number: AF334474) was amplified using PCR. The amplified gene was inserted between the NcoI and XhoI sites of pET-28a (+) plasmid (Novagen) for expression as a C-terminal 6 × histidine tag. The extract was applied to a Ni Sepharose™ 6 Fast Flow column (GE Healthcare) and purified p90 recombinant protein was eluted with 50 mM imidazole in 8 M urea-PBS. p90 was diluted in PBS to a final concentration of 0.5 μg/ml. p90 signals were evaluated by calculating the difference in absorbance.[18] The s-p90-Ab titers were divided as follows: negative OD values (<0.130) and positive values (>0.130).

HCA25a

For HCA25a recombinant protein, full-length cDNA of HCA25a (GenBank accession number: AF469043) was amplified using PCR. The amplified gene was inserted between the NcoI and XhoI sites of pET-28a (+) plasmid (Novagen) for expression as a C-terminal 6 × histidine tag. The extract was applied to a Ni Sepharose™ 6 Fast Flow column (GE Healthcare), and the column was washed with 100 mM imidazole in PBS. Purified HCA25a recombinant protein was eluted with 500 mM imidazole in PBS. HCA25a was diluted in PBS to a final concentration of 1.0 μg/ml. HCA25a signals were evaluated by calculating the difference in absorbance.[19] The s-HCA25a-Ab titers were divided as follows: negative OD values (<0.729) and positive values (>0.729).

HSP40

For HSP40 recombinant protein, full-length cDNA of HSP40 (GenBank accession number: NM 006145) was amplified using PCR. The amplified gene was inserted between the NdeI and XhoI sites of pET-28a (+) plasmid (Novagen), which had a modification of NcoI to NdeI, for expression as a C-terminal 6 × histidine tag. The purified HSP40 recombinant protein was eluted with 200 mM imidazole in PBS. HSP40 was diluted in PBS to a final concentration of 2.0 μg/ml. HSP40 signal was evaluated by calculating the difference in absorbance values between the wells containing HSP40 and PBS.[20] The s-HSP40-Ab titers were divided as follows: negative OD values (<0.465) and positive values (>0.465).

HER2

For HER2 recombinant protein, full-length cDNA of HER2 (GenBank accession number: NM 004448) was amplified using PCR. The amplified gene was inserted between the HindIII and XhoI sites of pcDNA3.1/myc-His A (Invitrogen) for expression as a C-terminal 6 × histidine tag. This recombinant protein was expressed in HEK293 cells (JCRB Cell Bank Osaka Japan) using the TransIT-LT1 transfection reagent (MoBiTec, Goettingen, Germany). The cells were cultured with SFM II media (Invitrogen) and the cell culture supernatant was collected. The culture supernatant was applied to a TALON Metal Affinity Resin column (Clontech), and the column was washed with 10 mM imidazole in PBS. Purified HER2 recombinant protein was eluted with 200 mM imidazole in PBS. HER2 was diluted in PBS to a final concentration of 1.0 μg/ml. HER2 signals were evaluated by calculating the difference in absorbance.[21] The s-HER2-Ab titers were divided as follows: negative OD values (<0.145) and positive values (>0.145).

p62

For p62 recombinant protein, full-length cDNA of p62 (GenBank accession number: AF057352) was amplified using PCR. The amplified gene was inserted between the EcoRI and XhoI sites of pET-28a (+) plasmid (Novagen) for expression as an N-terminal 6 × histidine tag. The extract was applied to a Ni Sepharose™ 6 Fast Flow column (GE Healthcare), and purified p62 recombinant protein was eluted with 250 mM imidazole in 8 M urea-PBS. p62 was diluted in a binding buffer to a final concentration of 1.0 μg/ml. p62 signals were evaluated by calculating the difference in absorbance.[22] The s-p62-Ab titers were divided as follows: negative OD values (<0.412) and positive values (>0.412).

c-myc

For c-myc recombinant protein, full-length cDNA of c-myc (GenBank accession number: K02276) was amplified using PCR. The amplified gene was inserted between the NcoI and XhoI sites of pET-28a (+) plasmid (Novagen) for expression as a C-terminal 6 × histidine tag. The extract was applied to a TALON Metal Affinity Resin column (Clontech) and the purified c-myc recombinant protein was eluted with 50 mM imidazole in 8 M urea-PBS. c-myc was diluted in PBS to a final concentration of 1.0 μg/ml. c-myc signals were evaluated by calculating the difference in absorbance.[23–25] The s-c-myc-Ab titers were divided as follows: negative OD values (<0.164) and positive values (>0.164).

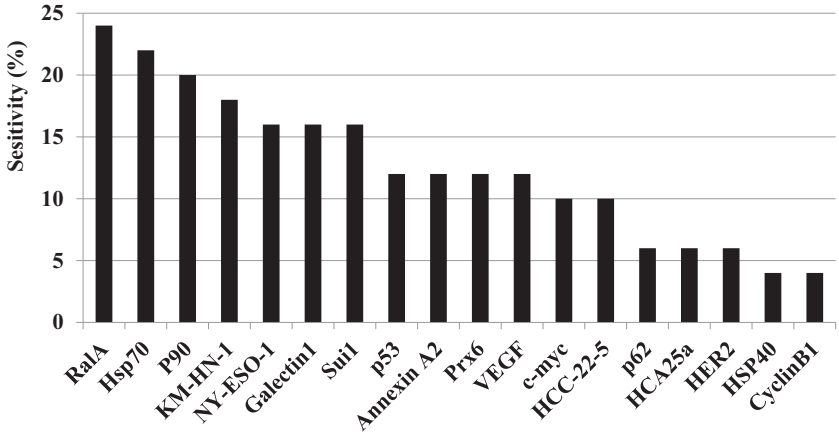


Fig. 1 Comparisons of the sensitivity of 18 serum autoantibodies in patients with hepatocellular carcinoma.

Cyclin B1

For Cyclin B1 recombinant protein, full-length cDNA of CyclinB1 (GenBank accession number: NM 031966) was amplified using PCR. The amplified gene was inserted between the NcoI and XhoI sites of pET-28a (+) plasmid (Novagen) for expression as a C-terminal 6 × histidine tag. First, the expressed CyclinB1 was purified as an inclusion body. To easily dissolve the inclusion body, it was suspended in 1% CHAPS solution (20 mTris, 150 mM NaCl, 1% CHAPS, pH 8). After suspension, the inclusion body was separated using centrifugation. The CHAPS-treated inclusion body was dissolved in 8 M urea-PBS. Cyclin B1 was diluted in a binding buffer to a final concentration of 0.5 μg/ml. CyclinB1 signals were evaluated by calculating the difference in absorbance.[24–27] The s-Cyclin B1-Ab titers were divided as follows: negative OD values (<0.331) and positive values (>0.331).

Enzyme-linked immunosorbent assay to detect serum antibodies

A standard protocol for ELISA was used as described previously.[6] Briefly, a 96-well microtiter plate (Immuno-Chemistry Technologies LLC, Bloomington, MN) was coated overnight at 4℃ with recombinant protein. The antigen-coated wells were blocked with gelatin post-coating solution at room temperature for 2 h. Human serum diluted (1 : 100) with serum diluent was added to the antigen-coated wells, and the mixture was incubated for 2 h at room temperature. Then, HRP-conjugated goat anti-human IgG (Caltag Laboratories, San Francisco, CA) was added at 1 : 4000 dilution. The substrate 22′ -azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS; Sigma-Aldrich, St. Louis, MO) was used to detect the immune complexes. The mean OD value at a wavelength of 405 nm was used for data analysis. The cutoff value designating a positive reaction was the mean OD value of 74 normal human serum samples plus 3 SD.

Results

In this study, the sensitivity of RalA autoantibody (24%) was the highest, followed by that of Hsp40 (22%) and P90 (20%). The sensitivity of p53 autoantibody was only 12% (Fig. 1). All specificities were above 95%. As a result, all false positive rates were less than 5% (supplemental Table 1). Only 20% patients were seronegative for all antibodies (Fig. 2). The remaining 80% patients were seropositive for at least one autoantibody. We found that 26% patients were positive for one antibody, 24% were positive for two antibodies, 14% were positive for three antibodies, and 16% were positive for more than three antibodies. Among 11 patients with stage I tumors, the positive rates of autoantibodies against RalA (36%) and/or KM-HN-1 (36%) were the highest (Table 1).

There were significant associations among the titers of pleural autoantibodies (Fig. 3). Patients who showed the highest titer for RalA also showed the highest titer for Sui1 and Annexin A2. Those who showed the highest titer for Hsp70 also showed the highest titer for HER2 and VEGF. Those who showed the highest titer for p90 also showed the highest titer for Prx6. Moreover, those who showed the highest titer for p53 also showed the highest titer for HCC-22-5. Thus, patients who showed the highest titer for some of the antigens were likely positive for other

Supplemental Table 1 Frequency of autoantibodies to tumor-associated antigens in hepatocellular carcinoma patients and normal controls.

Group	RalA	Hsp70	P90	KM-HN-1	NY-ESO-1	Galectin1
Sensitivity	24.0	22.0	20.0	18.0	16.0	16.0
Specificity	98.6	98.6	98.6	97.2	100	95.8
FPR	1.4	1.4	1.4	2.8	0	4.2
FNR	76.0	78.0	80.0	82.0	84.0	84.0

Group	Sui1	p53	Annexin A2	Prx6	VEGF	C-myc
Sensitivity	16.0	12.0	12.0	12.0	12.0	10.0
Specificity	98.6	97.2	98.6	98.6	98.6	95.8
FPR	1.4	2.8	1.4	1.4	1.4	4.2
FNR	84.0	88.0	88.0	88.0	88.0	90.0

Group	HCC-22-5	p62	HCA25a	HER2	HSP40	CyclinB1
Sensitivity	10.0	6.0	6.0	6.0	4.0	4.0
Specificity	100	98.6	95.8	97.2	97.2	97.2
FPR	0	1.4	4.2	2.8	2.8	2.8
FNR	90.0	94.0	94.0	94.0	96.0	96.0

FNR, false negative rate; FPR, false positive rate

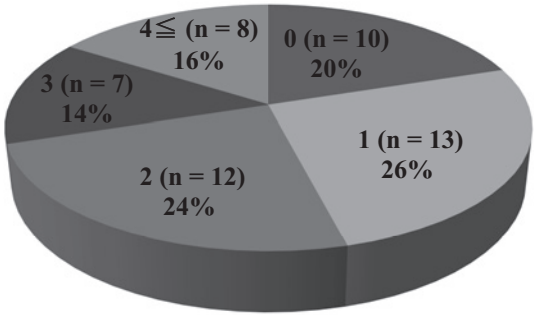


Fig. 2 Frequencies of the number of positive autoantibodies in patients with hepatocellular carcinoma.

Table 1 Number of seropositive patients for different tumor antigen combinations among 11 patients with stage I hepatocellular carcinoma.

Number of seropositive patients	Tumor antigens
4	RalA, KM-HN-1
3	HSP70, p90
2	HCC-22-5, Sui1,
1	NY-ESO-1, Annexin A2, Galectin-1, HCA25a, Prx6, VEGF, Hsp40, p53
0	P62, c-myc, CyclinB1, HER2

antigens (Supplemental Fig. 1).

Discussion

Some previous reports verified multiple serum tumor antibodies in HCC patients, but fewer reports have simultaneously verified 18 or more coherent serum autoantibodies.[24, 25, 28, 29] According to previous reports, the tumor suppressor protein p53 is one of the most highly immunogenic TAAs identified to date. The prevalence of serum p53 antibodies among HCC patients ranges from 12.2% to 73.07%.[30–32] Antibodies against p53 are present in patients with many types of cancers, except HCC, and may aid in the detection of cancer recurrence.[33] The reasons for the differences are unclear but may be associated with unidentified biological and geographical differences in the study populations.

The maximum number of positive autoantibodies in one patient was 14 (RalA, p90, KM-HN-1, Sui1, NY-ESO-1, Annexin A2, Galectin-1, HCC-22-5, p53, Prx6, c-myc, HCA25a, HER2, and VEGF). The mean numbers of positive autoantibodies according to tumor stage were as follows: 2.4 for stage I, 1.9 for stage II, and 3.2 for stage III. There was no difference in the number of positive autoantibodies among tumor stages (data not shown). According to tumor stage, the positive rates were 91% for stage I, 80% for stage II, and 67% for stage III. With stage I tumors, the positive rates of autoantibodies against RalA and/or KM-HN-1

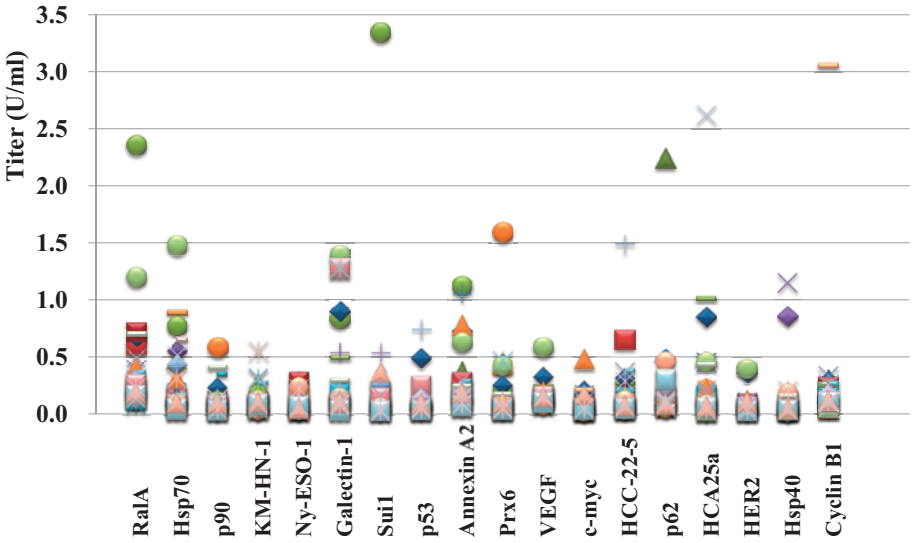
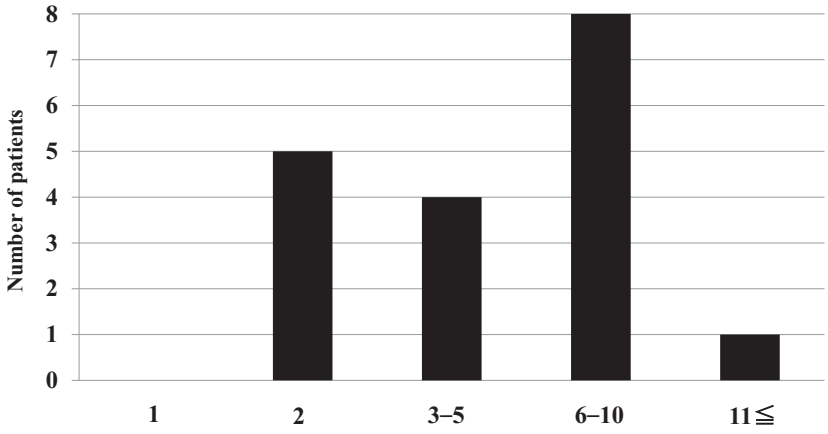


Fig. 3 Distribution of each titer of the 18 serum autoantibodies in patients with hepatocellular carcinoma.



Supplemental Fig. 1 Number of positive autoantibody results among patients with the highest titers for autoantibodies.
Number of positive antibodies in patients who have the maximum antibody titer against each tumor antigens.

were the highest. These findings in patients with stage I tumors were consistent with the results of a previous report.[10]

Such reactions against pleural antigens might be associated with immunogenicity. A long-term survival analysis, in prospective cohort, is required to clarify the impact of immunogenicity on patients' immune reaction to residual cancer cells. Precise follow-up data and analysis of recurrent timing and pattern will make suggestion on further utilities of autoantibodies in the patients with HCC.

The limitation of this study was that the examination of 18 types of serum antibodies was not practical. Further investigation should be required to develop combination assay with 4–5 types of serum autoantibodies to be more than 50% of sensitivity for practice. Currently, we are selecting several key serum autoantibodies combination to be high sensitivity rate enough for practice. For this further investigation, we have to examine a multi-institutional larger scale of study compared with the smaller scale of this study. Comparison with conventional serum markers, AFP and PIVKA-II, will be shown in the next study. The other limitation of this study was no comparison data with

hepatitis and/or cirrhosis patients. This perspective should be analyzed in further studies.

In conclusion, we developed 18 ELISA systems to detect autoantibodies and successfully profiled serum autoantibodies in Japanese patients with HCC. As pleural antibody responses were detected even in patients with stage I tumors, combination assay for the assessed autoantibodies may be useful to detect early-stage HCC.

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