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BRIEF ARTICLE

Serum manganese superoxide dismutase and thioredoxin are potential prognostic markers for hepatitis C virus-related hepatocellular carcinoma

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Abstract

AIM: To evaluate the clinical significance of oxidative stress markers in patients with hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC).

METHODS: Sixty-four consecutive patients who were admitted to Kagoshima University Medical and Dental Hospital were enrolled in this retrospective study. All patients had chronic liver disease (CLD) due to infection with HCV. Thirty patients with HCV-related HCC, 34 with HCV-related CLD without HCC (non-HCC), and 20 healthy volunteers (HVs) were enrolled. Possible associations between serum manganese superoxide dismutase (MnSOD) and thioredoxin (TRX) levels and clinical parameters or patient prognosis were analyzed over a mean follow-up period of 31.7 mo.

RESULTS: The serum MnSOD levels were significantly higher in patients with HCV-related HCC than in patients without HCC ($P = 0.03$) or HVs ($P < 0.001$). Similarly, serum TRX levels were also significantly higher in patients with HCV-related HCC than in patients without HCC ($P = 0.04$) or HVs ($P < 0.01$). However, serum levels of MnSOD and TRX were not correlated in patients with HCC. Among patients with HCC, the overall survival rate (OSR) was lower in patients with MnSOD levels $\geq$ 110 ng/mL than in patients with levels < 110 ng/mL ($P = 0.01$), and the OSR tended to be lower in patients with TRX levels < 80 ng/mL ($P = 0.05$). In addition, patient prognosis with HCC was poorest with serum MnSOD levels $\geq$ 110 ng/mL and serum TRX levels < 80 ng/mL. Furthermore, a multivariate analysis using a Cox proportional hazard model and serum levels of five factors (MnSOD, prothrombin time, serum albumin, serum α-fetoprotein (AFP), and serum des-γ-carboxy prothrombin) revealed that MnSOD levels $\geq$ 110 ng/mL (risk ratio: 4.12, 95% confidential interval: 1.22-13.88, $P = 0.02$) and AFP levels $\geq$ 40 ng/mL (risk ratio: 6.75; 95% confidential interval: 1.70-26.85, $P < 0.01$) were independent risk factors associated with a poor patient prognosis.

CONCLUSION: Serum MnSOD and TRX levels are potential clinical biomarkers that predict patient prognosis in HCV-related HCC.

Key words: Oxidative stress; Manganese superoxide dismutase; Thioredoxin; Hepatitis C virus; Hepatocellular carcinoma

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INTRODUCTION

As a significant cause of global cancer morbidity and mortality, hepatocellular carcinoma (HCC) is the fifth- and seventh-most frequently diagnosed cancer worldwide in men and women, respectively, and is the second- and sixth-most frequent cause of cancer deaths in men and women, respectively[1]. HCC is most frequently caused by persistent infection with hepatitis C or B virus. Early HCC diagnosis and better treatments have helped to improve the prognosis for patients with HCC. Also, interferon (IFN)-based treatments not only eliminate hepatitis C virus (HCV) infection, but also prevent HCC in patients with chronic hepatitis C (CHC)[2]. However, IFN-based therapies do not always effectively eliminate HCV infection or prevent HCC. Thus, biomarkers that are indicative of HCC pathological condition would have many clinical benefits, including aiding in the selection of the most appropriate treatment for a patient's disease.

Oxidative stress results from an imbalance in the production of reactive oxygen species (ROS) and the antioxidative defenses that maintain a cellular redox state. ROS include superoxide anions, hydrogen peroxide, hydroxyl radicals and nitric oxide, all of which are indispensable elements in many biochemical processes[3]. ROS are mainly derived from Kupffer and inflammatory cells in the liver[4], and upon exposure to other cells are thought to induce apoptosis, necrosis, inflammation, immune responses, fibrosis and tissue regeneration[5]. In liver disease, there is an overproduction of ROS from endogenous sources such as the mitochondria, peroxisomes, and activated inflammatory cells. In particular, ROS of mitochondrial origin were recently reported to be elevated in patients with alcoholic liver disease, non-alcoholic steatohepatitis (NASH)[6,7] and HCV-related chronic liver disease (CLD)[8]. Conversely, cells are protected from oxidative stress by intracellular antioxidants such as glutathione (GSH) and thioredoxin (TRX) and by various antioxidant enzymes such as superoxide dismutase (SOD), GSH peroxidase, catalase, and heme oxygenase-1[9-11]. Collectively, the relative expression levels of these molecules may serve as biomarkers for various liver diseases, including HCV-related HCC.

Manganese SOD (MnSOD) is an antioxidant enzyme that catalyzes the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. We have previously demonstrated that MnSOD expression was induced in primary cultured hepatocytes that were loaded with hydrogen peroxide *in vitro* and that serum MnSOD levels can be used to distinguish between NASH and simple steatosis in patients with nonalcoholic fatty liver disease[7]. However, the clinical significance of serum MnSOD levels in HCV-related CLD has not been fully investigated.

TRX was originally discovered in *Escherichia coli* as a proton donor for ribonucleotide reductase[12]. Subsequently, the human *TRX* gene was cloned as an adult T-cell leukemia-derived factor and was originally described as an interleukin-2 receptor inducer present in the cell culture supernatant of human T-lymphotropic virus type-1 -transformed cells[13]. TRX expression is induced by various oxidative stressors in patients with acquired immunodeficiency syndrome[14], Sjögren's syndrome[15], rheumatoid arthritis[16], and malignant neoplasms[17,18]. Previous studies have reported that serum TRX is an oxidative stress marker and that serum TRX levels increase in patients with HCV-related CLD during liver fibrosis progression[19]. In addition, serum TRX levels are reported to be elevated in patients with NASH compared to patients with simple steatosis[20]. However, the clinical significance of elevated TRX levels among patients infected with HCV in relation to HCC diagnosis and prognosis has not been elucidated.

In this study, we aimed to clarify the clinical significance of serum levels of MnSOD and TRX in patients with HCV-related CLD, and in particular among patients with HCC.

MATERIALS AND METHODS

Patients

Sixty-four consecutive patients who were admitted to Kagoshima University Medical and Dental Hospital between December 2006 and November 2008 were enrolled in this retrospective study. All patients had CLD due to an HCV infection and were diagnosed with HCC (30 patients; HCC group) or without HCC (34 patients; non-HCC group). Twenty healthy volunteers (HVs) were also enrolled in this study.

In this study, HCC was diagnosed based on findings from abdominal ultrasound, abdominal computed tomography, and serum levels of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP, also known as PIVKA-II). Patients were excluded from this study if they were positive for hepatitis B surface antigen; other types of hepatitis, including autoimmune hepatitis and alcoholic liver disease; or other malignancies.

The study endpoint was patient death, the available follow-up date, or December 31, 2010. Patient follow-up

periods ranged from 5.1 to 44.6 mo, with a mean observation time of 31.7 mo. Informed consent was obtained from all study patients and healthy controls. This study was approved by the ethical committees of Kagoshima University Graduate School of Medical and Dental Sciences and Kagoshima University Medical and Dental Hospital.

Laboratory markers

The clinical laboratory parameters assessed included platelet count (Plt), prothrombin time (PT), albumin (Alb), total bilirubin (T-Bil), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), AFP and DCP. The serologically defined HCV genotype (HCV serotype) was determined using a serological genotyping assay kit (Immunocheck F-HCV Grouping; International Reagents Co., Tokyo, Japan). If the HCV serotype could not be determined, the HCV genotype was evaluated using the HCV Core Genotype assay (SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, while genotypes 2a and 2b were included with serotype II. No other HCV genotype was detected in this study population. HCV RNA titers were quantified using either quantitative RT-PCR (Amplicor monitor version 2, Roche, Tokyo, Japan) or the Cobas TaqMan PCR assay (Roche, Tokyo, Japan). Patients were categorized as having a high viral load if their values were 100 KIU/mL or greater based on quantitative RT-PCR analysis, or 5 log IU/mL or more based on the Cobas TaqMan PCR assay.

Evaluation of clinical stage

Hepatic function was assessed in the HCC group using Child-Pugh staging based on both clinical (ascites and encephalopathy) and laboratory (Alb, T-Bil, and PT) parameters. HCC clinical stage was assessed based on a patient's Cancer of the Liver Italian Program (CLIP) score, which was calculated by adding points for the following four variables: Child-Pugh stage, tumor morphology, AFP value, and portal venous invasion[21,22]. The Japan Integrated Staging (JIS) system[23,24], developed by the Liver Cancer Study Group of Japan and based on a combination of Child-Pugh stage and HCC TNM classification, was used to clinically stage HCC.

Serum MnSOD and TRX levels

Serum was obtained from peripheral blood samples by centrifugation at 4000 *g* for 5 min at room temperature. Serum samples were frozen at -80 ℃ until further use. Serum MnSOD or TRX levels were measured using the Human Superoxide Dismutase 2 (AbFRONTIER, Seoul, Korea) and human thioredoxin (Redox Bio Science, Kyoto, Japan) ELISAs, respectively.

Statistical analysis

Results are expressed as the mean and standard deviation. *P* values less than 0.05 were regarded as statistically significant. Statistical analyses were performed using the Fischer's exact test or the Mann-Whitney *U* test, as appropriate. The area under the curve (AUC) was calculated for the receiver operating characteristic (ROC) curve in order to measure the overall accuracy of the test. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of diagnostic test were additionally determined according to the protocol described previously[25]. Differences among the three groups were evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparison tests. Correlation coefficients were calculated using Spearman's rank correlation analysis. The Kaplan-Meier method was used to estimate death for each parameter that had been identified at enrollment, and the death distribution curves were compared using the log-rank test. Univariate and multivariate analyses of patient outcome risk ratios were performed using Cox's proportional hazards regression analyses. All statistical analyses were conducted using PASW Statistics v. 18 (SPSS Inc., Chicago, IL).

Table 1 Patient clinical characteristics

Characteristics	Non-HCC group (*n* = 34)	HCC group (*n* = 30)	*P* value[1]
Age (yr)	62.3 ± 11.0	72.2 ± 7.5	< 0.001
Sex (male/female)	10/24	21/9	< 0.01
Plt (× 10^4/μL)	17.0 ± 5.5	10.3 ± 5.2	< 0.001
PT (%)	99.7 ± 13.3	77.6 ± 11.8	< 0.001
Alb (g/dL)	4.3 ± 0.4	3.6 ± 0.6	< 0.001
T-Bil (mg/dL)	0.8 ± 0.3	1.5 ± 0.8	< 0.001
ALT (IU/L)	44.8 ± 30.2	52.0 ± 28.2	0.12
γ-GTP (IU/L)	31.3 ± 16.1	56.2 ± 44.3	< 0.01
AFP (ng/mL)	7.2 ± 22.8	85.9 ± 197.6	< 0.001
DCP (mAU/mL)	22.8 ± 14.7	485.5 ± 1982.6	0.001
HCV serotype group (1/2)	18/10 (*n* = 28)	21/3 (*n* = 24)	0.06
HCV RNA level (high/low)	28/5 (*n* = 33)	21/4 (*n* = 25)	0.99

Data are shown as the mean ± SD. *n*: Number of patients or the number of samples analyzed. [1]Differences between mean values were evaluated using either the Fischer's exact test or the Mann-Whitney *U* test, as appropriate. Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: alpha-fetoprotein; DCP: des-γ-carboxy prothrombin; HCV: Hepatitis C virus; RNA: Ribonucleic acid.

RESULTS

Patient characteristics and classification according to the presence of hepatocellular carcinoma

Table 1 summarizes the baseline clinical characteristics of the 64 patients who were classified based on the presence or absence of HCC. Age, sex, and clinical laboratory parameters, including Plt, PT, Alb, T-Bil, γ-GTP, AFP and DCP, were significantly different between these two groups.

Serum MnSOD and TRX levels in hepatocellular carcinoma patients

Serum MnSOD levels were significantly higher in patients with HCC compared to patients without HCC ($P = 0.03$) and HVs ($P < 0.001$) (Figure 1A). The serum TRX levels were also significantly higher in the HCC group compared to the non-HCC group ($P = 0.04$) and HV group ($P < 0.01$) (Figure 1B). However, there was no correlation between these two markers in the HCC group ($P = 0.28$, $r = 0.20$) (Figure 1C).

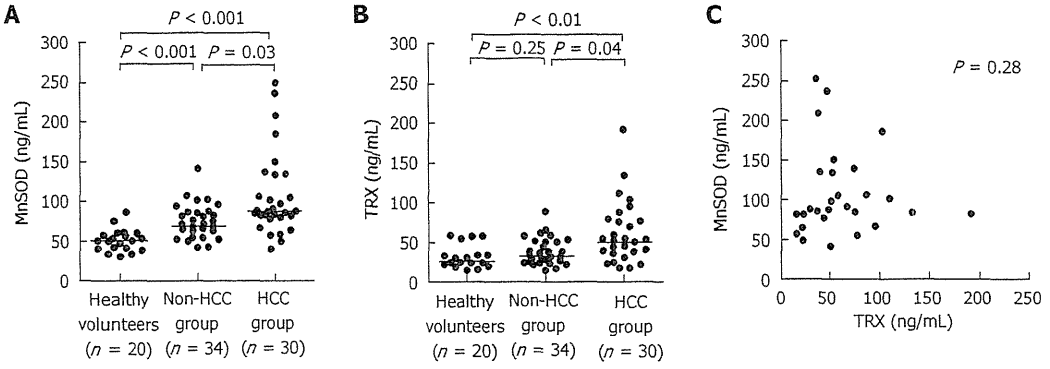

Figure 1 Serum levels of manganese superoxide dismutase and thioredoxin in the hepatocellular carcinoma, non-hepatocellular carcinoma and healthy volunteer groups. A: Serum manganese superoxide dismutase (MnSOD) levels were significantly higher in the hepatocellular carcinoma (HCC) group than in either the non-HCC group ($P = 0.03$) or the healthy volunteers (HV) group ($P < 0.001$); B: Serum thioredoxin (TRX) levels were also significantly higher in the HCC group than in either the non-HCC group ($P = 0.04$) or the HV group ($P < 0.01$); C: No significant correlation was detected between serum MnSOD and TRX levels in the HCC group.

Table 2 Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of manganese superoxide dismutase and α-fetoprotein serum levels for diagnosis of hepatocellular carcinoma in all patients (%)

Factors	Sensitivity	Specificity	PPV	NPV	Accuracy
MnSOD (≥ 110 ng/mL)	26.7	97.1	88.9	60.0	64.1
AFP (≥ 40 ng/mL)	33.3	97.1	90.9	62.3	67.2
Combination[1]	46.7	94.1	87.5	66.7	71.9

[1]MnSOD ≥ 110 ng/mL and/or AFP ≥ 40 ng/mL. PPV: Positive predictive value; NPV: Negative predictive value; MnSOD: Manganese superoxide dismutase; AFP: α-fetoprotein.

Table 3 Correlation between serum manganese superoxide dismutase or thioredoxin levels and laboratory data in the hepatocellular carcinoma group

Factors	HCC group (n = 30)			
	Serum MnSOD levels		Serum TRX levels	
	Correlation coefficient	P value	Correlation coefficient	P value
Age (yr)	-0.97	0.61	0.11	0.55
Plt (× 10^4/μL)	0.03	0.89	0.66	< 0.001
PT (%)	-0.36	0.05	0.12	0.53
Alb (g/dL)	-0.63	< 0.001	0.19	0.33
T-Bil (mg/dL)	0.25	0.18	0.05	0.79
ALT (IU/L)	0.12	0.52	0.15	0.42
γ-GTP (IU/L)	0.30	0.11	0.28	0.13
AFP (ng/mL)	0.38	0.04	0.11	0.57
DCP (mAU/mL)	0.57	0.001	0.12	0.52

P values were assessed by Spearman's rank correlation analysis. MnSOD: Manganese superoxide dismutase; TRX: Thioredoxin; HCC: Hepatocellular carcinoma; Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: α-fetoprotein; DCP: des-γ-carboxy prothrombin.

Diagnostic value of serum MnSOD and TRX levels for patients with hepatocellular carcinoma and hepatitis C virus infection

Serum AFP and DCP concentrations are established diagnostic markers for HCC. To evaluate the utility of MnSOD and TRX for the diagnosis of HCC, we measured AFP and DCP expression in addition to MnSOD and TRX expression. In an AUC-ROC analysis, AFP was the strongest diagnostic marker for HCC (AUC-ROC, 0.90). AUC-ROCs for MnSOD, TRX and DCP were 0.73, 0.77 and 0.77, respectively. Additional analyses showed that the accuracy of AFP (≥ 40 ng/mL) for diagnosis of HCC was higher than that of MnSOD (≥ 110 ng/mL) (Table 2), while the combination of AFP and MnSOD was a more accurate marker of HCC than either marker alone.

Association of serum MnSOD or TRX levels with laboratory data in the HCC group

Serum MnSOD levels for the 30 patients in the HCC group were positively correlated with serum AFP and DCP levels and were negatively correlated with serum Alb levels (Table 3). Serum MnSOD levels were also significantly higher in patients with two or more HCC tumors than in patients with a single HCC tumor [average ± SD (ng/mL), 125.4 ± 50.9 *vs* 87.4 ± 48.8, $P = 0.008$], although HCC tumor size was not associated with serum MnSOD levels. In addition, HCC patient serum MnSOD levels increased in parallel with the Child-Pugh stage, CLIP score and JIS score (Figure 2A-C). In contrast, serum TRX levels were only associated with platelet counts (Table 3). Serum TRX levels were not associated with HCC tumor number or size. Furthermore, there were no significant correlations between serum TRX levels for various scores (Figure 2D-F).

Overall survival rate based on serum MnSOD or TRX levels in the HCC group

In the HCC group, the overall patient survival rate was significantly lower ($P = 0.01$) in patients with MnSOD levels ≥ 110 ng/mL compared to patients with levels < 110 ng/mL (Figure 3A). In addition, the overall survival rate tended to be lower ($P = 0.05$) in patients with TRX levels < 80 ng/mL compared to those with levels ≥ 80 ng/mL (Figure 3B). Furthermore, among all HCC groups, patients who had both serum MnSOD levels ≥ 110

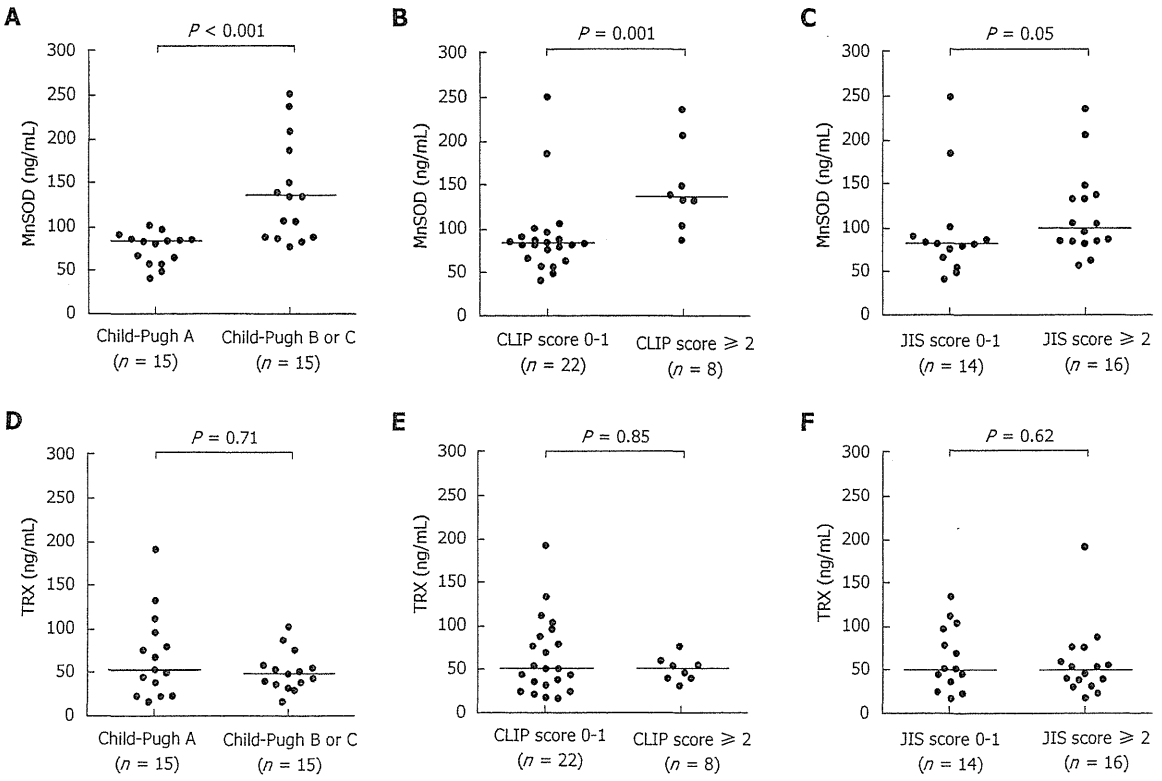

Figure 2 Clinical significance of serum manganese superoxide dismutase and thioredoxin levels in hepatocellular carcinoma. In the hepatocellular carcinoma (HCC) group, differences in serum manganese superoxide dismutase (MnSOD) and thioredoxin (TRX) levels were evaluated based on Child-Pugh stage, cancer of the liver italian program (CLIP) score and Japan integrated staging (JIS) score. A: Serum MnSOD levels were significantly higher in patients with Child-Pugh B or C compared to those with Child-Pugh A ($P < 0.001$); B: Serum MnSOD levels in patients with a CLIP score of 2 or greater were significantly higher compared to levels in patients with a CLIP score of 0 or 1 ($P = 0.001$); C: In addition, serum MnSOD levels tended to be higher in patients with a JIS score of 2 or greater compared to patients with a JIS score of 0 or 1 ($P = 0.05$); D-F: In contrast, serum TRX levels were not significantly different based on Child-Pugh stage, CLIP score or JIS score.

ng/mL and TRX levels < 80 ng/mL had a significantly poorer prognosis. Conversely, patients with a serum TRX level ≥ 80 ng/mL had a favorable prognosis, regardless of their serum MnSOD level (Figure 3C).

In addition to serum MnSOD and TRX levels, other possible prognostic factors were also investigated in the HCC group. A univariate analysis (log-rank test) revealed that the survival rate was significantly different between patients with high and low levels of MnSOD, PT, Alb, AFP and DCP, but not other factors such as TRX (Table 4). A multivariate analysis using a Cox proportional hazard model and five markers (MnSOD, PT, Alb, AFP and DCP) selected based on the results of the univariate analysis revealed that MnSOD levels ≥ 110 ng/mL and AFP levels ≥ 40 ng/mL were independent risk factors that were associated with a poor patient prognosis (Table 5). In addition, similar results were obtained from a similar multivariate analysis using the same five factors and TRX, supporting the finding that TRX is not an independent risk factor associated with HCC prognosis. Furthermore, patient Child-Pugh stage, CLIP score and JIS score, which were calculated based on several factors including clinical symptoms and laboratory data, were also prognostic factors for patients with HCC (Table 4). A multivariate analysis using the three markers of MnSOD, Child-Pugh stage and CLIP score indicated that Child-Pugh stage was also a significant prognostic factor (risk ratio: 6.19, 95% confidential interval: 1.33-28.95, $P = 0.02$).

DISCUSSION

HCV infection is the most important known contributor to the etiology of HCC. An increasing incidence of HCC has been largely attributed to a rise in HCV infections in the general population during the last 50 to 60 years[26]. During HCV infection, ROS production increases and persists throughout the infection. In addition, ROS are thought to play a major role in the pathogenesis of chronic inflammatory changes in the liver, leading to increased hepatic fibrosis and decreased hepatic function. In this study, we have shown that both serum MnSOD and TRX levels are elevated in patients with HCV-related HCC, with no correlation between these two markers. In addition, serum MnSOD and TRX levels were a useful predictor of overall patient survival. Serum MnSOD and TRX levels are reported to be biomarkers of oxidative stress in several diseases, including liver disease[7,14,17,19,27-29]. There were a small number of enrolled patients in this study and other contributors to liver diseases such as chronic hepatitis B infection should be further evaluated. However, our

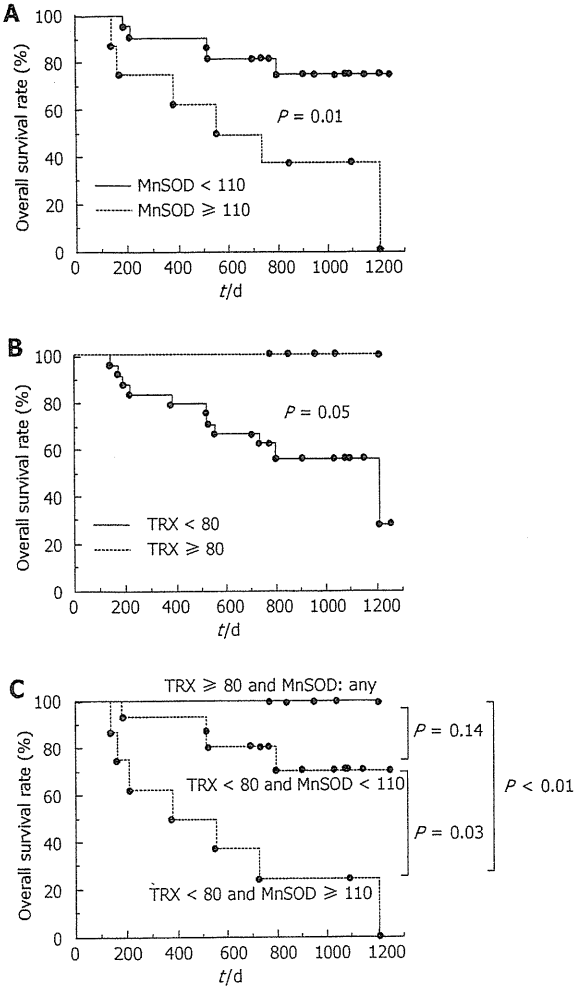

Figure 3 Overall hepatocellular carcinoma patient survival based on serum levels of manganese superoxide dismutase or thioredoxin. Overall survival was plotted using the Kaplan-Meier method after separation into two or three groups defined as follows: A: Manganese superoxide dismutase (MnSOD) < 110 ng/mL or ≥ 110 ng/mL; B: Thioredoxin (TRX) < 80 ng/mL or ≥ 80 ng/mL; TRX ≥ 80 ng/mL, TRX < 80 ng/mL; C: MnSOD < 110 ng/mL, or TRX < 80 ng/mL and MnSOD ≥ 110 ng/mL. The overall survival rate was lower in patients with MnSOD levels ≥ 110 ng/mL (P = 0.01) (A). Also, cumulative patient survival rate tended to be lower in patients with TRX levels < 80 ng/mL (P = 0.05) (B). Among these groups, patients with serum TRX levels < 80 ng/mL and serum MnSOD levels ≥ 110 ng/mL had the poorest prognosis (C).

Table 4 Univariate analysis of prognostic factors in the hepatocellular carcinoma group

Factors	Category	Number	P value[1]
Single marker			
MnSOD (ng/mL)	< 110/≥ 110	22/8	0.01
TRX (ng/mL)	< 80/≥ 80	24/6	0.05
Age (yr)	< 70/≥ 70	12/18	0.23
Plt (× 10^4/μL)	< 10/≥ 10	19/11	0.38
PT (%)	< 80/≥ 80	15/15	0.02
Alb (g/dL)	< 3.5/≥ 3.5	15/15	0.02
T-Bil (mg/dL)	< 1.5/≥ 1.5	18/12	0.34
ALT (IU/L)	< 40/≥ 40	11/19	0.58
γ-GTP (IU/L)	< 50/≥ 50	17/13	0.98
AFP (ng/mL)	< 40/≥ 40	20/10	< 0.01
DCP (mAU/mL)	< 40/≥ 40	16/14	0.02
Staging system			
Child-Pugh stage	A/≥ B	16/14	< 0.01
CLIP score	0-1/≥ 2	22/8	0.01
JIS score	0-1/≥ 2	14/16	0.41

[1]P values were assessed using the log-rank test. MnSOD: Manganese superoxide dismutase; TRX: Thioredoxin; Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: Alpha-fetoprotein; DCP: Serum des-γ-carboxy prothrombin; CLIP: Cancer of the Liver Italian Program; JIS: Japan Integrated Staging.

Table 5 Multivariate analysis of prognostic factors in the hepatocellular carcinoma group

Factors	Risk ratio	95% CI	P value
MnSOD (≥ 110 ng/mL)	4.12	1.22-13.88	0.02
AFP(≥ 40 ng/mL)	6.75	1.70-26.85	< 0.01

95% CI: 95% confidence interval; MnSOD: Manganese superoxide dismutase; AFP: α-fetoprotein.

study has clearly demonstrated the clinical significance of these markers in patients with HCV-related HCC.

Serum MnSOD and TRX levels should both reflect hepatic oxidative stress. The results of the current study showed that both of these markers were increased in the HCC group relative to levels in the non-HCC group and the HV group (Figure 1A and B). However, there was no correlation between these two markers in the HCC group (Figure 1C). MnSOD is primarily localized to the mitochondrial matrix[3] and abnormal mitochondrial morphologies are frequently observed in CHC[8]. Therefore, MnSOD may be an indicator of mitochondrial disorders that are induced by oxidative stress. On the other hand, there are two TRX proteins, cytoplasmic TRX1 and mitochondrial TRX2[30]. TRX1 negatively regulates the apoptosis signal-regulating kinase 1 (ASK1)-c-Jun N-terminal kinase/P38 apoptotic pathway by binding to and inhibiting the kinase activity of ASK1, which plays an important role in ROS-induced cellular responses[31]. TRX2 is an essential regulator of mitochondrial ROS levels that has been associated with mitochondrial outer membrane permeability[32]. In the present study, we examined the serum levels of TRX1, but not TRX2, using a sandwich ELISA. Thus, the MnSOD and TRX proteins that were examined in this study have different origins in the mitochondria and cytoplasm, respectively, which could contribute to the lack of correlation between these two markers.

Several studies have shown that the HCV core protein directly inhibits the electron transport system and modulates apoptosis, transcription, and cell signaling[33]. Abdalla *et al*[34] reported that expression of not only the HCV core protein but also the HCV NS proteins increases ROS and further showed that the presence of these proteins can increase endogenous expression levels of antioxidant enzymes and prooxidants such as MnSOD. Several reports have shown that serum MnSOD levels in patients with HCV-related CLD[35-37] are associated with

various clinical findings, such as fibrosis and hepatic oxidative stress. However, the significance of serum MnSOD levels has not been fully examined in patients with HCC. We previously reported that serum MnSOD levels may be correlated with fibrosis in patients with NAFLD[7]. In addition, serum MnSOD levels decreased in patients with CHC after administration of an interferon-based treatment (data not shown). These results indicate that serum MnSOD levels are likely associated with hepatic fibrosis or oxidative stress in patients with CHC. In the present study, however, MnSOD levels were not associated with platelet counts, which is a simple predictor of hepatic fibrosis in this patient population[38]. Thus, advanced hepatic fibrosis or oxidative stress may be one reason why serum MnSOD levels have diagnostic and prognostic utility with HCC, but other mechanisms should also be considered.

The present study revealed that serum MnSOD levels were significantly higher in the HCC group than in the non-HCC group (Figure 1A). In the HCC group, serum MnSOD levels were negatively correlated with serum Alb and tended to negatively correlate with PT (Table 3); these results showed an association between MnSOD and Child-Pugh stage (Figure 2A). It is known that in humans, MnSOD activity is comparatively higher in the liver compared to other tissues[39]. In addition, although a previous immunohistochemical study showed that MnSOD expression was higher in both cancerous and non-cancerous liver tissues from patients with HCC, this positive immunoreactivity was strongly observed in non-cancerous liver tissues, especially in normal hepatocytes surrounding HCC, regenerative small hepatocytes in the tumor boundary, and mononuclear inflammatory cells in necroinflammatory lesions[40]. Furthermore, ROS are overproduced by Kupffer cells and inflammatory cells in liver disease[5,41]. In the present study, serum MnSOD levels were also positively correlated with the serum tumor markers AFP and DCP (Table 3) and with Child-Pugh stage and CLIP score (Figure 2). These results indicate that increased MnSOD expression reflects hepatocyte oxidative stress and correlates with decreased hepatic function, increased hepatic fibrosis and ROS production by inflammatory cells in liver cirrhosis. These features comprise the main background characteristics leading to HCC and may be associated with the indirect effects of liver cancer progression. These associations may also explain why serum MnSOD levels predicted the overall survival of patients with HCC.

It was previously reported that serum levels of TRX, which is a stress-induced protein, increase relative to the degree of hepatic fibrosis, and that high serum concentrations of TRX may indicate advanced hepatic fibrosis[19,20]. In contrast, it has also been reported that a higher degree of hepatic fibrosis is associated with lower platelet counts[38]. Therefore, the present study may present a conflict, since results indicated that serum TRX level was positively correlated with platelet count. A previous report showed that the survival rate following LPS plus GalN-induced hepatitis was much higher in transgenic mice overexpressing TRX than in wild-type mice, and that thioacetamide-induced hepatic fibrosis was suppressed in TRX transgenic mice compared to wild-type mice[42]. Although it is still unclear why TRX and platelet counts are positively correlated, we speculate that elevated serum TRX in patients with HCC and advanced hepatic fibrosis potentially improves overall survival by suppressing oxidative stress[43]. In addition, patients with HCC, low levels of TRX, and high levels of MnSOD, which may be indicative of excessive oxidative stress without TRX attenuation, have the poorest prognosis. This result supports the hypotheses presented above. In order to better assess these findings, future studies are needed that incorporate sequential observations of serum TRX and MnSOD levels over time in patients with chronic hepatitis, cirrhosis and HCC.

Serum MnSOD and TRX may be useful biomarkers for HCC diagnosis (Figure 1). AFP is also a diagnostic marker for HCC, and the present results indicate that AFP can be used to distinguish between patients with and without HCC (Table 2). However, AFP is not a sufficiently sensitive marker for identification of the majority of patients with small HCCs[44,45], and AFP testing is not currently included in the recommendations for HCC surveillance in the updated HCC guidelines published by the American Association for the Study of Liver Disease[46]. Therefore, clinicians and clinical researchers should consider using MnSOD and TRX as diagnostic biomarkers for early HCC or as additional markers in a HCC surveillance program using ultrasonography or AFP. In addition, it is highly important to know whether these markers decrease in response to HCC therapy and reductions in tumor burden. These markers also may have utility in patients on a transplant waiting list who are treated with neo-adjuvant therapy for tumor downstaging.

Our study demonstrated that elevated serum AFP level is indicative of a poor prognosis for patients with HCC (Table 4), as was previously reported[47]. The CLIP score, which is calculated based on four factors such as the AFP value, was also useful to predict the prognosis of HCC patients in this study as well as in a previous report[48]. Other markers such as the protein survivin have been reported as poor prognostic factors for HCC[49]. Similarly, MnSOD was an independent predictive factor for overall survival in the HCC group (Figure 3A, Table 5). Although TRX was not an independent predictor of overall survival in patients with HCC (Table 4), we speculate that a combination assay using both MnSOD and TRX could be used to predict overall patient survival. It will be important to conduct further prospective evaluations of each individual marker as well as a combination of these markers using a large number of patients.

In conclusion, serum MnSOD and TRX levels increased as HCV-related chronic liver disease progressed, especially among patients with HCC. Although there was no correlation between serum levels of MnSOD and TRX, higher serum MnSOD levels and lower TRX levels in patients with HCC trended towards an indication of poor

patient prognosis. These results suggest that serum Mn-SOD and TRX levels are not only a potential biomarker for HCV-related progressed liver disease, but may also serve as prognostic markers in HCC.

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COMMENTS

Background

During hepatitis C virus (HCV) infection, production of reactive oxygen species (ROS) is persistently increased throughout HCV infection. ROS are thought to play an important role in the pathogenesis of chronic inflammatory changes in the liver, which may lead to the development of hepatic fibrosis, decreased hepatic function or hepatocellular carcinoma (HCC). However, there is little information currently available regarding serum oxidative stress markers in patients with HCV-related HCC.

Research frontiers

Cells are protected from oxidative stress by antioxidant enzymes such as superoxide dismutase (SOD) and by intracellular antioxidants such as thioredoxin (TRX). Serum manganese SOD (MnSOD) and TRX are thought to be biomarkers for various liver diseases, including HCV-related liver disease, but these possibilities have not been fully investigated. In this study, the authors demonstrated the clinical significance of serum levels of MnSOD and TRX in patients with HCV-related HCC.

Innovations and breakthroughs

Although there was no correlation between serum levels of MnSOD and TRX, serum levels of both markers increased as HCV-related chronic liver disease progressed, and in particular among patients with HCC. In addition, higher serum MnSOD levels and lower TRX levels tended to indicate a poor prognosis among patients with HCC.

Applications

Serum MnSOD and TRX levels are not only potential biomarkers for progression of HCV-related liver disease, but they may also serve as prognostic markers for patients with HCC. Therefore, clinicians should consider using serum levels of MnSOD and TRX as diagnostic biomarkers for early HCC or as additional markers in HCC surveillance programs. In addition, it will be important to know whether these markers change after therapy for liver disease, including HCC.

Peer review

Oxidative stress is closely associated with carcinogenesis. If oxidative stress markers could be useful in predicting clinical outcome in chronic hepatitis C and HCV-related HCC, they would provide us with a practical and informative tool. However, there are some limitations of this investigation, including a relatively small number of patients studied. Thus, the overall assessment is "good".

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Highly sensitive lens culinaris agglutinin-reactive α-fetoprotein is useful for early detection of hepatocellular carcinoma in patients with chronic liver disease

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Abstract. The fucosylated fraction of α-fetoprotein (AFP-L3) is a specific marker for hepatocellular carcinoma (HCC). However, conventional AFP-L3% (c-AFP-L3%) has not always been reliable in cases with low serum α-fetoprotein (AFP) levels. In this study, we evaluated the clinical utility of a newly developed assay, highly sensitive AFP-L3% (hs-AFP-L3%). Subjects included 74 patients with benign liver disease (BLD), including chronic hepatitis and cirrhosis, and 94 with HCC. Serum hs-AFP-L3% was significantly higher than c-AFP-L3% in patients with early-stage HCC (solitary or <20 mm in diameter). Additionally, hs-AFP-L3% was significantly increased in patients with well-differentiated HCC. In patients with serum AFP <20 ng/ml, the sensitivities of c-AFP-L3% and hs-AFP-L3% were 12.5 and 44.6%, respectively, at a cut-off value of 5%. In 59 BLD patients with serum AFP <20 ng/ml, the HCC-positive rate in patients with hs-AFP-L3% ≥5% was significantly higher compared to those with hs-AFP-L3% <5% during the follow-up period (median, 35 months; range, 5-48 months). Importantly, none of the BLD patients with both serum AFP <20 ng/ml and hs-AFP-L3% <5% developed HCC. These results indicated that hs-AFP-L3% is useful for early detection of HCC in BLD patients, even for those with serum AFP <20 ng/ml. Furthermore, since hs-AFP-L3% increases before HCC is detectable by various advanced imaging modalities, this assay may help identify BLD patients with a higher risk of HCC.

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Key words: α-fetoprotein, α-fetoprotein fucosylated fraction L3, hepatocellular carcinoma, hepatocarcinogenesis

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world, and the third most common cause of cancer-related death (1). Although it is more common in Asia and Africa, its incidence in the United States has increased over the past two decades, largely due to the spread of hepatitis C (HCV) infection, which is an underlying risk factor (2). Early detection of HCC increases the potential for curative treatment and improves prognosis. Several methods developed for the diagnosis of HCC, including evaluation of serum markers, ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI), have been tested clinically. α-fetoprotein (AFP) and des-γ carboxy prothrombin (DCP), serum proteins that are elevated in HCC, are the most widely used markers. Although routine screening offers the best chance for early tumor detection, the reported sensitivities and specificities of elevated serum AFP and DCP levels vary significantly (3-8). Furthermore, serum AFP levels increase in only 30-40% of patients with HCC, especially early in the disease process (5). Additionally, an increase in serum AFP is also seen in patients with non-cancerous conditions, including cirrhosis or exacerbation of chronic hepatitis (9). AFP-L3, the lectin lens culinaris agglutinin-bound fraction, is one of the three glycoforms of AFP, and is the major glycoform elevated in the serum of HCC patients. The reported sensitivities of AFP-L3 as a method of detecting HCC range from 75-97% with specificities of 90-92% (10,11). In cases of HCC, however, high percentage of AFP-L3 is closely associated with poor differentiation and biologically malignant characteristics, including portal vein invasion, of neoplastic cells (11,12). Therefore, it is not clear how useful this test is for the early detection of HCC. Additionally, measurement of AFP-L3 has not always been reliable for serum samples with low total AFP concentration, as determined by conventional lectin affinity system (LiBASys) (13).

Recently, a novel automated immunoassay for AFP-L3 has been developed. The new method uses on-chip electrokinetic reaction and separation by affinity electrophoresis (micro-total

analysis system; μ-TAS) (14). In patients with an AFP level of ≥20 μg/ml, μ-TAS AFP-L3% correlated well with LiBASys AFP-L3% (15). Furthermore, this system has enabled the accurate measurement of AFP-L3% at very low AFP concentrations. Therefore, in this retrospective study, we investigated the clinical utility of the new highly sensitive μ-TAS AFP-L3% assay for diagnosis of HCC in a population of patients with HCC or benign liver diseases (BLD), including chronic hepatitis or cirrhosis.

Patients and methods

Patients. Between December 2006 and September 2010, frozen serum samples were obtained from 94 patients with HCC, as well as from 74 patients with BLD, who had chronic hepatitis or liver cirrhosis, but not HCC (Table I). All patients met the eligibility criteria (availability of stored serum samples and written informed consent). Among the BLD patients, 20 were positive for hepatitis B surface antigen (HBsAg), 43 were positive for anti-hepatitis C virus (HCV) antibody, and 11 were negative for either HBsAg or anti-HCV antibody. The BLD patients were followed after serum sampling for 32.8±12.3 months (median, 35; range, 5-48); liver imaging was performed by US at 6- to 12-month intervals in most patients with chronic hepatitis, and CT, MRI, or US was performed at 3- to 6-month intervals in patients with liver cirrhosis.

HCC patients were diagnosed using imaging modalities such as US, MRI and CT during hepatic arteriography. Vascular invasion was evaluated by imaging modalities. In some cases that showed atypical features upon imaging, ultrasound-guided biopsies were performed. Based on imaging findings, tumor stage was ranked using the tumor-node-metastasis (TMN) staging system of the Liver Cancer Study Group of Japan (16,17): T1 (fulfilling the following three conditions: solitary, 2 cm, no vessel invasion), T2 (fulfilling two of the three conditions), T3 (fulfilling one of the three conditions), T4 (fulfilling none of the three conditions or showing presence of distant metastasis); N0 (no lymph node metastasis), N1 (metastasis to lymph nodes); M0 (no distant metastasis), M1 (distant metastasis); stage 1 (T1N0M0), stage II (T2N0M0), stage III (T3N0M0), and stage IV (T4N0M0 or any TN1M0, or any TN0-1M1).

Measurement of serum AFP and AFP-L3%. For the HCC group, AFP and AFP-L3% were measured in the same sample obtained at the time of HCC diagnosis, before any treatment. For the BLD without HCC group, measurements were made at the time of diagnosis of chronic liver disease. Highly sensitive AFP-L3% (hs-AFP-L3%) were measured by a microchip capillary electrophoresis and liquid-phase binding assay on a μ-TASWako i30 auto analyzer (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (15). Conventional AFP-L3% (c-AFP-L3%) was examined using a column chromatography and liquid-phase binding assay on a LiBASys auto analyzer (Wako Pure Chemical Industries, Ltd.) (13). The analytical sensitivity of the μ-TASWako i30 auto analyzer is 0.3 μg/ml AFP; the AFP-L3% can be measured when AFP-L3 is over 0.3 μg/ml. Although the analytical sensitivity of the LiBASys is 0.8 μg/ml AFP, AFP-L3% cannot be measured at AFP <10 ng/ml. Therefore, the correlation between μ-TAS-L3% and LiBA-L3% was poor at AFP <20 ng/ml.

Statistical analysis. We used the Mann-Whitney U test, Z test and Chi-square test for evaluation of the statistical significance of each finding. SPSS version 17.0J (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis; $p<0.05$ was considered to indicate statistical significance.

Results

Clinical feature of patients. The demographics, etiology of liver disease, hepatic functional reserve ranked by Child-Pugh classification, tumor stage, tumor size and tumor number of the study patients are summarized in Table I. The HCC group included 94 patients: 35 patients with stage I, 35 with stage II, 14 with stage III, and 10 with stage IV; thus, ~75% of HCC cases were stage I or II. The incidence of cirrhosis in HCC patients (55.3%) was significantly higher than in BLD (25.7%), whereas the hepatic reserve expressed by Child-Pugh classification of HCC patients was significantly preserved compared with BLD patients.

Serum AFP levels in patients with HCC were significantly higher than those with BLD (Table I and Fig. 1A). hs-AFP-L3% was measurable in 47.3 and 78.7% of patients with BLD and HCC, respectively, whereas c-AFP-L3% was detected in 31.1 and 63.8% of patients. Thus, hs-AFP-L3% was significantly higher than c-AFP-L3% in both BLD and HCC patients (Table I and Fig. 1B). Since a cut-off value of 5% has been reported to be useful for diagnosis of HCC using hs-AFP-L3% (18), the cut-off value for AFP-L3% was set at 5% in the present study. The sensitivity and specificity of hs-AFP-L3% were 57.0 and 63.5%, respectively, whereas those of c-AFP-L3% were 40.4 and 81.1%.

hs-AFP-L3% significantly increases in HCC patients at early stage. Next, we analyzed serum AFP levels, c-AFP-L3% and hs-AFP-L3%, and compared early and advanced stages of HCC (Fig. 2). When compared with HCC patients with stage I or II cancer, serum AFP levels were significantly increased in patients with stage III and IV disease (Fig. 2A). Both c-AFP-L3% and hs-AFP-L3% in HCC patients with advanced stages were also significantly higher than in patients with early stages (Fig. 2B). Although 86% of HCC patients with stage I (n=35) exhibited serum AFP <20 ng/ml, c-AFP-L3% and hs-AFP-L3% were measurable in 46 and 69% of these patients, respectively; hs-AFP-L3% was significantly higher than c-AFP-L3%. Consequently, in HCC patients at stage I, the sensitivity of c-AFP-L3% or hs-AFP-L3% at a cut-off level of 5% were 17.1 or 48.6%, respectively.

Next, we evaluated the relationship between AFP-L3% and tumor number or size (Fig. 3). hs-AFP-L3% was significantly higher than c-AFP-L3%, even in patients with single or small HCC (<20 mm in diameter) (Fig. 3). Conversely, when compared to HCC patients with solitary or small HCC, both c-AFP-L3% and hs-AFP-L3% were increased in cases with multiple or ≥20 mm HCC, and there was no statistical difference between c-AFP-L3% and hs-AFP-L3%. These results indicate that hs-AFP-L3% is a useful biomarker for detecting early-stage HCC.

An increase in hs-AFP-L3% is observed in both BLD and HCC patients with AFP <20 ng/ml. We analyzed c-AFP-L3%

Table I. Clinical features of patients with BLD and HCC.

	BLD (n=74)	HCC (n=94)	p-value
Age	56.23±13.88	65.76±12.98[a]	<0.001
Gender (male/female)	30/44	56/38[a]	0.015
CH/LC	55/19	42/52[a]	<0.001
HBV/HCV/NBNC	20/43/11	5/61/28[a]	<0.001
Child-Pugh class (A/B/C/unknown)	39/5/4/26	75/19/0/0[a]	<0.001
TNM stage (I/II/III/IV)		35/35/14/10	
Tumor size (mean ± SD)		22.35±16.42	
<20 mm/≥20 mm		58/36	
Tumor number (single/multiple)		50/44	
AFP (ng/ml)	46.17±163.6	2871.5±9882.7[a]	<0.001
c-AFP-L3%	2.96±6.45	18.19±26.95[a]	<0.001
hs-AFP-L3%	3.84±5.59	21.12±29.01[a]	<0.001
Platelet count (x10^4/μl)	14.98±6.82	11.39±4.73[a]	0.001
AST (IU/l)	70.55±95.87	55.78±22.92	0.099
ALT (IU/l)	85.38±144.71	48.28±24.13	0.783

BLD, benign liver disease; HCC, hepatocellular carcinoma; CH, chronic hepatitis; LC, liver cirrhosis; HBV, hepatitis B virus; HCV, hepatitis C virus; hs-AFP-L3%, hypersensitive-AFP-L3%; c-AFP-L3%, conventional-AFP-L3%.

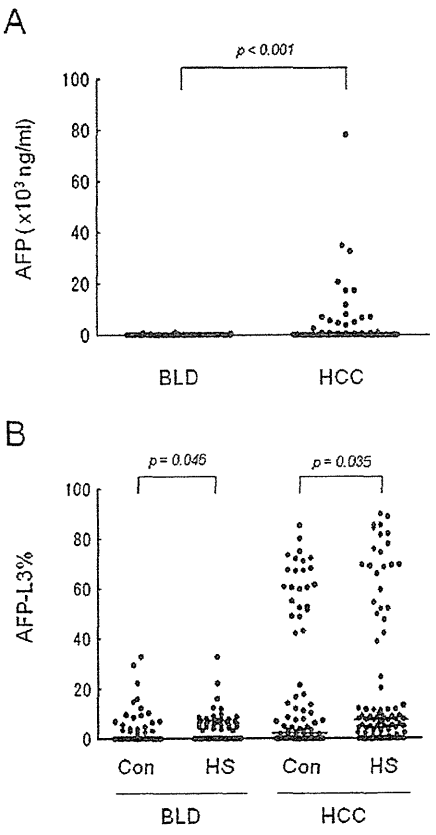

Figure 1. Serum levels of AFP, c-AFP-L3% and hs-AFP-L3% in patients with BLD or HCC. (A) Serum AFP concentrations in HCC patients (n=94) were significantly higher than those in BLD (n=74). (B) hs-AFP-L3% (HS) significantly increased in comparison with c-AFP-L3% (Con) in both BLD and HCC patients.

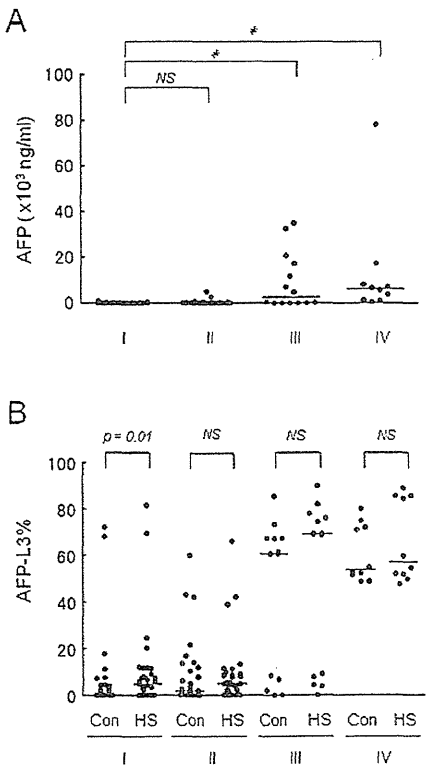

Figure 2. Serum levels of AFP, c-AFP-L3% and hs-AFP-L3% in patients with early or advanced HCC. (A) Serum AFP levels in HCC patients at stage III (n=14) or IV (n=10) were significantly higher than those at stage I (n=35) or II (n=35). *p<0.05. (B) hs-AFP-L3% (HS) was significantly higher than c-AFP-L3% (Con) in patients with HCC at stage I, whereas there was no significant difference between c- and hs-AFP-L3% in HCC patients at stages II, III and IV.

Table II. Clinical features of BLD and HCC patients with AFP <20 ng/ml.

	BLD (n=59)	HCC (n=56)	p-value
Age	56.78±13.51	68.88±12.05[a]	<0.001
Gender (male/female)	23/36	26/30	0.422
CH/LC	45/14	25/31[a]	0.001
HBV/HCV/NBNC	14/35/10	5/32/19[a]	0.008
Child-Pugh class (A/B/C/unknown)	31/4/1/23	50/6/0/0[a]	<0.001
TNM stage (I/II/III/IV)		30/21/5/0	
Tumor size (mean ± SD)		16.16±11.59	
<20 mm/≥ 20 mm		47/9	
Tumor number (single/multiple)		35/21	
AFP (ng/ml)	4.68±3.6	8.92±5.23[a]	<0.001
c-AFP-L3%	0.83±3.92	1.86±3.16[a]	0.002
hs-AFP-L3%	2.7±5.15	4.86±5.19[a]	0.003
Platelet count (x10^4/μl)	15.93±6.67	11.93±4.49[a]	0.001
AST (IU/l)	43.91±25.72	54.32±21.61[a]	0.003
ALT (IU/l)	49.21±51.7	48.66±24.41	0.184

BLD, benign liver disease; HCC, hepatocellular carcinoma; CH, chronic hepatitis; LC, liver cirrhosis; HBV, hepatitis B virus; HCV, hepatitis C virus; hs-AFP-L3%, hypersensitive-AFP-L3%; c-AFP-L3%, conventional-AFP-L3%.

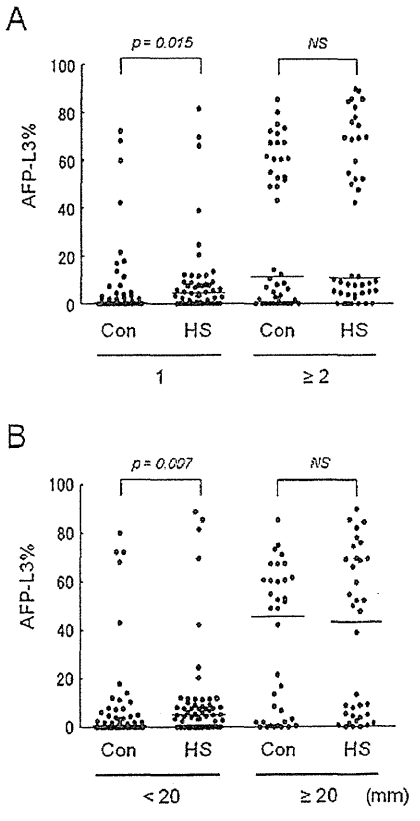

Figure 3. hs-AFP-L3% significantly increased in patients with solitary or small HCC, but not multiple or HCC ≥20 mm in diameter. (A) hs-AFP-L3% (HS) was significantly higher than c-AFP-L3% (Con) in patients with solitary HCC (n=50), but not in patients with multiple HCC (n=44). (B) hs-AFP-L3% significantly increased in comparison with c-AFP-L3% in patients with small HCC (<20 mm in diameter) (n=58), but not in patients with large HCC (≥20 mm) (n=36).

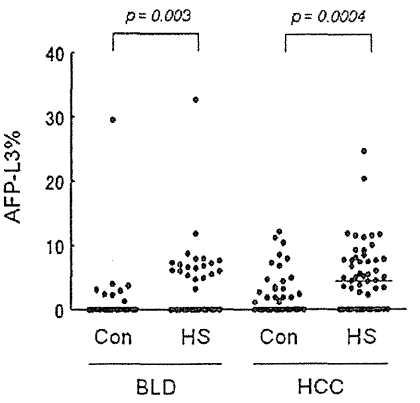

Figure 4. Higher levels of hs-AFP-L3% were observed in both BLD and HCC patients with serum AFP <20 ng/ml. c-AFP-L3% (Con) and hs-AFP-L3% (HS) in BLD and HCC patients with AFP <20 ng/ml (n=59 and 56, respectively) were analyzed. c-AFP-L3% was detectable in 13.6 and 39.3% of BLD and HCC patients, respectively, whereas hs-AFP-L3% was measurable in 33.9 and 64.3% of BLD and HCC patients, respectively; hs-AFP-L3% was significantly higher than c-AFP-L3%.

and hs-AFP-L3% in BLD and HCC patients with AFP <20 ng/ml (Table II). Forty-seven of 56 (83.4%) HCC patients exhibited small HCCs (<20 mm in diameter); 35 patients (62.5%) exhibited solitary tumors. c-AFP-L3% was detectable in 13.6 and 39.3% of BLD and HCC patients, respectively. Conversely, hs-AFP-L3% was measurable in 33.9 and 64.3% of BLD and HCC patients, respectively, and the levels of hs-AFP-L3% were significantly higher than those of c-AFP-L3% [BLD: mean ± SD (range) 0.83±3.92 (1.3-29.5) vs. 2.70±5.15%, p=0.003, and HCC: 1.86±3.16 (1.1-12.1) vs. 4.86±5.19% (2.3-24.6), p=0.004] (Fig. 4). The sensitivity and specificity of hs-AFP-L3%

Table III. Characterization of seven BLD patients, who developed HCC.

Case no.	1	2	3	4	5	6	7
Age	58	70	63	70	53	60	59
Gender	M	F	F	F	M	M	F
CH/LC	LC	CH	LC	LC	LC	LC	CH
HCV/NBNC	HCV	HCV	HCV	NBNC	HCV	HCV	HCV
AFP (ng/ml)	5.3	8.3	10.7	10.9	27.8	28.5	32.0
c-AFP-L3%	ND	ND	29.5	4.9	15.9	12.2	3.4
hs-AFP-L3%	6.0	7.0	32.6	8.4	12.2	9.6	3.7
ALT (IU/l)	31	48	23	39	41	65	116
Months until HCC detection	13	31	5	13	18	8	31

F, female; M, male; ND, not detectable.

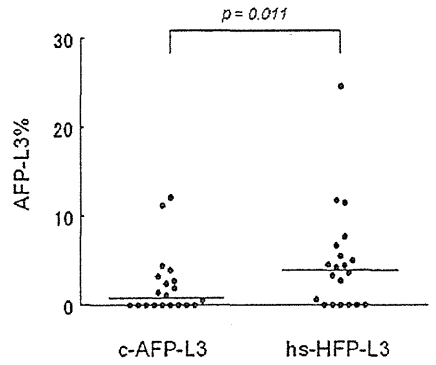

Figure 5. Patients with well-differentiated HCC showed an increase in hs-AFP-L3%. hs-AFP-L3% (HS) was significantly higher than c-AFP-L3% in patients with well-differentiated HCC; this was confirmed by histological examination.

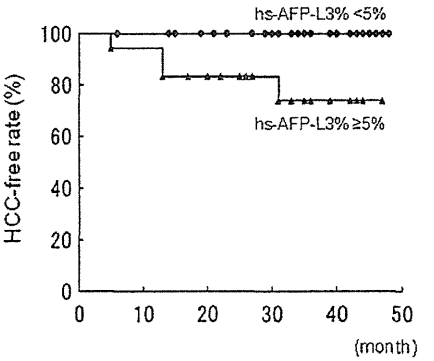

Figure 6. No patients with both serum AFP <20 ng/ml and hs-AFP-L3% <5% developed HCC. Patients with BLD (n=74) were periodically followed by US, CT, or MRI during the follow-up period (median, 35 months; range, 5-48 months). In cases of BLD with AFP <20 ng/ml (n=59), HCC was newly detected in 4 patients with hs-AFP-L3% ≥5%. The HCC-free rate in patients with hs-AFP-L3% ≥5% (▲) was significantly higher than in patients with hs-AFP-L3% <5% (●) (log-rank test and Wilcoxon test; p=0.0012 and p=0.0017, respectively). Importantly, no patients with hs-AFP-L3% <5% developed HCC.

at a cut-off level of 5% were 44.6 and 71.2%, whereas those of c-AFP-L3% were 12.5 and 98.3%, respectively. These results suggest that hs-AFP-L3% is useful for early detection of HCC, even when serum AFP is <20 ng/ml.

Serum hs-AFP-L3% increases in patients with well-differentiated HCC. Most HCC, initially present as well-differentiated HCC, develops in patients with chronic liver disease. Therefore, we evaluated c-AFP-L3% and hs-AFP-L3% in 20 patients with well-differentiated HCC, which was confirmed by histological examination. Fifteen patients (75.0%) exhibited small HCCs (<20 mm), and 9 (45.0%) suffered from liver cirrhosis. Serum AFP was 14.2±12.4 ng/ml (1.4-54.1), and 18 patients (90%) exhibited serum AFP levels <20 ng/ml. hs-AFP-L3% was measurable in 14 patients (70%), while 11 patients (55%) exhibited detectable levels of c-AFP-L3% (Fig. 5). Consequently, hs-AFP-L3% was significantly higher than c-AFP-L3% [4.81±5.91 (0.6-24.6) vs. 2.24±3.53% (0.5-12.1), p=0.011]. These results support the possible utility of hs-AFP-L3% for detection of early-stage HCC.

hs-AFP-L3% increases prior to detection of HCC in patients with BLD. Seven of 74 patients with BLD developed HCC during the follow-up period (median, 35 months; range, 5-48) (Table III). Five patients suffered from liver cirrhosis, and 6 exhibited hepatitis C virus infection. Two of the patients with chronic hepatitis required a longer period (31 months) for appearance of HCC than did the 5 patients with cirrhosis (5-18 months). Five patients exhibited measurable c-AFP-L3%, and an increase in c-AFP-L3% (≥5%) was observed in 3 patients. In contrast, hs-AFP-L3% was measurable in all 7 patients prior to detection of HCC, and 6 patients (85.7%) exhibited hs-AFP-L3% ≥5%. In 59 BLD patients with serum AFP <20 ng/ml, 4 patients developed HCC (Table III). An increase in c-AFP-L3% (≥5%) was observed only in 1 patient, who developed HCC during the follow-up period, whereas the other three patients exhibited undetectable levels or <5% of c-AFP-L3%. Conversely, all 4 patients with serum AFP <20 ng/ml exhibited an increase in hs-AFP-L3% (≥5%) prior to detection of HCC.

Next, we analyzed the HCC-free rate in BLD patients with serum AFP <20 ng/ml during the follow-up period (Fig. 6). The HCC-free rate in patients with hs-AFP-L3% ≥5% was

significantly higher than those with hs-AFP-L3% <5%. Of importance, HCC was not detected in BLD patients with both serum AFP <20 ng/ml and hs-AFP-L3% <5%, whereas 3 out of 58 patients with both serum AFP <20 ng/ml and <5% of c-AFP-L3% developed HCC. These results suggest that an increased hs-AFP-L3% allows prediction of HCC development; measurement of hs-AFP-L3% is useful for selecting BLD patients with higher risk of HCC.

Discussion

Most HCC occurs in patients with chronic liver diseases, especially cirrhosis. Therefore, periodical measurement of tumor markers for HCC, such as AFP and DCP, is recommended in patients who are at high risk for HCC. However, recent advances in diagnostic imaging techniques, including US, CT and MRI, facilitate the detection of small and early-stage HCC (19-21), resulting in an increase in the number of HCC patients diagnosed without an observed increase in serum AFP. Indeed, the 18th survey and follow-up study of primary liver cancer in Japan has reported that most patients with HCC exhibited low levels of serum AFP, <15 ng/ml. Additionally, although AFP-L3% status is known to be a specific marker for HCC, measurement of c-AFP-L3% has not always been reliable in patients with AFP <20 ng/ml.

In this study, we investigated the clinical utility of hs-AFP-L3%, which was measured by a newly developed and highly sensitive method, μ-TAS, in patients with BLD and HCC. Here, we showed that although most HCC patients with stage I cancer did not exhibit an increase in serum AFP levels (≥20 ng/ml), hs-AFP-L3% was measurable in ~70% of the patients, and was significantly increased in comparison with c-AFP-L3% (Fig. 2). Since hs-AFP-L3% is reliable even when serum AFP is <20 ng/ml, it is possible to set the cut-off value for hs-AFP-L3% at 5-7% (18,22,23). We show here that at a cut-off level of 5%, the sensitivity and specificity of hs-AFP-L3% were 44.6 and 71.2%, respectively, in HCC patients with serum AFP <20 ng/ml (Fig. 4). Recent investigations have shown that diagnostic sensitivity of hs-AFP-L3% at a cut-off level of 5 or 7% was 41.5 or 41.1%, respectively, in HCC patients with serum AFP <20 ng/ml (18,22). Therefore, our findings in this study support the specificity of hs-AFP-L3% in patients with serum AFP <20 ng/ml, as previously reported.

The sensitivity of c-AFP-L3% is relatively low (22.2-38.6%) in early-stage HCCs <20 mm in diameter (24,25). In this study, although the sensitivity of c-AFP-L3% was <20% in patients with HCC at stage I, hs-AFP-L3% was significantly higher than c-AFP-L3% in patients with solitary or small (<20 mm) HCC or with stage I HCC (Figs. 2 and 3); consequently, ~50% of HCC patients at stage I exhibited hs-AFP-L3% ≥5%. Additionally, in patients with well-differentiated HCC, hs-AFP-L3% was also significantly higher than c-AFP-L3%. Conversely, patients with stage III or IV HCC (multiple or larger (≥20 mm) tumors) exhibited an increase in both hs- and c-AFP-L3%, with no statistical difference. HCC initially develops as well-differentiated HCC, and then progresses to moderately- to poorly-differentiated HCC via a process of dedifferentiation. Thus, an increase in hs-AFP-L3% in patients with well-differentiated HCC and early-stage HCC supports the conclusion that measurement of hs-AFP-L3% is useful for early detection of HCC.

HCC often develops in patients with chronic infection of hepatitis B or C virus; especially in patients with chronic HCV infection, the annual incidence of HCC increases as a function of the stage of liver fibrosis, from 0.5% at stages F0 to F1 to 7.9% at stage F4 (cirrhosis) (26). Recently, Tateyama *et al* demonstrated that elevated AFP levels are a risk factor for the development of HCC in patients with HCV infection; the 10-year cumulative incidence rates of HCC in the patients with AFP levels of <6, 6-20 and ≥20 ng/ml at entry were 6.0, 24.6 and 47.3%, respectively, and that AFP levels may be used as a non-invasive and predictive marker in place of stage of fibrosis (27). In this study, all 7 BLD patients who developed HCC during the follow-up period exhibited measurable hs-AFP-L3% prior to detection of HCC, and 6 patients exhibited hs-AFP-L3% ≥5%. Of particular note, even when serum AFP levels increased to up to 20 ng/ml, HCC was not detected in patients with hs-AFP-L3% <5% (Fig. 6).

Although prolonged observation will be required in order to clarify whether hs-AFP-L3% is useful for prediction of HCC, the findings presented here indicated that hs-AFP-L3% is useful for early detection of HCC in BLD patients even with serum AFP <20 ng/ml, and also that an increase in hs-AFP-L3% prior to detection of HCC by various advanced imaging modalities may contribute to more precisely identifying BLD patients with a higher risk of HCC.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The complement component C3a fragment is a potential biomarker for hepatitis C virus-related hepatocellular carcinoma

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Abstract
Background Hepatocellular carcinoma (HCC) has a high mortality rate, and early detection of HCC improves patient survival. However, the molecular diagnostic markers for early HCC have not been fully elucidated. The aim of this study was to identify novel diagnostic markers for HCC.
Methods Serum protein profiles of 45 hepatitis C virus infection (HCV)-related HCC patients (HCV-HCC) were compared to 42 HCV-related chronic liver disease patients without HCC (HCV-CLD) and 21 healthy volunteers using the ProteinChip SELDI system. One of the identified proteins was evaluated as a diagnostic marker for HCC in patients with HCV.
Results Five protein peaks (4067, 4470, 7564, 7929, and 8130 m/z) had p-values less than 1×10^{-7} and were significantly increased in the sera of HCV-HCC patients compared to HCV-CLD patients and healthy volunteers. Among these proteins, an 8130 m/z peak was the most differentially expressed and identified as the complement component 3a (C3a) fragment. For HCV-HCC and HCV-CLD, the relative intensity of this C3a fragment had the best area under the ROC curve [0.70], followed by des-γ-carboxy prothrombin (DCP) [0.68], lectin-bound alpha fetoprotein (AFP-L3) [0.58] and AFP [0.53] for HCC. A combined analysis of the C3a fragment, AFP and DCP led to a 98% positive identification rate. In addition, the measurable C3a fragment in some HCC patients was not only significantly higher in the year of HCC onset compared to the pre-onset year, but also decreased after treatment.
Conclusions The 8130 m/z C3a fragment is a potential marker for the early detection of HCV-related HCC.

Keywords Hepatocellular carcinoma · Complement component C3a · Serum proteomics · Serum biomarkers · Proteinchip SELDI system · Hepatitis C virus

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Introduction

Hepatocellular carcinoma (HCC) is reportedly the third most frequent cause of global cancer-related deaths, and the incidence of HCC is increasing worldwide [1, 2]. The clearly established risk factor for HCC is chronic hepatitis C virus (HCV) infection [3].

To date, both ultrasonography and serum tumor markers such as the alpha fetoprotein (AFP), and des-γ-carboxy prothrombin (DCP) assay are the principle methods for screening and detecting HCC. Routine screening is the best method to detect early HCC and improve patient survival; however, elevated serum AFP and DCP levels have insufficient sensitivity and specificity, respectively. The sensitivity and specificity of serum elevated AFP levels were reported to range from 39–64% and 76–91%, while those of the serum elevated DCP levels were 41–77% and 72–98%, respectively [4–9]. In addition, it was recently reported that only a small percentage of small HCC tumors were diagnosed based on AFP and DCP [6, 10]. The lens culinaris agglutinin-reactive fraction of AFP (lectin-bound AFP or AFP-L3) has been reported to be elevated in the serum of HCC patients. Although AFP-L3 has a high range of specificity for detecting HCC, the sensitivity is low [11, 12]. The ability to detect early HCC, prior to the onset of clinical symptoms, leads to curative treatment and significantly improves the disease prognosis. Thus, additional biochemical markers are necessary for the specific detection of early HCC.

Serum profiling using a proteomic approach is thought to be a useful technique to detect or predict early HCC in chronic liver disease patients. Studies using the ProteinChip SELDI system, which is a powerful tool to discover new biomarkers, have shown that this method may be successfully used to diagnose HCC. Zinkin et al. [13], Schwegler et al. [14] and our research group [15] previously detected early HCC using the profile of several protein peaks that were identified by the ProteinChip SELDI system. Paradis et al. [16] reported the highest discriminating peak (8900 Da), which was identified as the V10 fragment of vitronectin. Furthermore, Lee et al. [17] described complement 3a, which had a molecular weight of approximately 8900 Da, as a novel marker of HCC. Therefore, using this proteomic approach to identify specific proteins may not only help establish simple methods to detect HCC, but also further our understanding of the molecular mechanisms of hepatocarcinogenesis and facilitate the development of novel cancer therapies. Therefore, this study assessed and compared the protein expression profiles in the sera of HCC patients in order to identify a more useful biomarker of HCC-associated HCV infection using proteomic approach.

Materials and methods

Samples

Eighty-seven patients [45 HCC patients and 42 patients with chronic liver diseases without HCC (CLD)] with HCV infection were selected to participate in this study (Table 1). These patients provided informed consent. Serum samples were collected by the Faculty of Medicine, University of Miyazaki (Miyazaki, Japan), and some patients were in a hyperendemic HCV area with a cohort study in Miyazaki [18]. The sera of all patients with and without HCC, which was confirmed by abdominal ultrasonography or computed tomography, were obtained prior to treatment. All of the sera samples from HCV-infected patients were analyzed in a previous study [15]. In addition, sera from 10 HCV-HCC patients who were diagnosed with HCC within 1 or 2 years and sera from five patients who had received radiofrequency ablation (RFA), percutaneous ethanol injection therapy (PEIT) and/or transarterial chemoembolization (TACE) for HCC were collected through a cohort study in Miyazaki. We also analyzed the sera of 21 healthy volunteers without HCC as controls. After freezing and thawing once, all samples were separated into 50–100 μl aliquots and refrozen at −80°C. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Miyazaki, Kagoshima University Graduate School of Medical and Dental Sciences, and Harvard School of Public Health and Boston University School of Public Health.

Table 1 Patient characteristics

	HCC[a]	CLD[b]	*p* value
Patients (male/female)	45 (40/5)	42 (40/2)	–
Age	73.6 [63–85]	61.8 [41–83]	<0.0001
PLT[c] ($\times 10^4$/ul)	12.5 ± 5.8	8.4 ± 4.6	0.001
Albumin (g/dl)	3.8 ± 0.8	4.2 ± 1.6	0.8
ALT[d] (IU/l)	57.7 ± 28.3	52.8 ± 37.5	0.7
AFP[e] (ng/ml)	311 ± 1144	51.6 ± 36.1 (38)	0.008
DCP[f] (mAU/ml)	235 ± 605 (44)	37.1 ± 59.8 (39)	<0.0001
HA[g] (ng/ml)	388 ± 446 (40)	280 ± 272 (27)	0.6
Diameter of HCC (mm)	23.2 [10–40]	–	–
TNM stage[h] (I/II/III/IV)	24/18/3/0	–	–

Data are shown as the means ± SD or means [range] (numbers)

[a] Hepatocellular carcinoma

[b] Chronic liver disease

[c] Platelet counts

[d] Alanine aminotransferase

[e] Alpha fetoprotein

[f] Des-γ-carboxy prothrombin

[g] Hyaluronic acid

[h] TNM; primary tumor/lymph node/distant metastasis

SELDI-TOF/MS analysis of sera

Expression difference mapping analysis profiles of the samples were obtained using weak cation-exchange (CM10) ProteinChip Arrays (Bio-Rad Laboratories). Arrays were analyzed by ProteinChip reader as previously reported [15]. In addition, the laser intensity ranged from 220 to 245, with a detector sensitivity of 8, and spectra ranging from 1300 to 150000 m/z were selected for analysis in this study.

Separation of candidate biomarker (8.1 k m/z)

The purification strategy was determined by the ProteinChip Arrays. Two hundred microliters of sera from HCV-HCC patients were diluted 5-fold into 50 mM Na-phosphate buffer, pH 7.0, and loaded onto a CM-Ceramic HyperD F spin column (Bio-Rad Laboratories). After equilibrating with the same buffer, the samples were eluted with a stepwise sodium chloride gradient from 0, 200, 300, and 1000 mM. The elution was desalinated and concentrated using a centrifugal concentrator (VIVA-SPIN, Vivascience, Hannover, Germany), and the purification progress was monitored using NP20 arrays. The flow-through fraction was dialyzed and then separated by 16.5% tricine one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The SDS–PAGE samples were run in tricine sodium dodecyl sulfate buffer according to the manufacturer's instructions and then stained with Coomassie brilliant blue (CBB).

Identification of the candidate biomarker (8.1 k m/z)

Gel pieces containing the target 8.1 k m/z protein were excised. The excised bands were reduced and alkylated for 30 min at room temperature, and then digested with trypsin (Modified Sequence Grade, Roche Diagnostics, Basel, Switzerland) in Tris–HCl, pH 8.0, for 20 h at 35°. The reaction solution was applied to NP20 arrays and allowed to air dry. To identify the protein, the digested peptides were purified by high-performance liquid chromatography (HPLC; MAGIC 2002; Michrom Bioresources Inc., Auburn, CA) and analyzed by Q-Tof2 (Micromass; Waters Ltd., Hertsfordshire, UK). The HPLC solvent consisted of solvent A (2% acetonitlile/0.1% formic acid) and B (90% acetonitlile/0.1% formic acid). The digested peptides were separated with a linear gradient from 10 to 50% solvent B with a flow rate of 400 nl/min using HPLC [19]. Mass spectral data were searched with Mascot (http://www.matrixscience.com) to identify proteins based on the peptide mass [20, 21].

Immunodepletion assay

For immunodepletion, serum samples were prepared as follows. Sera (250 µl) from HCC patients were diluted 5-fold in 50 mM Tris–HCl buffer, pH 8.0, and loaded onto a CM-Sepharose Fast Flow spin column (GE Healthcare Bio-Sciences Corp., NJ). After equilibration with the same buffer, the samples were eluted with a stepwise sodium chloride gradient from 0, 500, and 1000 mM. The elution from each NaCl concentration was monitored using NP20 arrays. To prepare the antibodies for immunodepletion, 6 µl anti-human C3 antibody, which detected C3 and C3a expression, or anti-C4a antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 20 µl Interaction Discovery Mapping (IDM) affinity beads (Bio-Rad Laboratories) and Protein A (Sigma Chemical Co, St. Louis, MO) over night at 4° with shaking. These beads were centrifuged, and the supernatant was discarded. The beads were washed with 50 mM phosphate buffer (pH 7.0), and 3 µl of the prepared serum sample was incubated with 15 µl IDM affinity beads with shaking for 2 h at 4°. As a negative control, 3 µl sample was incubated with IDM affinity beads and Protein A with an anti-C4a antibody or without antibody. After the incubation, the samples were cleared by centrifugation, and 5 µl of each supernatant was analyzed on NP20 ProteinChip arrays in a PBS II reader.

Cell culture and SELDI-TOF/MS analysis of culture supernatants

The human hepatocarcinoma cell line HuH-7 and human hepatoblastoma cell line HepG2 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin G, and 100 mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA). Before starting the experiments, the cells were cultured on 96-well microplates in medium without FBS for 24 h. After washing with FBS-free media, the cells were cultured for 24 h with FBS-free media with or without 500 µg/ml of C3a (Calbiochem, San Diego, CA). The supernatants were collected by centrifugation and analyzed for the expression of 8.1 k m/z using the ProteinChip system.

Statistical analysis

Values are shown as the means ± SD. Statistical differences, including laboratory data and individual peaks in SELDI TOF/MS, were determined using the Mann–Whitney U test. Values of $p < 0.05$ were considered statistically significant. The discriminatory power for each putative marker was described via receiver operating characteristics

(ROC) area under the curve (AUC). These statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA), SPSS software (SPSS Inc., Chicago, IL), JMP software, or Ciphergen ProteinChip Software, version 3.0.2.

Results

Profiling sera from HCC patients and healthy controls

We analyzed the sera of all patients with HCV-HCC or HCV-CLD and healthy controls without HCC using the CM10 ProteinChip array to identity the most differential protein peak. Peaks were automatically detected using the Ciphergen ProteinChip Software 3.0.2. following baseline subtraction as described previously [15, 22]. This analysis identified 178 protein peak clusters, as seen in the spectrum representations from the three groups (HCV-HCC, HCV-CLD, and healthy control) in the 3000- to 15000-m/z range. Peak expressions were increased for 18 proteins and decreased for 14 proteins in sera from HCV-HCC patients compared to HCV-CLD patients. Compared to healthy subjects, 68 protein peaks were increased, and 16 protein peak intensities were decreased in the sera of HCV-HCC patients. Five protein peaks (4067, 4470, 7564, 7929, and 8130 m/z) had a p-value less than 1×10^{-7} and were significantly increased in the sera of HCC patients compared to the sera of HCV-CLD patients and healthy volunteers. In particular, an 8130 m/z peak was the most significantly different peak and had the most differential expression profile between patients with HCV-HCC and with HCV-CLD.

Purification and identification of the 8.1 k m/z peak

We optimized the adsorption and desorption conditions on the arrays using an HCV-HCC patient serum sample and healthy volunteer serum sample in order to determine a procedure to purify the target 8.1 k m/z protein. The optimal pH for retention of the 8.1 k m/z protein was a *pI* value of approximately 7.0 on the CM10 arrays, which indicates that weak cation-exchange sorbents and buffer pH should be fixed for further experiments. The target protein was eluted by increasing the sodium chloride concentrations in a Na-phosphate buffer and was eluted in the 1000 mM sodium chloride fraction. The concentrated serum protein that was eluted with 1000 mM sodium chloride was applied to SDS–PAGE for further separation. The 8.1 k m/z protein was identified and excised by in-gel trypsin digestion for identification. The peptide sequences were analyzed using liquid chromatography (LC)-MS/MS and then examined by a database search with Mascot. The digested peptides matched human complement C3a (Fig. 1).

After reacting the HCC sera with anti-complement C3a or anti-C4 antibodies or without antibody, the supernatants were analyzed by the SELDI ProteinChip system for immunodepletion. Analysis of the supernatant showed that only the 8.1 k m/z peak corresponding to complement C3a

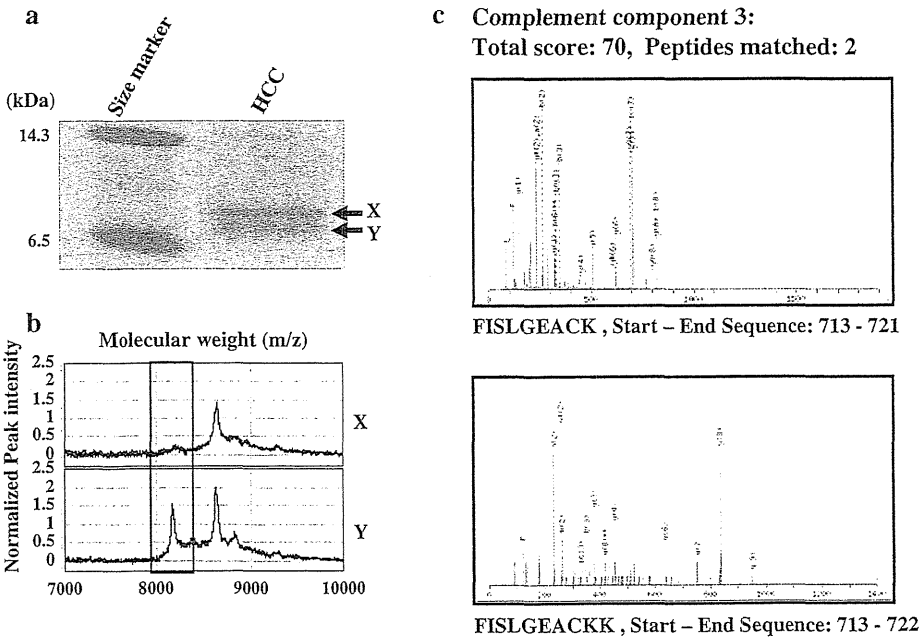

Fig. 1 **a** Partially purified proteins were separated by SDS–PAGE using serum samples from HCV-HCC patients. The Coomassie-stained SDS-PAGE gel shows two clear bands at approximately 8 kDa (*X* and *Y*). **b** After each band (*X* and *Y*) was excised from the gel, the proteins were extracted and analyzed using the ProteinChip system. The target protein in the excised band was detected, and the 8.1 k m/z peak corresponded only to the "*Y*" band contained in gel. **c** The excised "*Y*" band was alkylated and digested using trypsin. The peptides were collected and subjected to LC–MS/MS analysis. The proteins, which were derived from complement C3a, were identified using a database search