Table III. Patient characteristics in cohort-based population with HCV infection.

Characteristics	PNALT-2 (n=37)	CHC-2 (n=30)	P-value[a]
Age	75.6±6.5	70.4±6.6	<0.01
Gender (male/female)	8/29	15/15	0.02
HCV core antigen (fmol/l)	6,042±4,295	4,553±3,546	0.27
HCV serotype (I/II)	14/23	24/6	<0.001
Platelet count ($x10^4/\mu l$)	22.3±5.3	11.8±3.8	<0.001
AST (IU/l)	28.3±8.0	100.1±81.8	<0.001
ALT (IU/l)	19.5±6.0	96.9±81.8	<0.001
γ-GTP (IU/l)	15.1±10.6 (n=31)	56.2±46.7 (n=20)	<0.001
T-Cho (mg/dl)	181.8±29.3 (n=31)	158.8±27.1 (n=29)	0.02
Albumin (g/dl)	4.4±0.3 (n=33)	4.1±0.5 (n=29)	<0.01

HCV, hepatitis C virus; PNALT, persistently normal alanine aminotransferase; CHC, chronic hepatitis C; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyltranspeptidase; T-Cho, total cholesterol; n, number of patients or the number of samples analyzed. Data are presented as the means ± standard deviation or number. [a]Differences between mean values were evaluated using either the Fisher's exact test or the Mann-Whitney U test, as appropriate.

Table IV. Correlation between serum C4a levels and blood laboratory parameters in PNALT subjects.

Parameter	Correlation coefficient	P-value[a]
HCV core antigen	0.06	0.73
White blood cell	-0.06	0.72
Hematocrit	-0.06	0.12
Platelet	0.12	0.51
Albumin	0.03	0.88
γ-globulin	-0.05	0.77
AST	-0.39	0.02
ALT	-0.47	<0.01
Total-bilirubin	-0.07	0.69
Total cholesterol	0.05	0.76
Ferritin	-0.18	0.30
Hyaluronic acid	-0.23	0.17
Type IV collagen	-0.04	0.82
α-fetoprotein	-0.29	0.09
DCP	-0.07	0.69

PNALT, persistently normal alanine aminotransferase; HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; DCP, des-γ-carboxy prothrombin. [a]P-values were assessed by Spearman's rank correlation analysis.

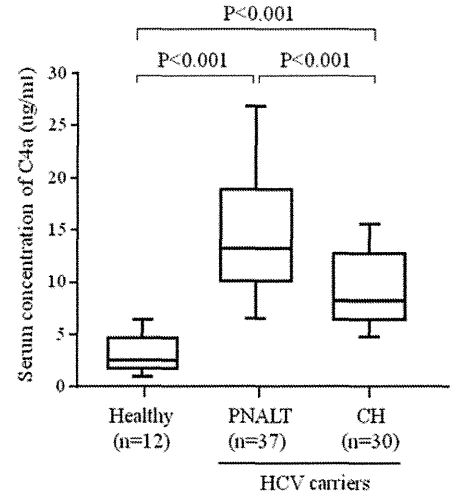

Figure 2. Serum concentrations of C4a determined by enzyme-linked immnosorbent assay in HCV carriers and healthy controls. Serum C4a levels were significantly higher in HCV carriers with PNALT than in HCV carriers with chronic hepatitis or healthy controls (P<0.001). Boxes indicate the median ± 25th percentile, the lower bar indicates the 10th percentile, and the upper bar indicates the 90th percentile. HCV, hepatitis C virus; PNALT, persistently normal alanine aminotransferase; CH, chronic hepatitis; C4a, complement component 4a.

and total cholesterol levels were significantly higher in the PNALT-2 group than in the CHC-2 group. By contrast, serum AST, ALT and γ-GTP levels were lower in the PNALT-2 group (Table III). In the cohort-based population, serum concentrations of C4a, as determined by ELISA, were significantly higher in the PNALT-2 group than in the CHC-2 group and healthy controls (Fig. 2).

Serum C4a levels in the PNALT-2 group correlated significantly with serum AST and ALT levels, but not with HCVcAg levels or other blood laboratory parameters (Table IV). In addition, a significant negative correlation between serum C4a and ALT levels was observed in the population as a whole (Fig. 3, r=-0.35, P=0.03) and in PNALT patients

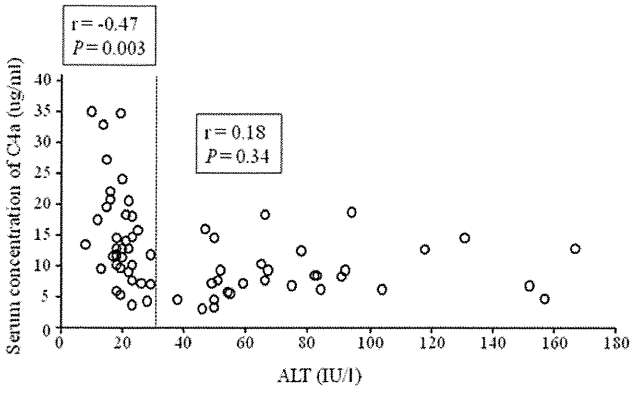

Figure 3. Association between serum C4a and ALT levels in all HCV carriers (including PNALT and chronic hepatitis). Serum C4a levels negatively correlated with serum ALT levels (r=-0.35, P=0.03). This correlation was observed in subjects with normal ALT (≤30 IU/l) (r=-0.47, P=0.003) but not in those with abnormal ALT (>30 IU/l). C4a, complement component 4a; HCV, hepatitis C virus; ALT, alanine aminotransferase; PNALT, persistently normal ALT.

(Table IV; ALT ≤30 IU/l; Fig. 3), but not in CHC-2 patients (ALT >30 IU/l; Fig. 3).

Discussion

HCV is not thought to be directly cytopathic to hepatocytes, and a T helper (Th)1-type or cytotoxic T lymphocyte (CTL) response is critically involved in HCV-mediated liver injury (15). Therefore, it is conceivable that various suppressor mechanisms exist against Th1-type immune responses in HCV carriers with PNALT, which may be distinct from those in CHC patients with active liver inflammation (16). However, few studies have focused on PNALT using a serum proteomic approach. In this study of HCV carriers, a number of proteins were detected which were differentially expressed between PNALT and CHC patients. Of these, the C4 fragment was identified by peptide mass fingerprint (PMF) methods following a Mascot search, and serum levels of C4a, which correlated with the protein peak of the identified C4 fragment, were higher in PNALT than in CHC patients, as determined by ELISA. In addition, serum C4a levels correlated with ALT levels in the PNALT but not CHC patients.

Following acute HCV infection, approximately 70% of individuals remain positive for both anti-HCV Ab and HCV RNA, and are defined as HCV carriers. By contrast, approximately 30% of acutely infected individuals clear the HCV and remain positive for anti-HCV Ab but negative for HCV RNA. We confirmed that serum C4a levels in those who cleared the virus were similar to healthy controls (data not shown). Serum C4a levels were higher in CHC patients and individuals with PNALT compared to healthy controls. Therefore, serum C4a levels appear to be at least elevated by existing HCV infection, although serum C4a levels did not correlate with serum HCVcAg levels in individuals with PNALT (Table IV). It was previously reported that serum L-ficolin levels were increased in HCV patients, and that this protein only recognized and bound to glycoproteins E1 and E2 of the HCV envelope, but also activated the complement lectin pathway-mediated cytolytic activity in HCV-infected hepatocytes (17). In the lectin pathway, mannose-binding lectin (MBL)-associated serine protease-2 (MASP-2) cleaves C4, releasing C4a and generating C4b (18). Thus, C4a levels should increase in HCV carriers compared to healthy controls by post-translational mechanisms.

Previous studies have reported decreased serum C4 levels in patients with CHC (19,20). Recently, the HCV core protein and non-structural 5A protein (NS5A) were reported to transcriptionally downregulate C4 expression by modulating the expression of upstream stimulating factor 1 and IFN regulatory factor 1, respectively (21). Thus, serum C4 protein levels are decreased in HCV patients compared to healthy controls as a result of altered transcriptional regulation (22). Although the mechanism of C4a variation in HCV carriers has not been elucidated, our study suggests that serum C4a levels in HCV carriers with PNALT should be dominantly affected by post-translational mechanisms, but patients with CHC may be affected by both translational (downregulation) and post-translational (upregulation) mechanisms.

In CHC, decreased specific C4 activity without C3 consumption suggests complement activation leading to the N-terminal cleavage of C4 with the production of C4a (20). Another study demonstrated increased C4a levels in CHC patients without a significant increase in the levels of C3a (23). Avirutnan *et al* reported that flaviviruses, such as dengue virus, use their non-structural protein, NS1, to attenuate complement activation by directly interacting with C4, leading to viral persistence (24). Although the mechanisms responsible for HCV persistence or PNALT in HCV carriers are not well understood, the interactions between HCV and the host immune system are thought to play a pivotal role in patients with HCV infection.

The majority of individuals with PNALT have minimal or mild inflammation and absent or minimal fibrosis, and follow-up studies have shown disease stability with minimal fibrosis progression over the years, leading to a favorable prognosis. However, cirrhosis and HCC are occasionally observed in HCV carriers with normal ALT levels (25). In addition, some patients with PNALT may develop ALT elevation over time (4), and these individuals may be at increased risk of the significant progression of fibrosis. Long-term observation or liver biopsy has not always been performed; serum C4a levels may be a diagnostic marker for advanced fibrosis or a predictor for ALT elevation in PNALT. These issues should be subject to further analysis.

It has been reported that in HCV carriers with PNALT or normal ALT levels, IFN-based therapy is safe and efficacious (26,27). However, the decision whether or not to treat HCV carriers should be made with the specific clinical setting in mind (28). In addition, it is recommended that serum ALT levels be kept <30 IU/l to prevent the occurrence of HCC (29). If serum C4a levels are an indicator of disease prognosis, HCV carriers with low serum C4a levels may have to be treated despite ALT elevation. More significantly, elucidating the mechanism underlying the association between serum C4a and ALT levels should lead to new approaches to the treatment of HCV carriers with PNALT.

In conclusion, host factors such as C4a differ between HCV carriers with PNALT and CHC patients with elevated ALT levels. Proteomic approaches could greatly contribute to elucidate the host factor in PNALT patients as more differ-

ences are discovered. Identification of these and other proteins will help clarify the mechanism and may improve clinical outcomes of HCV carriers.

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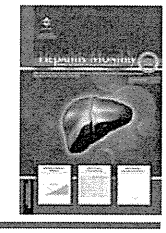

Clinical Features of Hepatitis C Virus Carriers With Persistently Normal Alanine Aminotransferase Levels

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ABSTRACT

Hepatitis C virus (HCV) infection causes chronic hepatitis, which frequently leads to hepatic fibrosis and hepatocellular carcinoma (HCC). Alanine aminotransferase (ALT) is a biomarker of hepatocyte injury and is associated with the progression of hepatic fibrosis. Advanced hepatic fibrosis also predisposes HCV carriers to a risk of HCC. In contrast, some cases with persistent HCV infection have normal ALT levels that persist for a long time, and these HCV carriers have no or mild hepatitis and hepatic fibrosis. These HCV carriers are defined as persistent normal ALT (PNALT) cases and their risk of HCC is low compared to HCV carriers with abnormal ALT. However, there are various definitions of normal ALT and PNALT, and advanced hepatic fibrosis may be missed without a liver biopsy. In addition, there is also a risk of ALT elevation in HCV carriers with PNALT, which increases the risk of progression to hepatic fibrosis and HCC. Most HCV carriers with PNALT have asymptomatic or nonspecific symptoms. HCV carriers with PNALT are also considered to be responsive to interferon-based treatment. Thus, assessment of hepatic fibrosis is important in HCV carriers, and the eradication of HCV infection is more likely in HCV carriers with evidence of hepatic fibrosis, regardless of their ALT levels.

▸ *Implication for health policy/practice/research/medical education:*
Epidemiological studies have suggested a linkage between alanine aminotransferase (ALT) levels and hepatic fibrosis or hepatocellularcarcinoma (HCC) in hepatitis C virus (HCV) carriers. However, clinical features of HCV carriers with persistent normal ALT levels (PNALT) are not fully elucidated. This review focuses on the PNALT in HCV carriers and clinical significance of hepatic fibrosis in these carriers in order to bring out some common opinions on how to manage such HCV carriers with PNALT.

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1. Context

Hepatitis C virus (HCV) causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) at high rates (1-3). However, some cases remain asymptomatic with normal levels of alanine aminotransferase (ALT) after HCV infection and detection of the infection in these cases may only occur through screening, such as with an anti-HCV antibody test. The ALT level usually rises in hepatitis (4), but it is normal in approximately 20-30% of HCV carriers (5, 6). Previous studies have shown that elevated ALT levels predict an increased rate of HCV-associated HCC in a community-based population and that serial measurements to identify persistent ALT abnormality may be useful in determining the HCC risk (7, 8). Thus, HCV carriers with normal ALT levels were previously not indicated for antiviral therapy. In contrast, it has been shown that hepatic inflammation and fibrosis are histologically present in many HCV carriers, even though their ALT level is normal, and these HCV carriers are candidates for antiviral therapy. These conflicting issues may arise from ambiguity around the definition of normal ALT and with the natural course of HCV carriers with persistent normal ALT (PNALT). In this review, which is based on a search for articles using terms such as persistent ALT, normal ALT, and HCV, we describe the characteristics, natural course, and treatment in HCV carriers with PNALT.

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2. Evidence Acquisition

2.1. Epidemiologic Features of HCV Carriers

Many HCV carriers are asymptomatic. According to American Association for the Study of Liver Diseases (AASLD) practice guidelines, drug users, patients with a high risk for HCV infection (patients with HIV infection or hemophilia treated with coagulation factor preparations before 1987, and patients with hemodialysis or ALT abnormality), patients who received blood transfusion or organ transplantation before July 1992, children born to HCV-infected mothers, medical workers exposed to needles used for HCV-infected patients or to the mucosa of HCV-infected patients, and people who have had a sexual relationship with an HCV carrier should be screened for a HCV infection (9). More than 170 million people worldwide are thought to be infected with the HCV. HCV infection progresses to a chronic state in 60-85% of infected people and may develop into liver cirrhosis and HCC after 20-35 years (3, 10). Poynard *et al.* reported that 33% of patients with a HCV infection had an expected median time to cirrhosis of less than 20 years without treatment (11). Other studies have also shown a rate of progression to cirrhosis of between 1.5 and 16 years following blood transfusion in approximately 20% of cases (12). The rate of progression to HCC was reported to be approximately 2% per year (13, 14). In addition, about 1.4 million people (approximately 1% of HCV carriers) are estimated to die annually due to liver cirrhosis or HCC (1, 3). We previously reported that 70 out of 758 HCV carriers died due to a liver-related cause over an average of 8.2 years of follow-up (a rate of 1% per year) (8). The important point is that those rates may be mainly due to the diversity in the design of the studies themselves. Host factors and environmental factors affect the progression of hepatic inflammation and fibrosis. Host factors include; advanced age at the time of infection, male gender, excess alcohol consumption, excess iron intake, cigarette smoking, obesity, complications with diabetes, fatty liver and metabolic syndrome, and coinfection with human immunodeficiency virus (HIV), hepatitis B virus (HBV), and *Schistosomiasis* (3, 15). In addition, hepatic fibrosis in liver grafts shows rapid progression after liver transplantation in hepatitis C patients (16, 17), although the mechanism for this remains unclear. In contrast, the progression of hepatic fibrosis may not be associated with HCV genotype or viral load (11, 18).

2.2. The Concept of ALT Normality and Definition for PNALT

Since ALT is released into the blood when the hepatocyte membrane is impaired or hepatocytes are destroyed, elevation of the serum ALT level is a useful marker indicating hepatocellular impairment (15). According to UpToDate ver. 19.3 (UpToDate, Inc. Waltham, MA), HCV infection with a normal ALT level is defined by the following (5); detectable HCV RNA and serum ALT concentration that is persistently within the normal range. The definition of PNALT includes the idea of "persistence", indicating maintenance of an ALT level that is below the upper limit of the normal value over a long period of time (e.g., over a six-month period). However, this definition has varied among the many reports on PNALT, which have used different periods of observation and upper limits of normal ALT that are often set at the highest value of the measurement instrument. Moreover, the ALT level varies depending on; age, gender, race, and body mass index (BMI) (9). Prati *et al.* investigated 6,835 blood donors with ALT levels within the standard range (male: ≤ 40 IU/l, female: ≤ 30 IU/l) who were negative for infections (HBV, HCV, and HIV), had no history of medication, with blood glucose, cholesterol, and triglyceride levels lower than the upper limits of the standard ranges, and had a BMI < 25 kg/m^2. The findings showed that 30 and 19 IU/l were appropriate as the upper limits of the normal ALT range in males and females, respectively (20). However, according to the AASLD Practice guidelines, PNALT is present when ALT is measured 2-3 times over 1-6 months and all values are < 40 IU/l (9). Thus, the standard ALT level is unclear and the criteria for PNALT vary among the different reports. Therefore, it is important to pay attention to the definition of a normal ALT level in order to evaluate the natural course of HCV carriers with PNALT.

2.3. Clinical Manifestations in HCV Carriers With PNALT

Most HCV carriers with PNALT are discovered incidentally when anti-HCV antibodies are found following a blood donation. Most of these patients have asymptomatic disease or nonspecific symptoms including; fatigue, weakness, and upper right quadrant pain (20). The frequency and severity of these symptoms have been reported to be similar among anti-HCV positive patients with normal serum ALT, mildly elevated values (< 2 times normal), and more marked elevation (> 2 times normal) (21). In contrast, Jamal *et al.* have suggested that there is a trend towards depression being more common in HCV carriers with abnormal ALT compared to those with normal ALT, although they found that there was no statistically significant difference in depressive symptoms (20). Therefore, depression, though nonspecific, might be an important clinical marker of a more severe disease.

2.4. Epidemiology, Clinical Course and Management in HCV Carriers With PNALT

The ALT level is within the normal range in about 30% of HCV carriers and lower than 2-fold the normal upper limit in 40% (5, 6), although what constitutes a normal ALT value is not completely clear. The frequency of PNALT is reported to be higher in HCV genotype 2 carriers and females (6, 22). Inflammation and fibrosis were shown to be histologically mild in approximately 70% of PNALT cases (23); however, fibrosis of stage 2 (F2) or more severe fibrosis was noted in 22% of histologically evaluated PNALT cases in another report (22). Therefore, severe fibrosis can be present in some cases in which their ALT level is

normal. This may be because ALT levels occasionally normalize in patients with liver cirrhosis. The rate of progression of hepatic fibrosis in patients with chronic hepatitis C is reported to be 0.1-0.13 units/year (11, 24). PNALT cases have been found to show only a slight histological progression over a 10-year follow-up period (25), with a reported rate of progression of fibrosis of 0.05 units per year (26, 27). These results suggest that fibrosis progresses more slowly in PNALT cases. During the long-term follow-up of patients with PNALT, ALT levels rose in 21.5% of cases 3-18 months after PNALT was judged to be present (6). Platelet count is thought to be a simple marker for hepatic fibrosis, and PNALT patients with severe fibrosis can be identified using the criterion of a platelet count ≤ 150,000/μL. In patients selected using this criterion and an ALT level of ≤ 30 IU/L for 5 years, Okanoue *et al.* showed that ALT ≤ 30 IU/L was maintained in only 14% of patients (28). In our study, 101 HCV carriers with PNALT (defined as ALT ≤ 34 IU/l) were surveyed between 1993 and 2000, and ALT levels rose in 31.8% of these patients over a 5-year observation period (29). Thus, there is a risk of ALT elevation in HCV carriers with PNALT, even if the ALT level has been continuously normal over several years and if the definition of PNALT includes the platelet count, in addition to the ALT level. Furthermore, we found that an ALT level of 20-34 IU/l [odds ratio (OR): 5.6], a serum ferritin level of ≥ 90 ng/ml (OR: 3.1), and a minor allele of the HFE gene (H63D, OR: 4.8) were independent risk factors for ALT elevation (29). Shiffman *et al.* found that the ALT level was maintained at about 20 IU/L in PNALT cases (30). Therefore, when the ALT level is ≤ 19 IU/L, it is unlikely to rise, and this suggests that 19 IU/L may be an appropriate upper limit for ALT in the definition of PNALT.

2.5. ALT levels in HCV Carriers with End Stage Renal Failure or HIV Coinfection

The population of patients with fibrosis progression, despite having ALT levels within the normal range, typically includes dialysis patients with HCV infection and HCV carriers with HIV coinfection. Dialysis patients are a high-risk group for HCV infection, with an HCV prevalence of about 13.5% among these patients (31). Dialysis patients also generally have a low ALT level, and we also found low mean ALT levels of 13.2 and 18.5 IU/l in 238 HCV-negative and HCV-positive dialysis patients (32). A comparison of ALT levels with histologically severe inflammation and fibrosis showed that the ALT levels were lower in patients with end-stage renal failure who were under dialysis, compared to patients with normal renal function (33). However, the cause of the low ALT level in end-stage renal failure patients is unclear. Among HIV-infected patients, 25% are reported to be infected with HCV (34), and the incidence of fibrosis progression in PNALT cases is nearly 2 times higher in patients with HIV-HCV coinfection than in those with a HCV infection alone (35). Thus, although histological progression is generally mild in HCV carriers with PNALT, hepatic fibrosis is more progressive in those patients with end-stage renal failure or an HIV coinfection.

2.6. Immunopathogenesis of HCV Carriers With PNALT

The mechanism for ALT elevation in HCV carriers is not yet fully elucidated, but may be associated with functional abnormalities of the immune cells, such as activated lymphocytes and NK cells in patients with chronic hepatitis C (36, 37). Dendritic cells (DCs) play a central role in the activation of immunocytes, and the numbers of myeloid DCs (mDCs) and plasmacytaid DCs (pDCs) are smaller in patients with chronic hepatitis C than in subjects without a HCV infection. A comparison of PNALT cases and chronic hepatitis C patients showed no difference in the number of mDCs or pDCs, but a reduced DC function was associated with a higher ALT level (38). In contrast, many immunosuppressive regulatory T cells

Table 1. Diagnostic Accuracy of the Models for Predicting Liver Fibrosis Using Examination of Peripheral Blood and Serum Chemistry

Model Name (Reference)	Variables	Etiology	Fibrosis Stage	Cut off Values	AUROC [a]
APRI [a] (44)	AST [a], Plt [a]	HCV	Ishak score 0-2 Ishak score 3-6	< 0.5 ≥ 1.5	0.80-0.88
FIB-4 (45)	Age, AST, Plt, ALT [a]	HCV/HIV	Ishak score 0-3 Ishak score 4-6	< 1.45 ≥ 3.25	0.765
Forns Index (46)	Plt, GGT [a], age, cholesterol	HCV	F0-1 F2-4	> 4.2 > 6.9	0.81-0.86
Fibro Index (47)	Plt, AST, γ globulin	HCV	F0-1 F2-3	≤ 1.25 ≥ 2.25	0.83
FibroTest (48)	α2-MC [a], α2 globulin (or haptoglobin), γ-globulin, apolipoprotein A1, GGT, Total bilirubin	HCV	F0-1 F2-4	0-0.1 0.6-1.0	0.836-0.870
SHASTA Index (49)	HA [a], AST, albumin	HCV/HIV	Ishak score 0-2 Ishak score 3-6	< 0.3 > 0.8	0.878
Hepascore (50)	HA, α2-MC, GGT, age, gender	HCV	F0-2 F3-4	< 0.5 ≥ 0.5	0.82-0.90

[a] Abbreviations: α2-MC, α2-macroglobulin; ALT, alanine aminotransferase; AUROC, area under receiver operating characteristic curve; AST, aspartate aminotransferase APRI, AST-platelet ratio index; GGT, gamma-glutamyltransferase;HA, hyaluronic acid; Plt, platelet

Table 2. Diagnostic Accuracy for Predicting Liver Fibrosis Using Transient Elastography or Acoustic Radiation Force Impulse

Technique	(Reference)	Etiology	Fibrosis Stage	Cut off Values	AUROC [a]
TE [a]	(51)	HCV [a]	F≥2	8.7	0.79
			F≥3	9.56	0.91
			F=4	14.52	0.99
	(52)	HCV	F≥2	7.1	0.88
			F≥3	9.5	0.95
			F=4	12.5	0.95
	(53)	CLD [a]	F=4	14.6	0.95
	(54)	HCV	F≥2	7.1	0.88
			F≥3	9.6	0.90
			F=4	11.6-16.9	0.90
	(55)	HCV	F≥1	5.2	0.902
			F≥2	8.1	0.941
			F≥3	9.6	0.926
			F=4	13.1	0.945
ARFI [a]	(56)	HCV	F≥2	1.215	0.902
			F≥3	1.54	0.993
			F=4	1.94	0.993
	(57)	CLD [a]	F≥2	1.34	0.94
			F≥3	1.44	0.94
			F=4	1.80	0.96
	(55)	HCV	F≥1	1.19	0.709
			F≥2	1.34	0.851
			F≥3	1.61	0.869
			F=4	2.00	0.911

[a] Abbreviations: ARFI, acoustic radiation force impulse; AUROC, area under receiver operating characteristic curve; CLD, chronic liver disease; HCV Hepatitis C virus; TE, transient elastography

(Treg) are present in HCV carriers and the inhibitory activity is stronger in PNALT cases than in patients with active hepatitis (39). These results indicate that therapy to regulate the functional abnormalities of immune cells may be valuable in patients with chronic hepatitis C in order to reduce the ALT level or hepatic inflammation. In addition, the frequency of DR13 in HCV-infected patients with a normal ALT level was significantly higher than that of HCV-infected patients with elevated ALT (42% *vs.* 4%, Pc < 0.003) (40). Thus, immunological analysis in HCV carriers with PNALT may lead to new therapies for patients with chronic hepatitis C.

2.7. Noninvasive Evaluation of Fibrosis in HCV Carriers With PNALT

In HCV carriers, the risk of HCC increases with the progression of hepatic fibrosis (41). Liver biopsy is the standard test for hepatic fibrosis, but it is invasive and causes complications at a rate of 1-3% and has a mortality rate of 1/10000-12000 (42). Moreover, sampling errors leading to the underestimation of liver cirrhosis have been reported in 14.5% of cases (43). Noninvasive tests for the evaluation of fibrosis have also been described, these include; combinations of peripheral blood and serum chemistry tests (44-48) and fibrosis markers (49, 50) (*Table 1*); elastography using transient elastography (TE FibroScan®) and acoustic radiation force impulse (ARFI) (51-57) (*Table 2*). Accuracy increases when several tests are used in combination, but the utility of these tests for the evaluation of hepatic fibrosis in PNALT cases remains uncertain.

2.8. PNALT and the Risk for HCC in HCV Carriers

Tanaka *et al.* investigated HCC development in 1,927 HCV antibody-positive blood donors and found that the incidence of HCC was lower in subjects with a low ALT level compared to those with a high ALT level (58). In our study, subjects were followed for an average of 8 years before HCC development, and a strong association between the ALT level and HCC development was found, with a significant association between a 20 IU/L higher ALT level and the subsequent incidence of HCC being observed [hazard ratio (HR) = 1.2] (7). The HCC risk was also much lower in the PNALT cases than in subjects with a persistent abnormal ALT level. Among 551 subjects with at least 4 repeated measurements of ALT, those with persistently abnormal ALT levels (n = 118) had a significantly increased rate of HCC compared to those with persistently normal ALT levels (n = 296) (HR = 23.2) (7). Kumada *et al.* also found that a high ALT level and low platelet count were strongly associated with HCC development (59). When a low ALT level persists for a prolonged period and the platelet count does not decrease, the HCC complication rate may be very low despite the persistence of a HCV infection. Collectively, these results show that the risk for HCC in subjects with PNALT is low among HCV carriers, including patients with chronic hepatitis and liver cirrhosis.

2.9. Insulin Resistance and Fatty Liver in HCV Carriers

In liver cirrhosis, insulin sensitivity decreases in peripheral tissue, for which pancreatic β cells compensate through the secretion of excess insulin, inducing hyperinsulinemia. Allison *et al.* found that the rate of diabetes complications was higher in liver cirrhosis associated with a HCV infection, than in that induced by other causes (60). The homeostasis model assessment-insulin resistance (HOMA-IR) value, which is an index of insulin resistance, was found to be significantly higher in stage 0 or 1 chronic hepatitis C patients with mild hepatic fibrosis than in healthy subjects, and HOMA-IR served as a predictor of the progression of hepatic fibrosis (61). Animal studies also suggest that the HCV core protein acts on the insulin signal transmission pathway and induces insulin resistance (62). However, it is unclear whether insulin resistance is present in PNALT cases without hepatic fibrosis. A high rate of fat deposition in the liver is caused by HCV infection (63), and fatty changes in the liver and insulin resistance are induced in transgenic mice expressing the HCV core protein (64). Castera *et al.* performed a liver biopsy at mean intervals of 48 months and observed that; male gender, histological stage, and the presence of advanced fatty changes were significant risk factors in promoting hepatic fibrosis, and that fatty change in the liver was an independent risk factor in multivariate analysis (65). Fatty change in the liver is particularly marked with genotype 3 viruses (66), and fatty change in the liver and insulin resistance have also been associated with the negative effects of interferon (IFN)-based therapy (67). Thus, insulin resistance and fat deposition in the liver, which are associated with hepatic fibrosis, should be less severe in PNALT cases compared to HCV patients with abnormal ALT, and this may produce a more favorable outcome for IFN-based treatment. However, the antiviral effect of IFN-based therapy in patients with normal ALT is comparable to that for patients with abnormal ALT, regardless of their background advantages (68, 69). Therefore, further studies of insulin resistance and fat deposition in the liver are needed in PNALT cases.

3. Results

3.1. Improved Outcomes With Antiviral Treatment for HCV Carriers

Treatment for HCV carriers has improved markedly in recent years. Combination therapy with pegylated (PEG) interferon (IFN) plus ribavirin achieves a sustained viral response (SVR) in approximately 50% of the most intractable genotype 1 patients and high viral load cases. The therapeutic effect is determined by; host, viral, and drug factors, and host factors include; age, gender, severity of hepatic fibrosis, fatty liver changes, and insulin resistance. In 2009, a study performed to identify single nucleotide polymorphisms (SNPs) that determine the effect of IFN-based therapy in chronic hepatitis C patients revealed the importance of a SNP near the IL28B gene (70). IL28B (also called IFNλ3) is thought to induce antiviral activity via the JAK-STAT pathway through phosphorylation of STAT1/STAT2 and the subsequent induction of the IFN-stimulating gene (ISG) (71). In addition to the genotype and viral load of HCV RNA, mutations of amino acids in the core (72) and the interferon sensitivity-determining region (ISDR) (73) have been reported as viral factors that determine the effect of IFN-based therapy. In multivariate analysis of host and viral factors, a high platelet count with mild hepatic fibrosis (F0-1), ≥ 2 ISDR mutations, and the IL28B SNP as the major allele were identified as factors associated with SVR (74). A lower γGTP level, and milder inflammation, fibrosis, and fatty changes were found in patients with the IL-28B SNP as the major allele, compared to those with this SNP as the minor allele (74). This suggests that the IL-28B SNP is involved in the progression of hepatitis C pathology, as well as in the therapeutic effect. These results indicate that SVR is likely in PNALT cases. However, there has been no report of an IL-28B SNP associated with PNALT. Drug factors that influence the therapeutic effect include; the type of IFN and the dose and duration of administration of IFN or ribavirin. Novel drugs, such as protease and polymerase inhibitors, will soon become available. In combination therapy with PEG-IFN, ribavirin and protease inhibitor, the overall SVR rate should increase to more than 60% in patients with genotype 1b and a high viral load. In addition, SVR was achieved in 84% of cases with IL-28B SNP as the major allele and glutamine or histidine at position 70 in the core protein, and in 50% of cases with IL-28B SNP as the minor allele and arginine at position 70 (75). These viral factors and host factors require further investigation in HCV carriers with PNALT.

3.2. Antiviral Treatment in HCV Carriers With PNALT

The treatment of chronic hepatitis C has improved significantly over the last few years. Detailed analysis of host factors including the IL28B SNP and viral factors may improve the accuracy of predicting IFN-based therapy effects. For example, this may be based on a prediction of the therapeutic effects using data mining of the blood test results (74, 76). Establishment of tailor-made therapies may be possible with further investigation of SNPs and other data for prediction of IFN-based treatments, including those for PNALT cases. Antiviral therapy for PNALT cases has been estimated to reduce the prevalence of liver cirrhosis and complications by 22% and 16%, respectively, and to decrease the mortality rate by 14% (77). The AASLD practice guidelines suggest that a decision to use PEG-IFN plus ribavirin therapy should be made based on; a histological evaluation, the possibility of adverse effects, prediction of therapeutic effects, and the presence of complications, regardless of the ALT level (9). In addition, Okanoue *et al.* reported that the combination of ALT and platelet counts is useful for evaluating the fibrosis stage in HCV carriers with normal serum ALT levels, and that most patients with platelet counts < 150000/

μL are candidates for antiviral therapy, especially those with ALT levels ≥ 31 U/L when the focus is placed on the inhibition of HCC development (27). Puoti *et al.* have also found that a rapid virological response was a predictor of sustained response in HCV carriers with PNALT (69). Thus, although further studies are required to determine whether antiviral therapy should be given in all PNALT cases, it seems likely that many HCV carriers with PNALT should be treated with IFN-based therapy. Tailor-made therapies may also be possible based on the accumulation of data for the prediction of IFN-based treatment efficacy in PNALT cases.

4. Conclusion

In this review, we have described the clinical characteristics of HCV carriers with PNALT. For HCV carriers, it is important to minimize complications such as liver cirrhosis and HCC through careful observation and treatment at the appropriate time. For PNALT cases, the course and prognosis should also be monitored and treatment should be considered at an early stage.

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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 $\geqslant$ 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 $\geqslant$ 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 $\geqslant$ 110 ng/mL (risk ratio: 4.12, 95% confidential interval: 1.22-13.88, $P = 0.02$) and AFP levels $\geqslant$ 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).

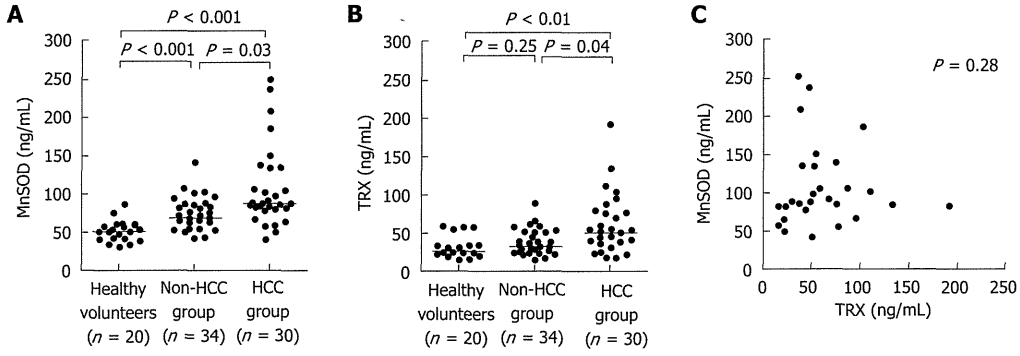

Figure 1 Serum levels of manganese superoxide dismutase and thioredoxin in the hepatocellular carcinoma, non-hepatocellular carcinoma and healthy volunteer groups. A: Serum manganese superoxide dismutase (MnSOD) levels were significantly higher in the hepatocellular carcinoma (HCC) group than in either 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

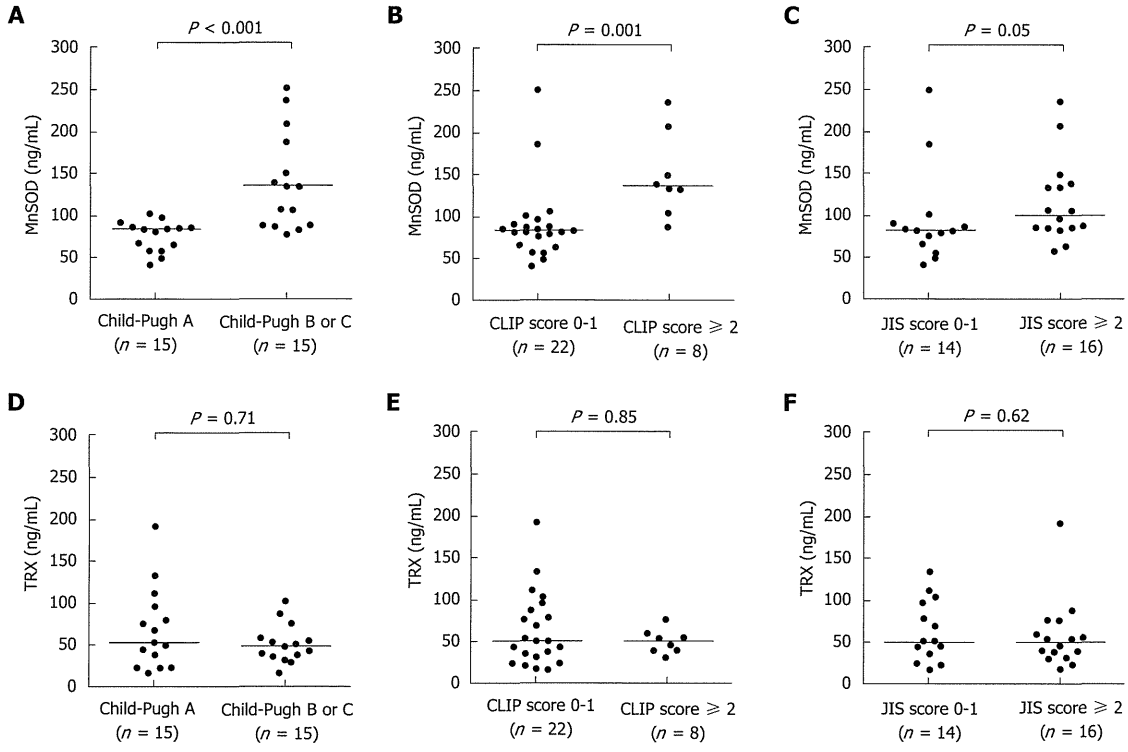

Figure 2 Clinical significance of serum manganese superoxide dismutase and thioredoxin levels in hepatocellular carcinoma. In the hepatocellular carcinoma (HCC) group, differences in serum manganese superoxide dismutase (MnSOD) and thioredoxin (TRX) levels were evaluated based on Child-Pugh stage, cancer 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

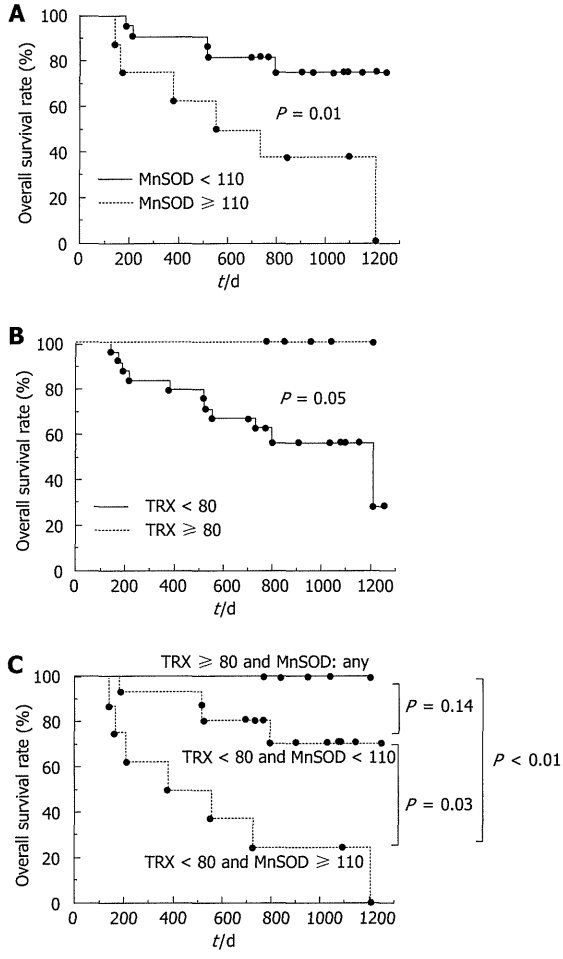

Figure 3 Overall hepatocellular carcinoma patient survival based on serum levels of manganese superoxide dismutase or thioredoxin. Overall survival was plotted using the Kaplan-Meier method after separation into two or three groups defined as follows: A: Manganese superoxide dismutase (MnSOD) < 110 ng/mL 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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