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Serum anti-Ku86 is a potential biomarker for early detection of hepatitis C virus-related hepatocellular carcinoma ☆

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ABSTRACT

Hepatocellular carcinoma (HCC), the predominant form of primary liver cancer, is one of the most common cancers worldwide and the third most common cause of cancer-related death. Imaging studies including ultrasound and computed tomography are recommended for early detection of HCC, but they are operator dependent, costly and involve radiation. Therefore, there is a need for simple and sensitive serum markers for the early detection of hepatocellular carcinoma (HCC). In our recent proteomic studies, a number of proteins overexpressed in HCC tissues were identified. We thought if the serum autoantibodies to these overexpressed proteins were detectable in HCC patients. Of these proteins, we focused on Ku86, a nuclear protein involved in multiple biological processes and aimed to assess the diagnostic value of serum anti-Ku86 in the early detection of HCC.

Serum samples were obtained prior to treatment from 58 consecutive patients with early or relatively early hepatitis C virus (HCV)-related HCC and 137 patients with HCV-related liver cirrhosis without evidence of HCC. Enzyme immunoassays were used to measure serum levels of autoantibodies.

Serum levels of anti-Ku86 antibodies were significantly elevated in HCC patients compared to those in liver cirrhosis patients (0.41 ± 0.28 vs. 0.18 ± 0.08 Abs at 450 nm, $P < 0001$). Setting the cut-off level to give 90% specificity, anti-Ku86 was positive in 60.7% of stage I solitary tumor <2 cm in diameter, whereas the sensitivities of alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II) were 17.8% and 21.4%, respectively. The results of ROC analyses indicated the better performance of anti-Ku86 for early detection of HCC. Serum anti-Ku86 levels decreased after surgical resection of the tumors in the 12 HCC cases tested, Elevation of anti-Ku86 in solid tumors other than liver was minimal.

Serum anti-Ku86 is a potential biomarker for early detection of HCV-related HCC. Further studies in a larger number of HCC patients with various etiologies are needed to further evaluate the diagnostic and pathophysiological roles of elevation of serum anti-Ku86 in early HCC.

Abbreviations: 2-DE, two-dimensional gel electrophoresis; 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; AFP, alpha-fetoprotein; AFP-L3, lectin lens culinaris agglutin bound fraction of AFP; CHC, clathrin heavy chain; CT, computed tomography; DCP, des-gamma-carboxy prothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MRI, magnetic resonance imaging; NASH, non-alcoholic steatohepatitis; PIVKA-II, protein induced by vitamin K absence or antagonist II; SELDI-TOF MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; MRI, magnetic resonance imaging; US, ultrasonography.

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1. Introduction

Hepatocellular carcinoma (HCC), the predominant form of primary liver cancer, is one of the most common cancers worldwide and the third most common cause of cancer-related death [1]. Chronic infection by hepatitis B virus (HBV) or hepatitis C virus (HCV) and cirrhosis of any cause are major risk factors for HCC development [2,3]. Approximately 80% of HCC cases are derived from HCV-associated chronic liver diseases in Japan [4]. It has been shown that HCC surveillance of subjects at risk can facilitate tumor detection at an early stage, which in turn may improve survival [5,6]. Ultrasound (US) has been recommended for HCC surveillance [7], but the efficacy of this approach is highly operator-dependent. Other imaging modalities such as computed tomography (CT) and magnetic resonance imaging (MRI) may be effective [8,9], but are costly and unsuitable as a first-line examination.

Given this background, there is a need for sensitive serum markers for early detection of HCC. Alpha-fetoprotein (AFP) has been most widely used for this purpose, but many small HCCs do not secrete a diagnostic level of AFP [10,11]. A recent study indicated that AFP lacks adequate sensitivity and specificity for effective early diagnosis of HCC [12]. Measurement of the lectin lens culinaris agglutin-bound fraction of AFP (AFP-L3) can improve the specificity [13]. Protein induced by vitamin K absence or antagonist II (PIVKA-II; also referred to as des-gamma-carboxy prothrombin (DCP)) is a tumor marker complementary to AFP, but elevated PIVKA-II levels are found in only 28–47.6% of HCCs smaller than 3 cm [14,15]. Markers such as glypican-3 have been proposed to be complementary to AFP [16], but this marker has yet to be widely used, as reviewed elsewhere [17,18]. Thus, currently available tumor markers for HCC are not satisfactory in terms of sensitivity and specificity, indicating the need for development of new HCC serum biomarkers.

Recent technological advances have made proteomics useful for discovery of markers in various fields of medicine, including the discovery and identification of biomarkers for HCC [19,20]. Autoantibodies against autologous tumor-associated antigens are also promising targets as biomarkers for early cancer detection [21,22].

We previously conducted proteome analyses to compare protein expression between surgically resected HCC tissues and adjacent nontumor tissues using agarose two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) [23]. Expression levels of 83 proteins were found to differ between tumor and non-tumor tissue, and immunoblot analysis showed significantly increased expression of clathrin heavy chain (CHC) and Ku86, and decreased expression of formiminotransferase cyclodeaminase, rhodanese, and vinculin in the tumor tissue [23]. It is possible that the upregulated proteins can elicit autoantibody formation. Indeed, it has been shown that HCC with higher levels of cyclin B1 expression elicit anti-cyclin B1 antibody levels [24]. To test whether the proteins overexpressed in HCC tissues in our proteomic study elicit autoimmunity in primary liver cancers, we determined the anti-CHC and anti-Ku86 antibody levels in HCV-related HCC patients in comparison with those in patients with chronic liver diseases without HCC or other forms of cancers.

2. Materials and methods

2.1. Subjects

Fifty-eight consecutive patients with early (Stage I, $N = 28$) and relatively early (Stage II, $N = 30$) HCV-related HCC (29 males and 29 females, 69.7 ± 8.6 years old) hospitalized in the Gastroenterology Unit of Chiba University Hospital between January, 2008 and December, 2010 were included in the study (Table 1). For comparison, 137 with liver cirrhosis (56 males and 81 females, 65.8 ± 11.0 years old) we encountered during the same period were also included. Serum samples were obtained prior to initial treatment. Staging of the tumors was based on International Union Against Cancer (UICC)-TNM classification. The diagnosis of HCC was based on typical findings in three-phase dynamic CT or MRI. In cases with inconclusive imaging findings, the diagnosis was confirmed histopathologically. In 12 cases, serum samples were obtained just before and 2 months after surgical resection of the tumors in the Department of Hepatobiliary Surgery at Chiba University Hospital.

For comparison, 48 patients with other gastrointestinal cancers were analyzed in the study, including 16 gastric, 16 colorectal and 16 pancreatic cancers (Table 1). Blood samples obtained at the time of diagnosis were immediately centrifuged and serum was stored at −80 °C until analysis. Blood samples were also obtained from 114 apparently healthy subjects. The mean age of the healthy controls was comparable to that of the HCC patients (Table 1). All the samples were handled and stored essentially with the same protocol.

Written informed consent was obtained from each subject. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committee of the Graduate School of Medicine, Chiba University.

2.2. Determination of serum anti-CHC and anti-Ku86 antibody levels

CHC (Sigma–Aldrich Japan, Japan) and Ku86 (Abnova Corporation, Taiwan) proteins dissolved in PBS buffer were dispensed into a 96-well polystyrene microtiter plate (Thermo Fisher Scientific, Japan) at 0.5 μg/well and incubated for 1 day at 4 °C. The plate was washed three times with PBS containing 0.05% Tween 20, coated with 1.5% BSA (Proliant, Ankeny, IA, USA) containing 10% sucrose for 1 day at 4 °C, and then kept at 4 °C until use. After washing the microtiter plate with PBS buffer containing 0.05% Tween 20, 100-μL aliquots of 100-fold-diluted serum samples were added to wells. The plates were incubated at 37 °C for 1 h and then washed three times. Mouse anti-human IgG conjugated to HRP in PBS containing 0.05% Tween 20 (100 μL) was added to

Table 1
Subjects studied.

	Mean age (years)	Sex		Tumor stage (UICC stage[a])			
		M	F	I	II	III	IV
HCC ($N = 58$)	70	29	29	28	30	0	0
Liver cirrhosis ($N = 137$)	66	56	81	–	–	–	–
Others cancers							
Stomach ($N = 16$)	70	8	8	7	4	3	2
Colorectal ($N = 16$)	65	8	8	5	6	4	1
Pancreas ($N = 16$)	68	8	8	1	1	3	11
Healthy controls ($N = 114$)	67	74	40	–	–	–	–

[a] Union for International Cancer Control; TNM Classification of malignant tumors.

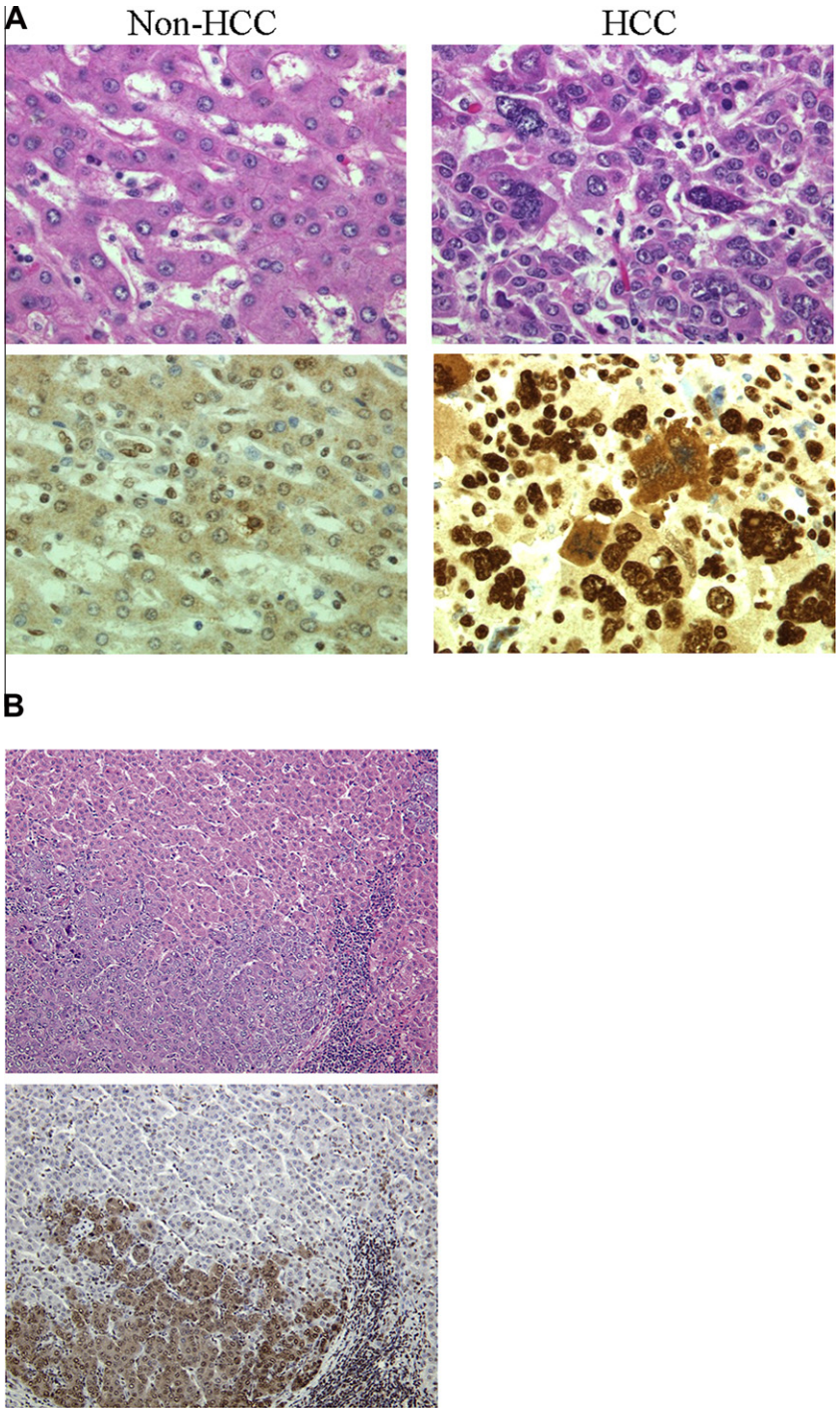

Fig. 1. Immunohistochemical analyses of Ku-86 in HCC and non-HCC tissues. (A) Surgically resected HCC tissues were stained with hematoxylin-eosin (upper panel) or anti-Ku86 antibody (lower panel). Strong staining of Ku86 was noted in the nuclei of tumor cells. In some tumor cells, faint staining was also seen in the cytoplasm. Similar results were obtained in four other comparisons. (B) HCC was distinguished from adjacent nontumor tissue by stronger staining of Ku86; hematoxylin-eosin (upper panel) and immunohistochemistry (lower panel).

each well and the plate was incubated at 37 °C for 1 h. The plate was washed three times and then 100 μL of TMB (3,3′,5,5′-tetramethylbenzidine) solution was added. After incubation at room temperature for 30 min, 100 μL of stop solution was added and absorbance at 450 nm was measured.

2.3. Immunohistochemistry of surgically obtained HCC tissues

Four-μm sections from paraffin tissue were fixed on slide glasses. Tissues were deparaffinized in xylene and rehydrated by reducing the concentration of ethanol (100%, 100%, and 70%, 5 min each). Antigen was unmasked with microwave irradiation for 5 min in pH 6.0 citric buffer three times. Anti-Ku86 antibody (Bio Matrix Research Inc., Chiba, Japan) was diluted 1:50 in blocking buffer (Dako Real™ Antibody Diluent; DAKO Japan, Kyoto, Japan). EnVision + system (DAKO Japan, Kyoto, Japan) was used to visualize tissue antigens. Tissue sections were counterstained with hematoxylin for 1 min. Protein expression, evaluated independently by two pathologists, was scored as negative (O), weak (1), moderate (2), and strong (3).

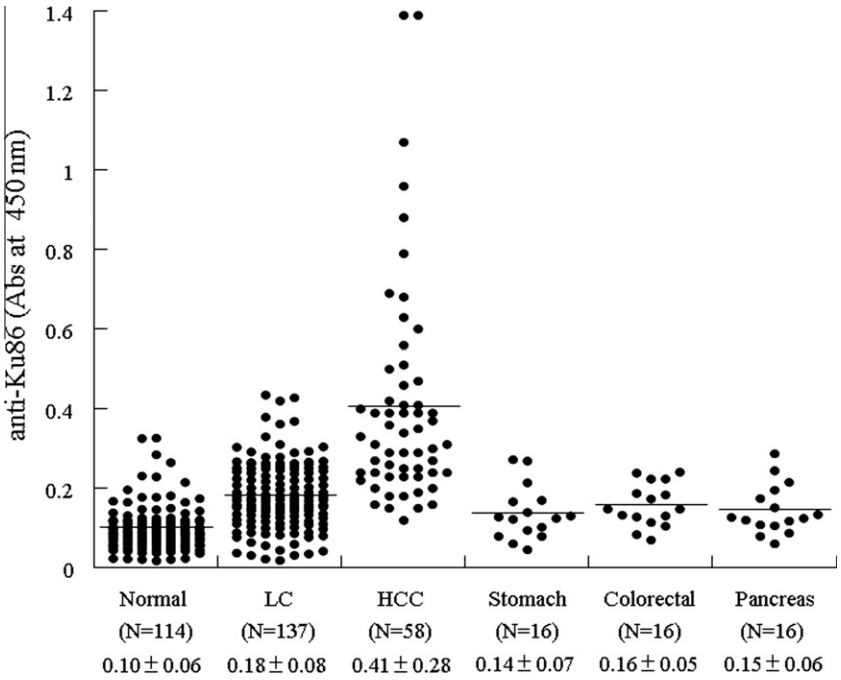

Fig. 2. Serum anti-Ku86 levels in patients with HCV-related chronic liver diseases, HCC, other gastrointestinal cancers and in healthy volunteers. Serum anti-Ku86 levels in HCC patients were significantly higher than those in patients with liver cirrhosis (LC) and other gastrointestinal cancers. Significance of the differences was assessed by Mann–Whitney *U*-test.

2.4. Data collection and statistical analysis

Serum levels of AFP and PIVKA-II were measured using commercial enzyme immunoassay kits (Fujirebio Inc., Tokyo, Japan), with cut-off values set at 40 ng/ml and 40 mAU/ml, respectively, to give 90% specificity in patients with liver cirrhosis. Numerical data are presented as the mean ± SD. The significance of differences in above analyses was examined using IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA). The overall diagnostic accuracies of each tumor marker were evaluated by receiver-operating characteristic (ROC) analysis using R statistical software, version 2.12.1 (http://www.r-project.org/) with the pROC add-on package. $P < 0.05$ was considered significant in all analyses.

3. Results

3.1. Immunohistochemistry of Ku86 in HCC tissues

Although staining of Ku86 in nontumor tissues was minimal, strong staining was noted in tumor tissues mainly in the nucleus. In some tumor cells, weak staining was also seen in the cytoplasm (Fig. 1A). Similar results were obtained in four other comparisons. It was noteworthy that HCC was distinguished from adjacent nontumor tissue by stronger staining of Ku86 (Fig. 1B).

3.2. Serum anti-Ku86 levels in patient groups

Serum anti-Ku86 levels in patient groups and healthy subjects are presented in Fig. 2. Serum anti-Ku86 levels were significantly higher in patients with HCC (0.42 ± 0.25) compared to those with liver cirrhosis (0.18 ± 0.08) (all $P < 0.001$). The levels in gastric, colorectal and pancreatic cancers were increased but were significantly lower than those of HCC ($P < 0.001$). In 12 cases, serum anti-Ku86 levels were determined just before and 2 months after surgical resection of the tumors. As shown in Fig. 3A, the levels significantly decreased after surgery (0.49 ± 0.33 vs. 0.19 ± 0.16, $P < 0.001$). Preoperative serum anti-Ku86 levels as related to expression levels of Ku86 assessed by immunohistochemistry are presented in Fig. 3B. Anti-Ku86 level tend to be higher in patients with greater Ku86 expression in HCC tissues.

3.3. Comparison with AFP and PIVKA-II (DCP)

There was no significant correlation of anti-Ku86 with the two conventional HCC tumor markers, AFP and PIVKA-II (Supplementary Fig 1).

Serum levels of AFP, PIVKA-II and anti-Ku86 in HCC patients are summarized in Table 2A. Also, serum levels of the three markers in all the 28 Stage I cases are shown in Table 2B.

The cut-off values of the three markers were all set at levels that gave 90% specificity compared with patients with liver cirrhosis: 0.28 Abs, 40 ng/ml, 40 mAU/ml for anti-Ku86, AFP, and PIVKA-II, respectively. In 28 HCC patients with solitary small (<2 cm) tumor (Stage I), serum anti-Ku86 levels were above the cut-off value in 17 cases (60.7% sensitivity). In these 28 patients, the sensitivities of AFP and PIVKA-II at cut-off levels that gave 90% specificity were 17.8% and 21.4%, respectively (Fig. 4A). Anti-Ku86 levels were above the cut-off level in 11 (61.1%) of 18 Stage I cases in which the serum levels of AFP and PIVKA-II were both below their respective cut-off values. Thus, combination assays of AFP, PIVKA-II and anti-Ku86 could detect 21 out of 28 Stage I HCC cases (Table 2B).

ROC curves for anti-Ku86, PIVKA-II, AFP and a combination of AFP and PIVKA-II in Stage I and Stage I–II HCC cases compared with LC patients are presented in Fig. 4B . The area under the curve (AUC) for anti-Ku86 was significantly greater than those for PIVKA-II, AFP and a combination of AFP and PIVKA-II ($P < 0.001$).

4. Discussion

The data presented in this study provides the first evidence that anti-Ku86 could be an early indicator of HCV-related HCC. The study is also a good example of potential HCC tumor marker

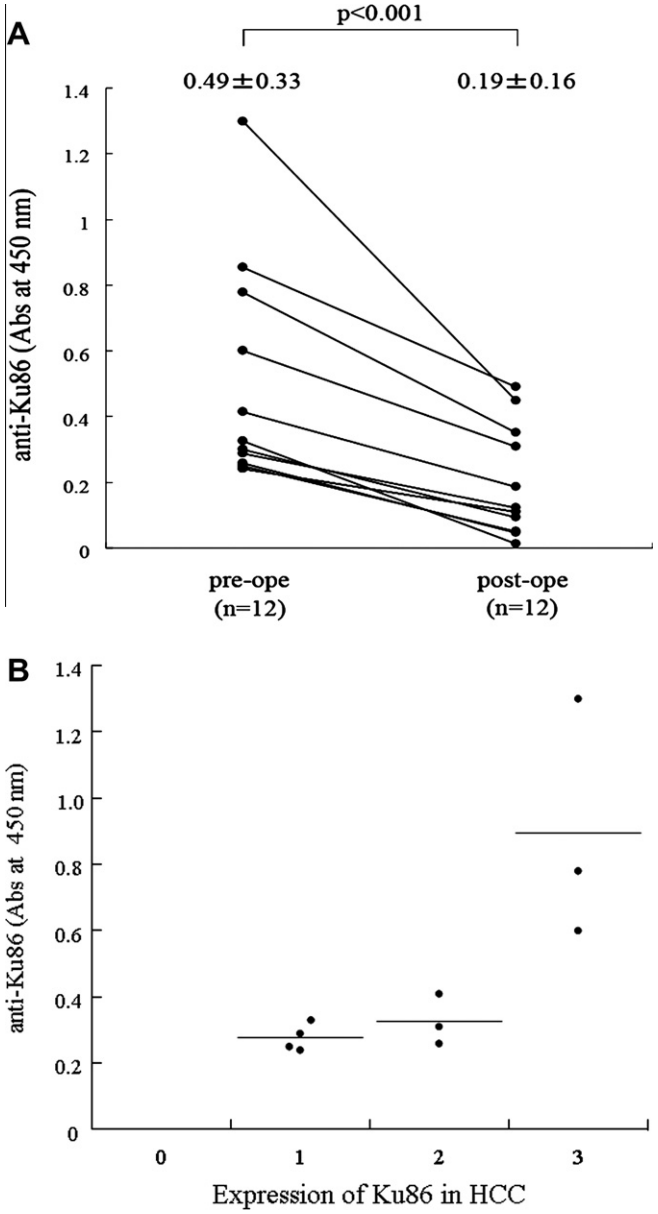

Fig. 3. Serum anti-Ku86 before and after surgical resection of tumors. (A) Serum anti-Ku86 levels before and 2 months after surgical resection of HCC in 12 cases. The levels significantly decreased after surgery ($P < 0.001$ assessed by the Wilcoxon signed rank sum test). (B) Preoperative serum anti-Ku86 levels as related to expression levels of Ku86 assessed by immunohistochemistry. Anti-Ku86 level tend to be higher in patients with greater Ku86 expression in HCC tissues.

Table 2A
Serum levels of AFP, PIVKA-II and anti-Ku86 in patients with hepatocellular carcinoma. (A) The mean values (mean + SD) of each marker in patients with liver cirrhosis and HCC.

	Liver cirrhosis ($N = 137$)	HCC	
		Stage I ($N = 28$)	Stage II ($N = 30$)
AFP (ng/mL)	14.2 ± 14.5	24.5 ± 47.5	473.9 ± 2010.6
PIVKA-II (mAU/mL)	21.6 ± 13.3	87.8 ± 218.6	136.7 ± 224.4
Anti-Ku86 (Abs)	0.18 ± 0.08	0.35 ± 0.17	0.46 ± 0.34

discovery originating from comprehensive proteome analysis of HCC tissues.

Table 2B
Serum AFP, PIVKA-II and anti-Ku86 levels in 28 patients with solitary and small (<2 cm) HCC (Stage I). Positive data are underlined.

No.	Age	Sex	Noncancerous tissue	Child-Pugh	Tumor size (mm)	AFP (ng/mL)	PIVKA-II (mAU/mL)	Anti-Ku86 (Abs)
1	73	Female	LC	B	14	5.6	14	0.50
2	69	Female	LC	A	20	4.8	12	0.18
3	80	Female	LC	A	19	14.3	30	0.19
4	74	Female	LC	A	20	28.7	20	0.34
5	61	Male	LC	C	20	43.8	12	0.96
6	81	Female	LC	C	20	9.0	586	0.41
7	73	Male	LC	A	17	8.6	1041	0.23
8	56	Female	LC	B	8	14.0	13	0.22
9	56	Male	LC	A	12	4.6	68	0.24
10	60	Male	LC	C	16	14.5	241	0.15
11	71	Female	LC	A	17	5.5	19	0.46
12	70	Male	LC	B	10	2.3	12	0.25
13	67	male	LC	A	7	6.6	16	0.56
14	76	Female	LC	A	10	8.7	15	0.40
15	80	Female	LC	B	14	227.7	50	0.60
16	79	Male	LC	A	15	137.9	18	0.24
17	61	Male	LC	B	8	40.5	20	0.37
18	81	Male	LC	A	12	6.4	26	0.39
19	76	Male	LC	B	10	41.7	13	0.29
20	51	Male	LC	A	12	7.1	12	0.24
21	81	Female	LC	B	10	12.9	10	0.30
22	75	Female	LC	A	17	6.1	15	0.35
23	63	Male	LC	A	12	7.8	33	0.36
24	76	Female	LC	A	18	6.5	20	0.18
25	71	Female	LC	B	17	5.5	19	0.33
26	75	Male	CH		14	3.6	37	0.47
27	69	Male	LC	A	17	2.4	17	0.23
28	49	Female	LC	A	11	8.9	78	0.29

LC, liver cirrhosis; CH, chronic hepatitis, Child-Pugh, Child-Pugh classification to indicate the severity of liver cirrhosis.

Although direct analyses of serum or plasma by mass spectrometry may provide biomarker candidates for a variety of diseases, the spectrum of observed proteins and peptides suggests that they are not easily applicable to early detection of solid tumors [25]. Glycomic and glycoproteomic approaches might be more promising [26,27].

We previously conducted proteome analyses to compare protein expression levels between surgically resected HCC tissues and adjacent non-tumor tissues using agarose 2D-DIGE [23]. Expression levels of 83 proteins differed between the tumor and non-tumor tissues, and immunoblotting showed significantly increased expression of clathrin heavy chain (CHC) and Ku86 in the tumor tissue [23]. Since autologous proteins overexpressed in tumor cells can be altered in a way that renders them immunogenic, we compared the serum anti-CHC and anti-Ku86 levels in HCV-related HCC patients with those in patients with liver cirrhosis without HCC. The results of preliminary experiments showed that the increase of serum anti-Ku86 in HCC sera was much greater than that of anti-CHC (data not shown). Therefore, in the current study, we focused on anti-Ku86.

The Ku complex is composed of two subunits of 70 and 86 kDa, which are designated as Ku70 and Ku86 (also referred to as Ku80), respectively [28]. Ku70 and Ku86 are the regulatory region of a DNA-dependent protein kinase that is involved in multiple biological processes, including DNA double-strand break repair, V(D)J recombination, telomere length maintenance, cell cycle progression, and transcriptional regulation [29]. We detected Ku86 overexpression in HCC by direct 2-DE proteome analysis of HCC tissues. Overexpression of Ku86 in HCC was also shown by Luk

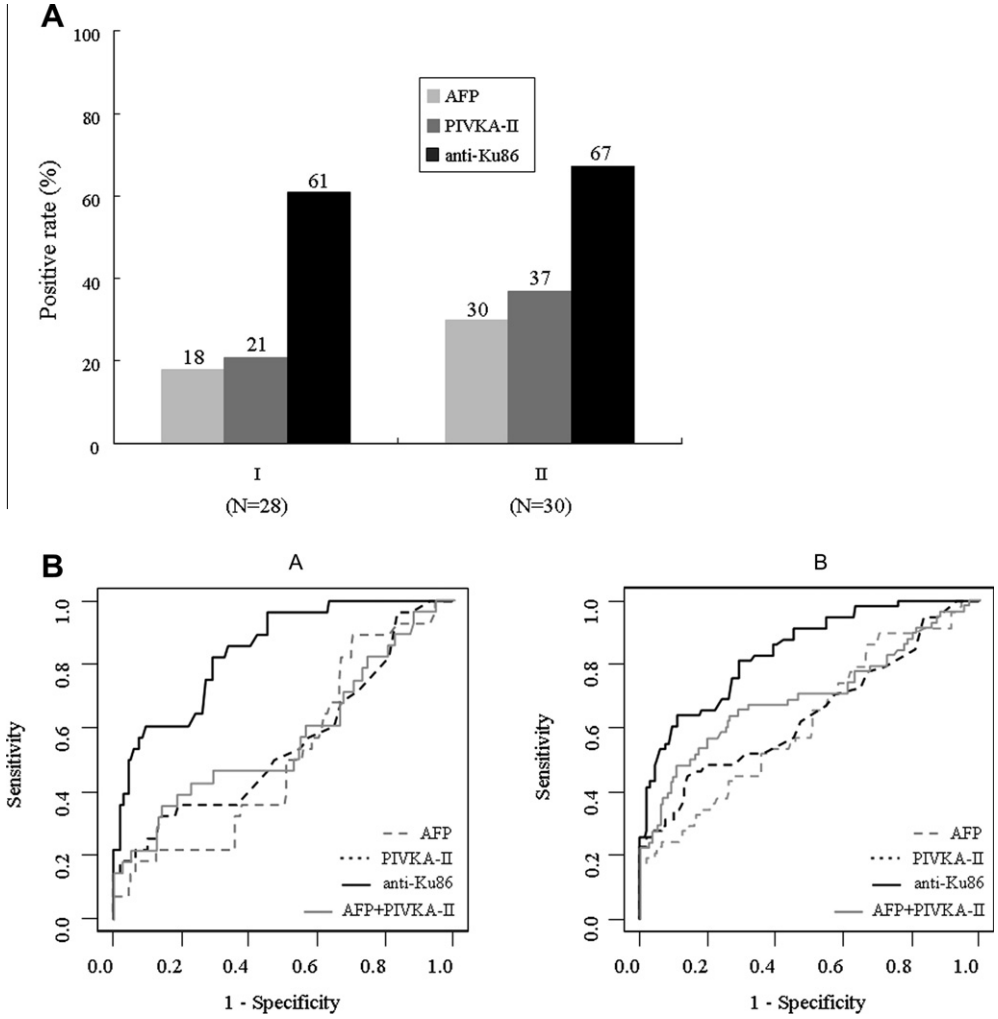

Fig. 4. Comparison of anti-Ku86 with the conventional tumor markers of HCC. (A) Sensitivity of AFP, PIVKA-II and anti-Ku86 in early (Stage I) and relatively early (Stage II) HCC cases. Sensitivities were obtained at cut-off levels that gave 90% specificity in cirrhotic patients without HCC: 40 ng/ml for AFP, 40 mAU/ml for PIVKA-II, and 0.28 Ab for anti-Ku86. The sensitivity of anti-Ku86 was significantly higher than those of AFP and PIVKA-II in Stage I and Stage II cases. The differences between anti-Ku86 and AFP or PIVKA-II in Stage I and Stage II cases were statistically significant ($P < 0.05$) as assessed by Fisher's exact test. (B) ROC curves for anti-Ku86, PIVKA-II, AFP in Stage I (early) and Stage I–II HCC cases, compared with LC patients. The area under the curve (AUC) for anti-Ku86 was significantly greater than those for PIVKA-II, AFP and a combination of AFP and PIVKA-II ($P < 0.001$).

et al. by a different approach [30]; using a murine monoclonal antibody generated against HCC samples, overexpression of the heterodimer Ku70/Ku80 (=Ku86) in the nucleus and/or cytoplasm was shown in HCC cell lines and in liver cancer tissues [30]. Ku70 is also present in the plasma membrane [31], which makes this antigen more accessible to the immune system.

There are many ways in which autologous proteins become immunogenic in tumor cells, including overexpression, mutation, misfolding, and aberrant degradation. In addition, proteins that are mislocalized during malignant transformation can also provoke a humoral response. Overexpressed proteins appear to increase the antigenic load in HCC, as in the case of cyclin B1 [24]. Indeed, in the present study, immunohistochemical staining of anti-Ku86 tended to be stronger in HCC cases in which preoperative serum anti-Ku86 levels were highly elevated. The possibility of a missense mutation of the *XRCC5* gene that codes for Ku86 should also be considered. The exact reasons of the increased antigenicity of Ku86 in HCC tissues remain to be clarified.

Autoantibodies have various characteristics and advantages as cancer biomarkers [21,22]. First, the immune response to tumor associated antigens (TAAs) can occur at a relatively early stage of carcinogenesis. Second, autoantibodies are stable and remain elevated for a relatively long period, in contrast to other biomarkers including TAAs themselves, which are less stable and rapidly degraded and cleared. Serum levels of the autoantibodies are also much higher than their respective TAAs, as a result of amplification by the immune system in response to a single autoantigen.

Based on a proteome analysis of HCC tissues, we have provided the first evidence that anti-Ku86 is a promising tumor marker for early detection of HCV-related HCC. Ku86 appears to develop antigenicity at a relatively early stage of tumorigenesis. Since mechanisms of hepatocarcinogenesis are variable depending on the etiology, it is possible that the antigenic potential of Ku86 differs in HCC of other etiologies. Therefore, a larger multicenter prospective study including HCC of various etiologies including HBV and non-alcoholic steatohepatitis (NASH) is required for further evaluation of the diagnostic and pathophysiological roles of elevation of serum anti-Ku86 in early HCC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.099.

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