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Reduced Organic Anion Transporter Expression Is a Risk Factor for Hepatocellular Carcinoma in Chronic Hepatitis C Patients: A Propensity Score Matching Study

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Key Words

Hepatocellular carcinoma · SLC22A7 · Organic anion transporter 2 · Chronic hepatitis C · Hepatocarcinogenesis

Abstract

Objectives: Recent reports indicated that reduced SLC22A7 (a gene-encoding organic anion transporter 2) expression in noncancerous liver tissue predicts hepatocellular carcinoma (HCC) recurrence after curative resection. Our study aimed to elucidate the association between SLC22A7 expression and HCC development in chronic hepatitis C patients. ***Methods:*** HCC recurrence after local ablation therapy and SLC22A7 expression in noncancerous liver tissue were analyzed in 20 patients. Subsequently, the association between de novo HCC development and SLC22A7 expression was examined at baseline in 38 hepatitis C patients without HCC who subsequently developed HCC as well as in 76 hepatitis C patients who did not develop HCC and were matched for age, gender and stage of fibrosis. ***Results:*** In the patients whose HCC had been cured, reduced SLC22A7 expression in noncancerous liver tissue was significantly associated with a high incidence of multifocal HCC recurrence. In patients without HCC at baseline, cumulative incidence of de novo HCC development was significantly higher with a reduced SLC22A7 expression than with a normal expression ($p = 0.01$). This difference remained significant among patients without known risk factors for HCC like age and advanced fibrosis. ***Conclusion:*** Reduced SLC22A7 expression in the liver indicates a significant risk for HCC development in chronic hepatitis C, independently of other risk factors.

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Introduction

Hepatocellular carcinoma (HCC) is the third most common cancer worldwide [1] and the most frequent primary liver cancer [2]. Chronic hepatitis C virus (HCV) infection is a major risk factor for developing HCC [3], increasing the risk by 17-fold when compared with healthy individuals [4, 5]. Among HCV-positive patients, several risk factors for HCC have been well documented, including age, obesity, sex, serum platelet count and stage of liver fibrosis [6–10]. Advanced fibrosis, in particular, is the most significant risk factor for HCC in chronic HCV patients. The response to interferon therapy is also related to HCC risk [11, 12], mainly because the treatment attenuates hepatitis in responsive individuals. However, despite the absence of known risk factors, younger patients and those with nonadvanced fibrosis also develop HCC. Thus, surveillance is insufficient and additional risk analyses are required for those chronic HCV patients without known risk factors for HCC.

As for curatively treated HCC patients, tumor differentiation or progenitor-cell feature markers of cancerous tissue have been identified as predictors of recurrence [13, 14]. In contrast, only several reports have mentioned the importance of background noncancerous liver tissue and the microenvironment; these are predictive of HCC recurrences [15, 16]. Moreover, no specific features of noncancerous liver tissue have been clarified to be associated with de novo HCC development.

A recent prospective study showed that reduced SLC22A7 (organic anion transporter 2, OAT2) activity in noncancerous liver tissue is associated with multifocal recurrence after curative resection, independently of age and stage of fibrosis [17]. Furthermore, this study revealed that reduced SLC22A7 expression indicates a high risk for poor prognosis [18]. This observation indicates that the function of the transporter in noncancerous liver tissue is related to hepatic carcinogenesis, which may explain HCC development in patients who have no other known risk factors.

In this study, the use of SLC22A7 as a biomarker for HCC recurrence after curative local ablation therapy was assessed in order to validate and extend previously reported observations. Subsequently, the propensity score matching method was used to match patients with and without HCC development as well as to elucidate the association between SLC22A7 expression in hepatitis tissue and the risk of HCC development in chronic HCV patients.

Patients and Methods

Distant Recurrence after Radio Frequency Ablation Therapy for HCC

Patients

To reveal the relationship between multifocal HCC recurrence and SLC22A7 expression in noncancerous liver tissue, we conducted a retrospective study enrolling patients who received curative local ablation therapy. Twenty of the patients who enrolled in this cohort fulfilled the following criteria: (1) their HCC was treated curatively by radio frequency ablation (RFA); (2) they were infected with HCV and (3) they underwent liver biopsy at least 6 months after curative RFA. Written informed consent was obtained from all patients. The study was approved by the Ethical Committee of the Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Data Collection and Histological Evaluation

Patient characteristics, treatment details and biochemical, hematological, virological and histological data were collected at enrollment.

Liver biopsy specimens were obtained using 13-gauge needles under laparoscopy or 15-gauge needles using an ultrasound guide. Liver biopsy specimens were scored by board-certified pathologists for stage of fibrosis and grade of inflammatory activity according to the classification by Desmet et al. [19].

Immunohistochemical Staining of SLC22A7

All liver biopsy specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with anti-OAT2 (SLC22A) antibody (kindly provided by Dr. Anzai) at a 1:20 dilution. Immunohistochemical (IHC) staining was performed using an automated immunostainer (Ventana XT System; Ventana Medical Systems Inc., Tucson, Ariz., USA), with the same procedure as the previous study [17]. Cell staining was evaluated along the entire length of the biopsy core (>30 high-power fields). Staining was graded according to the following score: ≤25% = reduced staining of cells and >25% = normal staining of cells (fig. 1). Scoring of SLC22A7 staining was performed independently by two hepatologists (K.M. and A.K.) who were blinded to the clinical outcome, and average scores were used for analysis.

Surveillance for HCC

Patients were examined for HCC every 3–6 months by abdominal ultrasonography, dynamic computed tomography or magnetic resonance imaging. Serum alpha-fetoprotein levels were measured every 3 months. HCC diagnosis was confirmed from needle biopsies, surgical resection specimens or according to the typical radiological hallmarks of early enhancement and delayed washout. The start date of follow-up was the date of liver biopsy and the end date was HCC development or the latest medical attendance.

Relationship between SLC22A7 and de novo HCC Development in Chronic HCV without HCC at Baseline

Patients

To elucidate the relationship between SLC22A7 and de novo hepatic carcinogenesis, we conducted a study in an independent cohort. A consort diagram of this study is shown in figure 2. Since 1992, 1,512 chronic HCV patients provided liver biopsies prior to interferon therapy at Musashino Red Cross Hospital. A total of 1,003 of these patients did not achieve a sustained virological re-

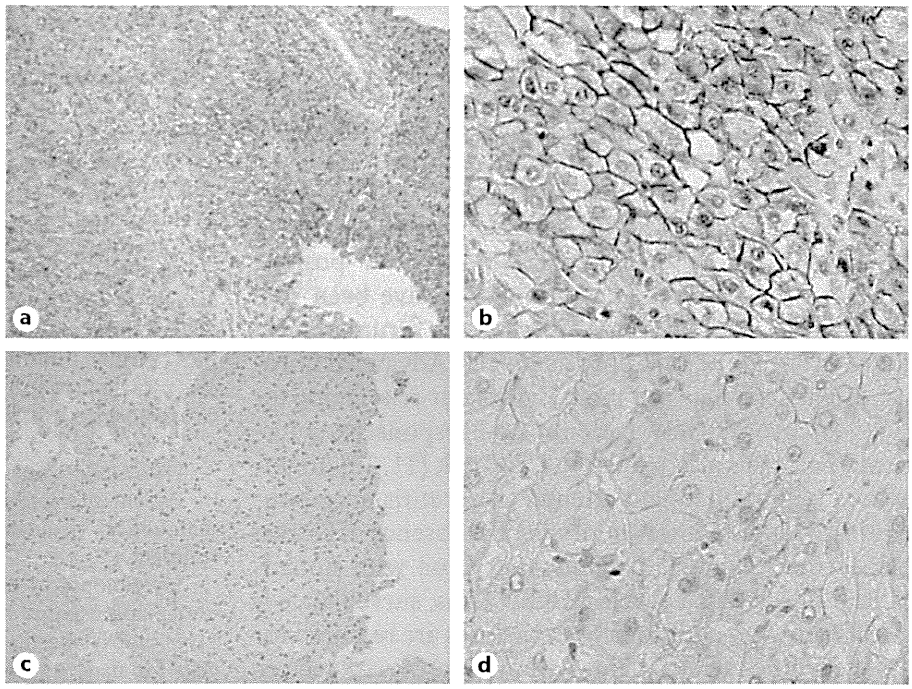

Fig. 1. IHC analysis of SLC22A7 in biopsy specimens. **a**, **b** Normal SLC22A7 expression (≥25% positive cells) **a** ×100. **b** ×400. **c**, **d** Reduced SLC22A7 expression (<25% positive cells). **c** ×100. **d** ×400.

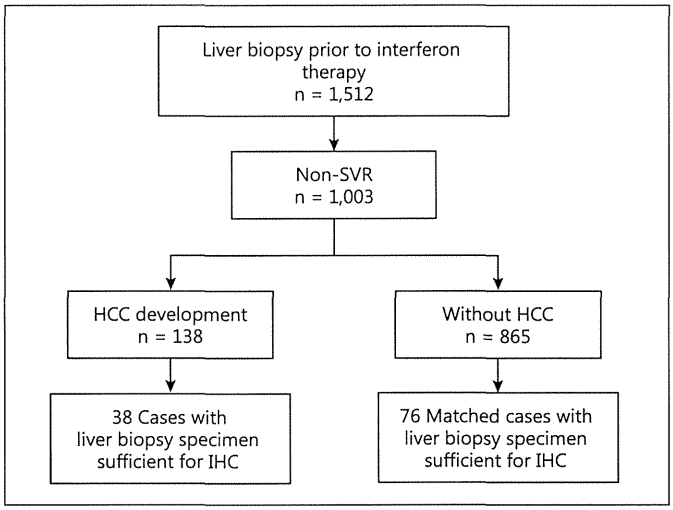

Fig. 2. Consort diagram of stratified analyses.

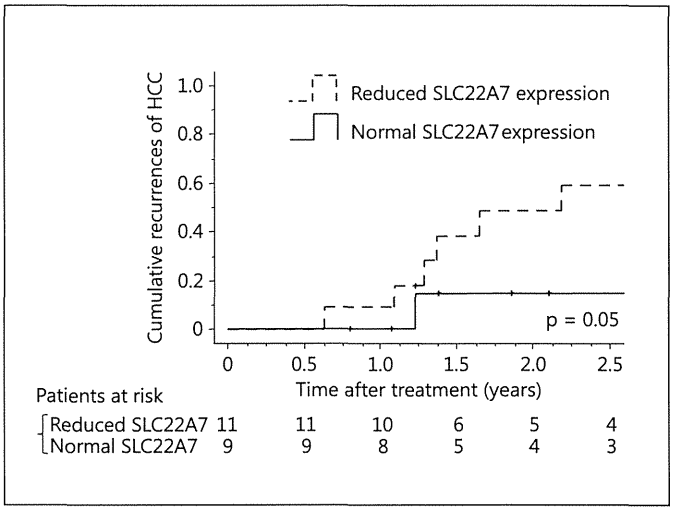

Fig. 3. Cumulative incidence of HCC recurrence after curative RFA was compared between patients with normal and reduced SLC22A7 expression.

sponse (SVR) to therapy and among these, 132 developed HCC. We enrolled 38 non-SVR patients who developed HCC and 76 matched non-SVR patients who did not develop HCC. Ninety-four patients who developed HCC were excluded because their liver biopsy specimens were of insufficient quality for IHC analyses. Matching was performed using a propensity score matching method. Histological evaluation, IHC staining and surveillance for HCC were performed as above. The average duration of follow-up was 6.6 years for all patients and 7.9 years for patients who did not develop HCC. As above, written informed consent was obtained from all patients and the study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Propensity Score Matching

In multivariate analyses of 1,003 non-SVR patients, age, gender and stage of fibrosis were independent risk factors for HCC development. Using this multivariate logistic regression analysis, pro-

Table 1. Baseline characteristics of patients who underwent RFA

	Normal SLC22A7 expression (n = 9)	Reduced SLC22A7 expression (n = 11)	p value
Age, years	66.5±5.0	62.9±4.1	0.09
Gender (M/F)	4/5	3/8	0.64
Fibrosis (F0–2/F3–4)	5/4	4/7	0.65
Mean tumor size, mm	20.4±11.3	18.8±6.0	0.91
Albumin, g/dl	4.0±0.3	3.9±0.3	0.71
Bilirubin, mg/dl	0.7±0.2	0.9±0.4	0.09
AST, IU/l	82.0±47.1	74.2±30.6	0.84
ALT, IU/l	80.7±50.2	75.1±33.0	0.85
Glucose, mg/dl	100.3±11.6	123.5±38.7	0.25
Cholesterol, mg/dl	164.0±21.5	166.6±33.8	0.93
Alpha fetoprotein, ng/ml[a]	6.8 (3.7–106)	19.3 (5.9–87.3)	0.46
DCP, mAU/ml[a]	32 (14–129)	15 (14–26)	0.15

ALT = Alanine aminotransferase; DCP = des-gamma-carboxy prothrombin.
[a] Values are shown with median and range.

Table 2. Baseline characteristics of patients enrolled in study 2

	HCC cases (n = 38)	Non-HCC matching cases (n = 76)	p value
Age, years	64.6±7.1	64.6±6.4	0.98
Gender (M/F)	19/19	39/37	0.99
Fibrosis (F0–2/F3–4)	15/23	31/45	0.84
BMI	23.8±3.1	23.5±3.2	0.60
Albumin, g/dl	3.9±0.3	4.1±0.3	0.007
Bilirubin, mg/dl	0.7±0.3	0.7±0.3	0.42
AST, IU/l	83.5±39.2	66.2±37.7	0.07
ALT, IU/l	92.4±45.9	76.8±56.6	0.29
GGT, IU/l	74.6±59.0	63.2±54.0	0.42
Platelets, $10^4/\mu l$	13.2±4.9	14.6±4.3	0.12
Glucose, mg/dl	116.8±20.9	112.4±24.1	0.16
Cholesterol, mg/dl	163.6±32.6	171.1±28.0	0.14

ALT = Alanine aminotransferase; BMI = body mass index; GGT = gamma-glutamyl transpeptidase.

pensity scores were calculated for each patient. These scores were used to match patients who developed HCC (HCC cases) with those who did not (non-HCC cases). Each HCC case was matched with 2 non-HCC cases whose propensity scores were similar to that of the HCC case (nearest-neighbor matching). Data analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, Ill., USA).

Statistical Analysis

Continuous variables are reported as the mean and standard deviation (SD) or median and categorical variables are shown as counts and proportions. Statistical significance was assessed using the Student t test (mean), the Mann-Whitney U test (median) or the Fisher exact test. In all tests, 2-sided p values were calculated and differences were considered statistically significant when $p < 0.05$. Statistically significant differences identified in univariate analyses were further assessed in multivariate logistic regression analysis. The stepwise and multivariate Cox proportional hazard models were used to explore independent factors that could be used to predict HCC development. Statistical analyses were performed using the SPSS software version 11.0.

Results

SLC22A7 Expression and Distant Recurrence after Curative RFA

Baseline characteristics of patients who received RFA are shown in table 1. No significant differences were observed between patients with normal SLC22A7 expression and those with reduced SLC22A7 expression. Figure 3 shows the cumulative rates of distant recurrences

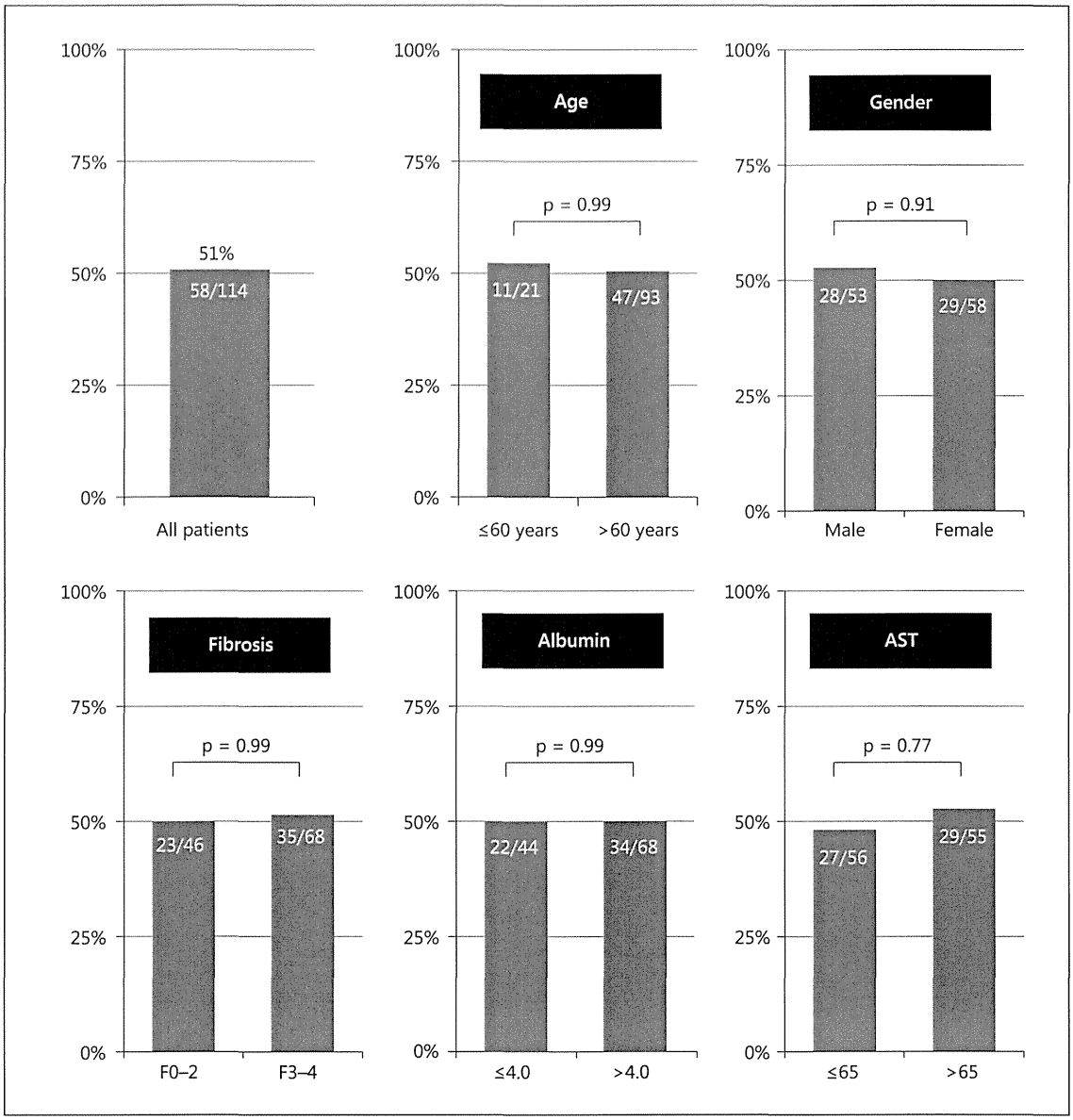

Fig. 4. Percentage of patients with normal SLC22A7 expression according to baseline clinical findings. No significant differences in the percentage of patients with normal SLC22A7 expression were observed after stratification by age, gender, fibrosis stage, albumin and/or AST.

after curative HCC treatment. Patients with reduced SLC22A7 expression had significantly higher rates of distant recurrence than those with normal SLC22A7 expression.

SLC22A7 Expression and de novo Hepatic Carcinogenesis in Chronic HCV Patients

Patient characteristics at the time of enrollment are shown in table 2. Age, gender and stage of liver fibrosis were matched using propensity scores. The distribution of serum albumin levels differed significantly between HCC cases and non-HCC cases. Serum aspartate aminotransferase (AST) levels were higher in patients with HCC than in those without HCC, although this was not statistically significant. Other factors, including body mass index, platelet count, serum glucose and serum cholesterol, which are known risk factors for HCC, were not significantly different between the patient groups.

Table 3. Factors associated with hepatic carcinogenesis according to the Cox proportional hazards model

Factors	Multivariable analysis	
	HR (95% CI)	p value
SLC22A7 (reduced expression)	3.49 (1.56–7.83)	0.002
Albumin (per 1 g/dl)	6.37 (1.56–25.6)	0.009

Normal SLC22A7 expression was found in 58 patients (51%) and reduced SLC22A7 expression was found in 56 patients. No significant differences in baseline characteristics were observed between these groups. When stratified by the matched risk factors age, gender and fibrosis stage, no significant differences were observed in the percentage of patients with normal SLC22A7 expression. Similarly, no significant differences were identified between the groups that were stratified by unmatched serum albumin and AST, which differed between HCC and non-HCC cases (fig. 4). In contrast, the percentage of patients with normal SLC22A7 expression was lower in HCC cases than in non-HCC cases (37 vs. 58%, respectively, $p = 0.05$). Furthermore, among patients aged <60 years, the percentage with normal SLC22A7 expression was significantly lower in HCC cases than in non-HCC cases ($p = 0.02$). This difference was observed in male patients ($p = 0.001$) and in patients with nonadvanced fibrosis (i.e. stages F0–2; $p = 0.05$; fig. 5). However, no significant differences were observed among patients aged >60 years, among female patients or among those with advanced fibrosis (i.e. stages F3–4).

The cumulative incidence of HCC was significantly higher in patients with reduced SLC22A7 expression than in those with normal SLC22A7 expression (33.9 vs. 13.8% after 5 years, respectively, $p = 0.01$). This difference remained significant in patients without a known risk of HCC development, such as older patients and those with advanced liver fibrosis (fig. 6). Importantly, in patients aged <60 years, the cumulative incidence of HCC after 5 years was 60 and 0% in those with reduced and normal SLC22A7 expression, respectively ($p = 0.02$). In patients with nonadvanced liver fibrosis, the cumulative incidence of HCC after 5 years was 31.3 and 12.0% in patients with reduced and normal SLC22A7 expression, respectively ($p = 0.02$). Because serum albumin levels differed between HCC and non-HCC cases, we assessed the cumulative incidence of HCC after stratification by this variable. Receiver operating characteristic analyses revealed that a level of 4.0 g/dl of serum albumin was the most appropriate cut-off for predicting HCC development. Therefore, we divided all cases into 2 groups with this cut-off. In patients with ≥4.0 g/dl of serum albumin, the cumulative incidence of HCC was significantly higher in patients with reduced SLC22A7 expression than in those with normal SLC22A7 expression (23.5 vs. 5.9% after 5 years, respectively, $p = 0.03$). In contrast, among patients with <4.0 g/dl of serum albumin, the cumulative incidence of HCC after 5 years was 50.0 and 22.7% in those with reduced and normal SLC22A7 expression, respectively ($p = 0.06$; fig. 6).

Multivariate analyses confirmed that serum albumin levels (odds ratio 3.1 and $p = 0.003$) and SLC22A7 expression (odds ratio 2.6 and $p = 0.01$) were independent risk factors for HCC in this cohort (table 3).

Discussion

This study demonstrates higher cumulative rates of multifocal HCC recurrence after curative treatment in patients with reduced SLC22A7 expression. Moreover, SLC22A7 expression in chronic HCV tissue specimens was a significant predictor for future development of HCC in chronic HCV patients. These analyses indicate the importance of SLC22A7 expression as a predictor of multifocal HCC, de novo and after curative treatment. In particular, among patients without known risk factors for HCC, the cumulative incidence of HCC was significantly higher in those with reduced SLC22A7 expression.

A recent study showed that reduced SLC22A7 expression is an independent risk factor for recurrence after HCC resection [17]. We hypothesized that SLC22A7 might be an IHC marker for the multifocal occurrence of HCC. Initially, we validated the previously reported utility of SLC22A7 as a biomarker for HCC recurrence after curative therapy in HCC patients treated with RFA instead of resection. Subsequently, we revealed a significant association between SLC22A7 expression in hepatitis tissue and the risk of future HCC in chronic HCV patients. Indeed, previous studies show several risk factors for HCC in these patients, including failure to achieve SVR, older age, male gender, obesity and advanced fibrosis and steatosis of the liver [20–22]. According to current data, assessments of transporter function in liver biopsies contribute an additional valuable predictor. This was further emphasized in patients who lacked known risk factors, such as older age and advanced fibrosis. Given the paucity of known risk factors for HCC among younger pa-

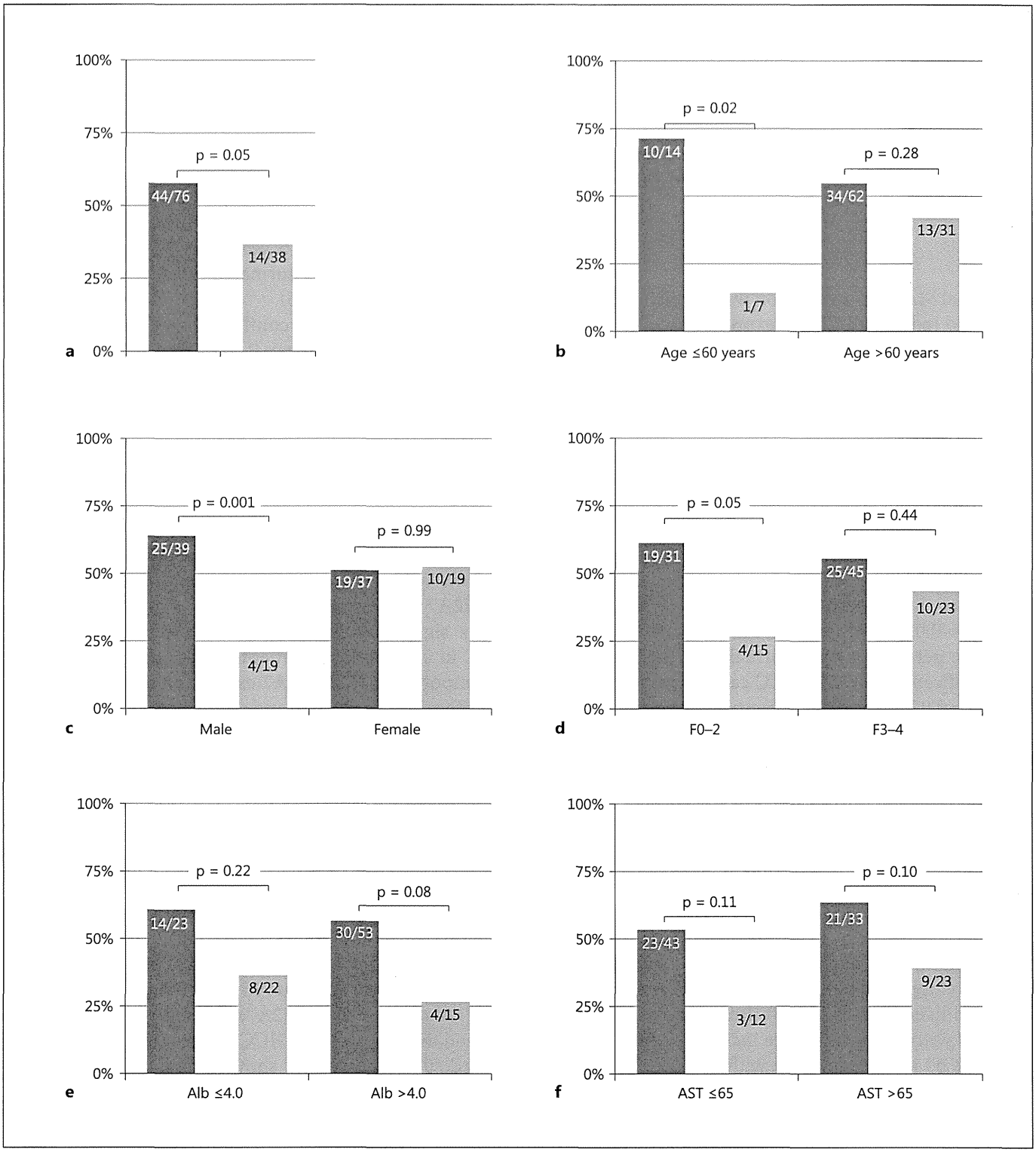

Fig. 5. Percentage of patients with normal SLC22A7 expression and HCC (**a**). SLC22A7 staining was compared between patients who did and did not develop HCC after stratification by age (**b**), gender (**c**), fibrosis stage (**d**), albumin (Alb, **e**) and AST levels (**f**). Light grey and dark grey bars represent patients with and without HCC, respectively.

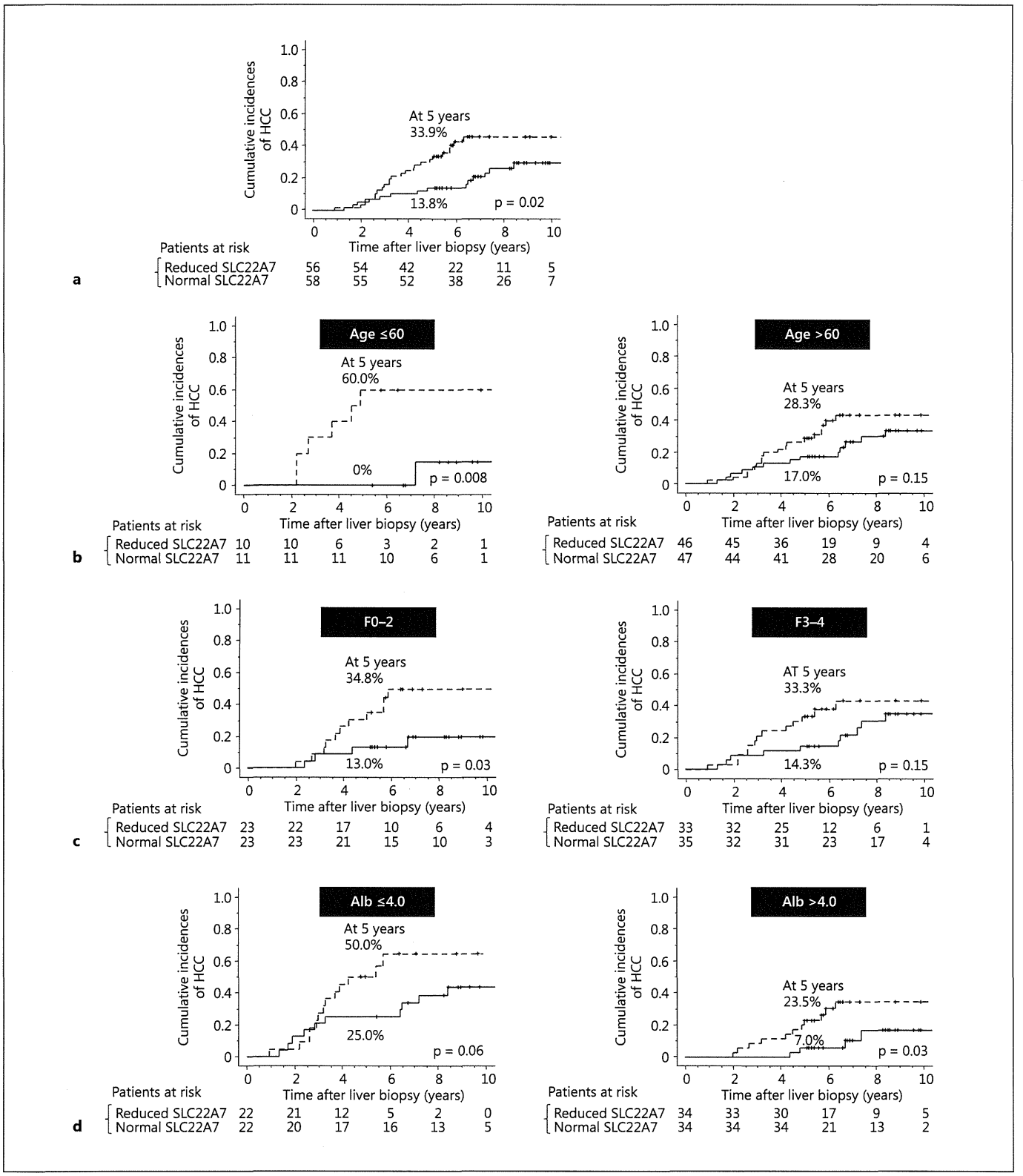

Fig. 6. Cumulative incidence of HCC according to SLC22A7 staining. **a** Comparison of the cumulative incidences of HCC in patients with normal (solid line) and reduced SLC22A7 expression (broken line). **b–d** The cumulative incidences of HCC after stratification by age (**b**), fibrosis stage (**c**) and albumin (Alb) level (**d**), respectively.

tients without advanced fibrosis, SLC22A7 expression can provide an important cost-effective screening tool. Moreover, we confirmed previous knowledge of low serum albumin levels as an independent risk factor for HCC development in patients matched for age, gender and stage of liver fibrosis. Nonetheless, in patients with higher serum albumin levels (≥4.0 g/dl), reduced SLC22A7 expression remained a significant independent risk factor for HCC.

The *SLC22A7* gene encodes OAT2, which is distributed mainly in the liver and kidney. As a protein predominantly expressed in the liver [23], OAT2 transports several antiviral drugs as well as prostaglandins. A recent study in rats showed that OAT2 is responsible for the uptake of orotic acid [24], which reportedly promotes liver carcinogenesis [25, 26]. In the clinical setting, orotic aciduria was also observed in HCC patients without liver cirrhosis [27]. Moreover, a previous study using gene-set enrichment analysis revealed that SLC22A7 expression is significantly correlated with mitochondrial oxidoreductase activity and fatty acid metabolism. Mitochondrial dysfunction and oxidative stress are considered key mechanisms for the development of HCC. Collectively, these studies indicate that reduced SLC22A7 expression promotes hepatic carcinogenesis by increasing the concentration of orotic acid around hepatocytes and promoting oxidative stress and mitochondrial dysfunction. Our study suggests that these microenvironmental changes might occur in patients with chronic HCV in an early stage. As for HCC recurrence after surgical resection, gene expression has been extensively investigated in tissues surrounding HCC [16, 28–30]. However, it remains unknown whether these signatures correlate with multifocal occurrence of HCC. Indeed, the precise mechanisms involved in the association between SLC22A7 expression and HCC development require further investigation.

In this study, personally gifted antibody was used for IHC. Staining performance of our antibody was similar to that of commercially available antibodies (Atlas Antibodies, Stockholm, Sweden) by a small pilot study (unpubl. data).

Our retrospective study design and low patient numbers must be acknowledged as limitations, particularly in the first study. However, this first study confirmed that our biopsy specimens were feasible for IHC analysis of SLC22A7, and we could therefore proceed to the larger matched-control study. To improve reproducibility, we conducted a propensity score matched study and only included patients who were HCV-positive and had not achieved SVR with interferon therapy, so our results may not pertain to chronic HCV patients who achieve SVR or patients with other chronic diseases of the liver. A larger prospective study will be required to confirm our results.

In conclusion, our study showed the importance of IHC staining for SLC22A7 as a predictive tool for HCC. We propose that patients with reduced SLC22A7 expression and lower serum albumin levels are candidates for intensive HCC surveillance, even if they do not exhibit other known risk factors.

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Original Article

Serum granulysin levels as a predictor of serious telaprevir-induced dermatological reactions

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Aim: Telaprevir-based therapy for chronic hepatitis C patients is effective; however, the high prevalence of dermatological reactions is an outstanding issue. The mechanism and characteristics of such adverse reactions are unclear; moreover, predictive factors remain unknown. Granulysin was recently reported to be upregulated in the blisters of patients with Stevens–Johnson syndrome (SJS). Therefore, we investigated the risk factors for severe telaprevir-induced dermatological reactions as well as the association between serum granulysin levels and the severity of such reactions.

Methods: A total of 89 patients who received telaprevir-based therapy and had complete clinical information were analyzed. We analyzed the associations between dermatological reactions and clinical factors. Next, we investigated the time-dependent changes in serum granulysin levels in five and 14 patients with grade 3 and non-grade 3 dermatological reactions, respectively.

Results: Of the 89 patients, 57 patients had dermatological reactions, including nine patients with grade 3. Univariate analysis revealed that grade 3 dermatological reactions were significantly associated with male sex. Moreover, serum granulysin levels were significantly associated with the severity of dermatological reactions. Three patients with grade 3 dermatological reaction had severe systemic manifestations including SJS, drug-induced hypersensitivity syndrome, and systemic lymphoid swelling and high-grade fever; all were hospitalized. Importantly, among the three patients, two patients' serum granulysin levels exceeded 8 ng/mL at onset and symptoms deteriorated within 6 days.

Conclusion: Male patients are at high risk for severe telaprevir-induced dermatological reactions. Moreover, serum granulysin levels are significantly associated with the severity of dermatological reactions and may be a predictive factor in patients treated with telaprevir-based therapy.

Key words: drug-induced hypersensitivity syndrome, granulysin, hepatitis C virus, telaprevir, toxic epidermal necrolysis

INTRODUCTION

HEPATITIS C IS a major pathogen causing liver cirrhosis and hepatocellular carcinoma worldwide. Until recently, standard therapies for chronic hepatitis C virus (HCV) genotype 1 infection were based on the combination of pegylated interferon (PEG IFN) and ribavirin (RBV); these combination therapies yield a sustained virological response (SVR) rate of approximately 50%.[1] Several classes of novel direct-acting antivirals

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(DAA) were recently developed and tested in clinical trials. Two first-generation HCV NS3/4A protease inhibitors, boceprevir[2,3] and telaprevir,[4–6] have been approved for the treatment of genotype 1 HCV infection. The inclusion of these agents in HCV treatment regimens has led to large improvements in treatment success rates.

Telaprevir, the first DAA, is administrated in combination with PEG IFN and RBV for 24 weeks, resulting in SVR rates up to 70–80%.[4,6–8] Although the telaprevir combination regimen is highly effective, the high frequency and severity of adverse events are outstanding issues limiting its use. Dermatological reactions are particularly prevalent, developing in 56–84.6% of patients treated with telaprevir, PEG IFN and RBV combination therapy.[9,10] Moreover, the prevalence of severe dermatological reactions including Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) and drug-induced hypersensitivity syndrome (DIHS) are substantially higher in patients treated with telaprevir-based therapy than PEG IFN and RBV combination therapy.[8,10] McHutchison *et al.* reported that 7% of patients treated with telaprevir, PEG IFN and RBV combination therapy discontinue therapy because of rash or pruritus in contrast to only 1% of patients treated with PEG IFN and RBV.[8] In some patients, serious skin reactions persist even after stopping all drugs.[10] However, the pathogenesis and clinical predictors of these adverse reactions are poorly understood.

Granulysin is a 15-kDa cationic cytolytic protein released by cytotoxic T lymphocytes and natural killer cells that induces apoptosis in target cells and has antimicrobial activities.[11] Serum levels of granulysin are elevated in primary virus infections including Epstein–Barr virus and parvovirus B19.[12] It was recently reported that serum granulysin levels are significantly elevated in patients with several types of severe dermatological lesions including SJS/TEN, which is the characteristic serious adverse event in telaprevir-containing regimens.[13,14]

Accordingly, the present study determined the risk factors for severe dermatological reactions in patients receiving telaprevir, PEG IFN and RBV combination therapy as well as the association between serum levels of granulysin and severe dermatological reactions.

METHODS

Patients and methods

IN THIS RETROSPECTIVE case–control study, at Hokkaido University Hospital and associated hospitals in the NORTE Study Group, between December 2011 and November 2013, a total of 123 patients positive for HCV genotype 1 with high serum HCV RNA titer (>5 log IU/mL) received PEG IFN, RBV and telaprevir combination therapy. Patients were excluded if they required hemodialysis or had a positive test result for serum hepatitis B surface antigen, co-infection with other HCV genotypes or HIV, evidence of autoimmune hepatitis or alcoholic hepatitis, or malignancy. Serum granulysin levels were analyzed in five healthy volunteers with no HCV, HIV or hepatitis B virus infection or any inflammatory diseases.

Written informed consent according to the process approved by the hospital's ethics committee was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of each participating hospital.

Study design and treatment regimen

Telaprevir 500 or 750 mg was typically administrated every 8 h after meals for 12 weeks. PEG IFN-α-2b (PegIntron; MSD, Tokyo, Japan) 1.5 IU/kg was administrated s.c. once per week for 24 weeks. RBV (Rebetol; MSD) was administrated for 24 weeks in two divided daily doses according to bodyweight: 600, 800 and 1000 mg for patients with bodyweights of less than 60, 60–80 and more than 80 kg, respectively. The doses of PEG IFN-α-2b, RBV and telaprevir were reduced at the attending physician's discretion on the basis of hemoglobin levels, decreased white blood cell or platelet counts, or adverse events.

During treatment, patients were assessed as outpatients at weeks 1, 2, 4, 6 and 8, and then every 4 weeks thereafter for the duration of treatment. Physical examinations and blood tests were performed at all time points.

Outcomes

The primary end-point was SVR, which was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. The secondary end-points were end-of-treatment virological responses (HCV RNA undetectable in serum) and rapid virological response (RVR), which was defined as undetectable serum HCV RNA at 4 weeks after the start of treatment. Dermatological reactions were classified according to severity in the same manner as in phase III trials in Japan.[10]

Serum granulysin measurement

To evaluate serum granulysin levels in chronic hepatitis C, we first measured serum granulysin levels in five

healthy volunteers and compared them with those of 20 chronic hepatitis C patients before treatment. Serum granulysin levels were measured at the onset of dermatological reactions (within 3 days of onset); if the symptoms worsened, the time when worsening occurred was adopted. Meanwhile, in patients with no dermatological reactions, the highest serum granulysin level during treatment was adopted.

Serum granulysin levels were measured by a sandwich enzyme-linked immunosorbent assay as described previously.[12,14,15] Briefly, plates coated with 5 mg/mL mouse antibody against human granulysin, RB1 antibody, were washed with phosphate-buffered saline containing 0.1% Tween-20. Next, they were blocked with 10% fetal bovine serum in washing buffer at room temperature for 2 h. The samples and standards (Recombinant Granulysin; R&D Systems, Minneapolis, MN, USA) were incubated for 2 h at room temperature. Next, they were reacted with 0.1 mg/mL biotinylated mouse antibody against human granulysin, RC8 antibody. The plates were subsequently treated with horseradish peroxidase-conjugated streptavidin (Roche Diagnostics, Basel, Switzerland). The plates were then incubated with tetramethyl-benzidine substrate (Sigma, St Louis, MO, USA), and 1 M sulfuric acid was then added. The optical density was measured at 450 nm using a microplate reader.

Diagnosis of dermatological reactions

Dermatological reactions were investigated throughout the 24-week administration period in the telaprevir-based combination therapy. Dermatological reactions were classified according to severity as follows. Grade 1 was defined as involvement of less than 50% of the body surface and no evidence of systemic symptoms. Grade 2 was defined as involvement of less than 50% of the body surface but with multiple or diffuse lesions or rashes with characteristic mild systemic symptoms or mucous membrane involvement with no ulceration/erosion. Grade 3 was defined as a generalized rash involving 50% or more of the body surface or a rash with any new significant systemic symptoms and considered to be related to the onset and/or progression of the rash. Life-threatening reactions included SJS, TEN, drug rash with eosinophilia and systemic symptoms (DRESS)/DIHS, erythema multiforme and other life-threatening symptoms, or patients presenting with features of serious disease.

When adverse skin reactions were detected, the attending physician classified the degree of severity and referred the patients to a dermatologist as needed. In principal, when grade 3 dermatological reactions occurred, the attending physician referred the patient to a dermatologist and discontinued telaprevir. When severe dermatological reactions including SJS/TEN and DRESS/DIHS were suspected, all drugs were discontinued immediately. SJS/TEN and DIHS were diagnosed by skin biopsy and according to disease criteria, respectively.

Statistical analysis

Categorical and continuous variables were analyzed by the χ^2-test and the unpaired Mann–Whitney *U*-test, respectively. All *P*-values were two-tailed, and the level of significance was set at $P < 0.05$. Multivariate logistic regression analysis with stepwise forward selection included variables showing $P < 0.05$ in univariate analyses.

The association between dermatological reactions and serum granulysin levels were evaluated by one-way ANOVA followed by Tukey's honestly significant difference test. All statistical analyses were performed using SPSS version 21.0 (IBM Japan, Tokyo, Japan).

RESULTS

Patients

WE INCLUDED 123 chronic hepatitis C patients who received telaprevir-based triple therapy. Of these, 89 patients who had proper information of dermatological adverse events were included. The baseline characteristics of patients are shown in Table 1.

Of these 89 patients, time-dependent changes of serum granulysin concentrations were measured in 20 who had had conserved serum, at least, at the pretreatment point, 1 and 2 weeks after commencement of therapy, 1 and 2 months after commencement of therapy, the onset point of dermatological adverse reaction and the worsening point if symptoms became worse.

Among the 89 patients, 64% (57/89) developed dermatological reactions, including nine with grade 3 reactions (Table 2). The characteristics of dermatological reactions by grade are shown in Table 2. Non-grade 3 dermatological reactions tended to occur early during treatment compared to grade 3 dermatological reactions.

Association between dermatological reactions and treatment outcomes

First, we determined whether dermatological reactions were associated with final treatment outcomes.

Table 1 Baseline characteristics of the participating patients

Total number	89
HCV genotype 1b (1b/others)	89/0
Age (years)†	60.0 (19–73)
Sex (male/female)	48/41
Bodyweight (kg)†	63.0 (32–97)
Baseline white blood cell count (/μL)†	4800 (1500–9800)
Baseline hemoglobin level (g/dL)†	13.5 (9.9–16.7)
Baseline platelet count ($\times 10^3$)†	15.9 (6.6–86)
Baseline ALT level (IU/L)†	40 (15–300)
Baseline HCV RNA level ($\log^{10}$ IU/mL)†	6.5 (3.2–7.6)
Initial telaprevir dose (1500/2250 mg)	20/89
Initial PEG IFN dose (1.5/<1.5 μg/kg)	775/14
Initial RBV dose (mg/kg)†	9.8 (2.2–15.5)
IL28B gene (rs8099917) (TT/non-TT/ ND)	51/22/16
HCV 70 core mutation (wild/ mutant/ND)	43/24/22
Previous treatment (naïve/relapse/NVR)	40/38/11

†Data are shown as median (range) values.
ALT, alanine transaminase; HCV, hepatitis C virus; IL28B, interleukin 28B; ND, not done; PEG IFN, pegylated interferon; RBV, ribavirin.

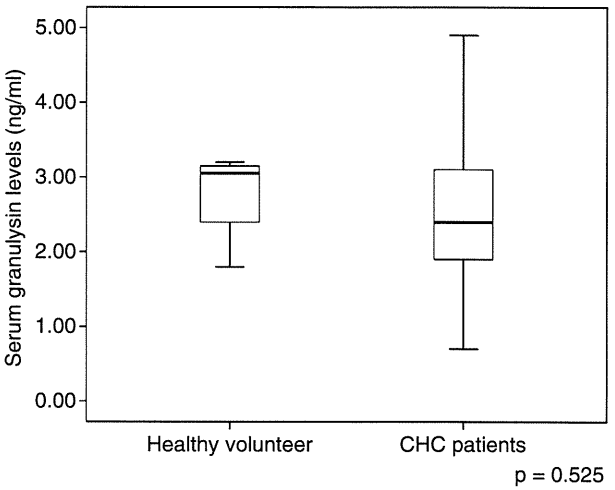

Figure 1 Serum granulysin levels of healthy volunteers and chronic hepatitis C patients. Serum granulysin levels were compared between five healthy volunteers and untreated 20 chronic hepatitis C patients. $P < 0.05$, Mann–Whitney *U*-test.

Univariate analyses identified baseline white blood cell and platelet counts, RVR, and non-grade 3 dermatological reactions significantly associated with SVR (Table 3). Among the nine patients with grade 3 dermatological reactions, three discontinued all treatment and six discontinued telaprevir administration; SVR was achieved in zero of the three (0%) and two of the six (33%), respectively.

Multivariate analysis showed that RVR and non-grade 3 dermatological reactions were significantly associated with SVR (Table 3).

Analysis of risk factors for telaprevir-induced dermatological reactions

Next, we analyzed the association between severe (i.e. grade 3) dermatological reactions and clinical parameters (Table 4). Univariate analysis showed that only sex was significantly associated with the grade 3 dermatological reactions ($P = 0.03$).

Serum granulysin levels in healthy subjects and chronic hepatitis C patients

As shown in Figure 1, serum granulysin levels did not differ significantly between healthy volunteers and chronic hepatitis C patients. Next, we evaluated the association between the severity of dermatological reactions and serum peak granulysin levels in 20 patients including five, four, five and six with grades 1, 2 and 3, and no dermatological events, respectively. One-way ANOVA showed that serum granulysin level was significantly associated with the severity of dermatological reactions ($P = 0.036$); in addition, Tukey's honestly significant difference test revealed that the serum

Table 2 Characteristics of the patients with each dermatological adverse event grade

	n	Age†	Sex (male/female)	Initial telaprevir dose (2250/1500)	Onset of DAR (days)
No DAR	32	61 (28–72)	15/17	26/6	
Grade 1	32	58 (19–73)	15/17	24/8	7 (3–50)
Grade 2	16	61 (44–73)	10/6	12/4	3.5 (1–56)
Grade 3	9	61 (48–65)	8/1	8/1	22 (1–60)

†Data are shown as median range) values.
DAR, dermatological adverse reaction

Table 3 Comparison of the clinical and laboratory characteristics of the patients with HCV infection based on therapeutic response

All patients	SVR	Non-SVR	Univariate analysis	Multivariate analysis		
$n = 89$	$n = 68$	$n = 21$	P	OR	95% CI	P
Age (years)†	60 (19–73)	62 (28–73)	0.402			
Sex (male/female)	37/31	11/10	0.870			
Bodyweight (kg)†	62 (39–97)	64 (32–87)	0.761			
Baseline white blood cells (/μL)†	5135 (1500–9800)	4200 (2490–7200)	0.048	0.492	(0.121–1.993)	0.320
Baseline hemoglobin level (g/dL)†	13.5 (10.5–16.7)	12.1 (9.9–15.4)	0.862			
Baseline platelet count ($\times 10^3$)†	16.7 (6.6–31.5)	12.8 (7.2–86)	0.025	0.388	(0.093–1.614)	0.193
Baseline ALT level (IU/L)†	37 (15–300)	53 (23–159)	0.070			
Baseline HCV RNA level ($\log^{10}$ IU/mL)†	6.7 (3.2–7.6)	6.4 (5.7–7.3)	0.812			
Baseline Cr level (mg/dL)	0.7 (0.5–1.3)	0.7 (0.5–0.9)	0.433			
Initial telaprevir dose (1500/2250 mg)	52/16	17/4	0.460			
Initial PEG IFN dose (1.5/<1.5 μg/kg)	58/10	17/4	0.430			
Initial RBV dose (mg/kg)†	9.9 (2.2–15.5)	9.5 (4.4–12.5)	0.546			
IL28B gene (rs8099917) (TT/non-TT/ND)	43/15/10	8/7/6	0.107			
Core 70 a.a. mutation (wild/mutant/ND)	36/16/16	7/8/6	0.108			
Previous treatment (naive/relapse/NVR)	34/28/6	6/10/5	0.095			
Rapid virological response (+/−)	60/8	10/11	<0.001	10.89	(2.838–41.83)	0.001
Grade 3 DAR (−/+)	66/2	14/7	<0.001	27.44	(3.718–202.5)	0.001

†Data are shown as median (range) values.
a.a., amino acid; ALT, alanine transaminase; CI, confidence interval; Cr, creatinine; DAR, dermatological adverse reaction; HCV, hepatitis C virus; IL28B, interleukin 28B; ND, not done; NVR, non-virological response; OR, odds ratio; PEG IFN, pegylated interferon; SVR, sustained virological response; RBV, ribavirin.

granulysin levels of patients with grade 3 dermatological reactions were significantly higher than those of patients with grade 1 or no dermatological reactions (both $P < 0.05$, Fig. 2).

Time-dependent changes in serum granulysin levels

We investigated the time-dependent changes in serum granulysin levels in five and 15 patients with grade 3 and non-grade 3 dermatological reactions, respectively (Fig. 3). Serum granulysin levels of patients with non-grade 3 dermatological reactions never exceeded 10 ng/ml. Of the five patients with grade 3 reactions, three had severe systemic manifestations that necessitated hospital admission: one each had SJS, DIHS, and systemic lymphoid swelling and high fever (>39 °C). All patients with grade 3 dermatological reactions with systemic manifestations had peak serum granulysin levels exceeding 10 ng/mL; importantly, the serum granulysin levels of two patients already exceeding 8 ng/mL at the onset of the reactions worsened within 6 days.

DISCUSSION

THE PRESENT STUDY demonstrates a significant association between telaprevir-induced dermatological reactions and elevated serum granulysin levels for the first time. Moreover, serum granulysin levels were significantly associated with the severity of dermatological reactions. Thus, the results indicate that serum granulysin level seems to be a useful predictor of telaprevir-induced dermatological reactions. Because the emergence of grade 3 dermatological reactions was significantly associated with non-SVR (Table 3), probably associated with high rate of treatment discontinuation, it is important to predict dermatological events in the early stage to achieve good treatment outcomes.

Table 4 Comparison of the clinical and laboratory characteristics of the patients based on the presence or absence of at least a grade 3 dermatological adverse event

All patients $n = 89$	Non-grade 3 $n = 80$	Grade ≥3 $n = 9$	Univariate analysis P
Age (years)†	60 (19–73)	61 (48–65)	0.453
Sex (male/female)	40/40	8/1	0.027
Bodyweight (kg)†	62 (32–97)	64 (51–87)	0.593
Baseline white blood cell count (/μL)†	4900 (1500–9800)	4700 (3000–7000)	0.876
Baseline hemoglobin level (g/dL)†	13.5 (9.9–16.7)	14.4 (12.1–15.4)	0.196
Baseline platelet count ($\times 10^3$)†	16.0 (6.6–86.0)	13.5 (10.4–22.5)	0.605
Baseline ALT level (IU/L)†	40(15–300)	37 (23–87)	0.765
Baseline Cr level (mg/dL)	0.7 (0.5–1.3)	0.8 (0.6–0.9)	0.123
Baseline HCV RNA level ($\log^{10}$ IU/mL)†	6.6 (3.2–7.6)	6.4 (5.7–7.1)	0.465
Initial telaprevir dose (1500/2250 mg)	62/18	7/2	0.675
Initial telaprevir/bodyweight (mg/kg)	33.7 (20–71.4)	30.0 (23.6–44.1)	0.563
Initial PEG IFN dose (1.5/<1.5 μg/kg)	66/14	9/0	0.198
Initial RBV dose (mg/kg)†	9.7 (2.2–15.5)	10.7 (7.7–12.9)	0.161
IL28B gene (rs8099917) (TT/non-TT/ND)	47/19/14	4/3/2	0.353
Core 70 a.a. mutation (wild/mutant/ND)	38/22/20	5/2/2	0.511
Previous treatment (naïve/relapse/NVR)	35/36/9	5/2/2	0.972
Onset of dermatological AE (days)	5 (1–75)	22 (1–60)	0.352

†Data are shown as median (range) values.
a.a., amino acid; AE, adverse event; ALT, alanine transaminase; Cr, creatinine; HCV, hepatitis C virus; IL28B, interleukin 28B; NVR, non-virological response; PEG IFN, pegylated interferon; RBV, ribavirin.

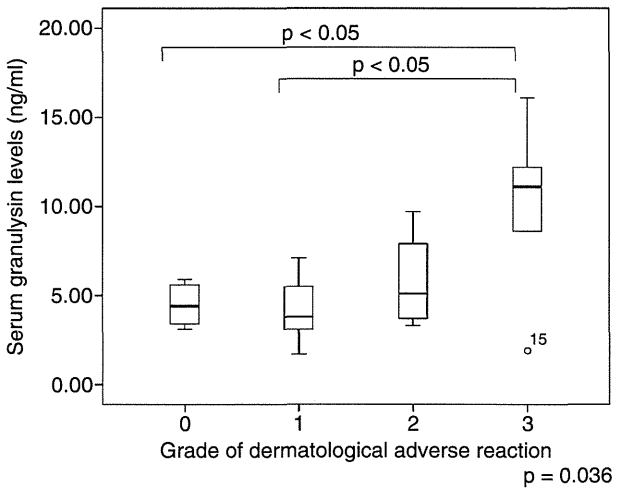

Figure 2 Association between dermatological adverse reaction severity and serum granulysin level. Serum granulysin levels were measured at the onset of dermatological reactions (i.e. within 3 days of onset); if the symptoms worsened, the time of worsening was adopted. In patients with no dermatological events, the highest serum granulysin level during treatment was adopted. $P < 0.05$, one-way ANOVA.

Recent genome-wide association studies have identified that genetic polymorphisms around the IL28B gene locus significantly associated with the outcome of PEG IFN and RBV combination therapy in HCV patients. Thus, PEG IFN and RBV combination therapy is ineffective in a subset of HCV-infected patients who have IL28B TG or GG genotypes, limiting the use of this therapy.[16] Therefore, novel drugs with different antiviral mechanisms were required. Accordingly, DAA were developed; they are mainly classified as NS3/4A protease inhibitors, or NS5B or NS5A inhibitors.[17] The NS3/4A serine protease inhibitor telaprevir, in combination with PEG IFN and RBV, has demonstrated the most promising results.[6–8] However, adverse events, especially severe dermatological reactions, develop more frequently in patients treated with telaprevir than those treated with only PEG IFN and RBV.

Little is known about the mechanisms of telaprevir-induced dermatological reactions. Reactions develop in patients treated with PEG IFN and RBV combination therapy[18,19] as well as telaprevir monotherapy.[20,21] It should be noted that the dermatological reactions in telaprevir monotherapy or PEG IFN and RBV therapy alone are generally mild.[7,8,20] However, dermatological

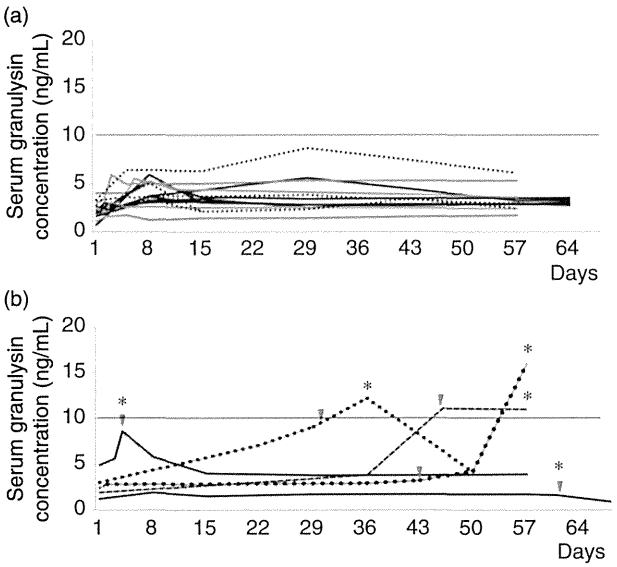

Figure 3 Association between time-dependent changes in serum granulysin levels and severe telaprevir-induced dermatological adverse reactions. (a) Time-dependent changes in serum granulysin levels patients with non-grade 3 dermatological reactions (three, five and six with grade 2, grade 1 and no reactions, respectively). The dashed line, gray line and black line indicate grade 2, grade 1 and no reaction, respectively. (b) Time-dependent changes in serum granulysin levels of five patients with grade 3 dermatological events. The dashed line indicates patients with severe systemic manifestations. Arrowheads indicate the onset of dermatological events and asterisks indicate the onset of grade 3 dermatological events.

reactions in telaprevir and PEG IFN/RBV combination therapy may be severe, indicating a synergistic effect. Severe dermatological events including SJS/TEN and DIHS have been reported in telaprevir-based triple therapy; these are life-threatening, and fatal cases have been reported.

The onset of grade 3 dermatological reactions tended to be later than non-grade 3 reactions, the same as in the study of Torii *et al.*[10] Taken together with the finding that male sex is a clinical risk factor, the results indicate that late-onset dermatological reactions in male patients treated with telaprevir-based triple therapy require more attention.

Roujeau *et al.* analyzed the risk factors for telaprevir-induced eczematous dermatitis and report that the incidence of telaprevir-related dermatitis was significantly higher age of more than 45 years, body mass index of less than 30 (kg/m^2), Caucasian ethnicity and treatment-naïve status.[9] While they analyzed the risk factors for telaprevir-induced eczematous dermatitis, the present study focused on the risk factors for severe telaprevir-induced dermatological reactions, because such reactions can affect treatment outcome (Table 2) and can be fatal. As mentioned above, male sex was significantly associated with grade 3 dermatological reactions. Sex is reported to be associated with the prevalence of some kinds of severe drug-induced dermatological events, although the underlying mechanism remains unknown.[22]

Fujita *et al.* report that serum granulysin levels are significantly elevated in SJS/TEN patients and thus may be a good predictive factor.[14] Therefore, we hypothesized that in telaprevir-based triple therapy for chronic hepatitis C patients, serum granulysin levels are associated with the severity of dermatological reactions and may thus be a predictive biomarker. However, Ogawa *et al.* report that serum granulysin levels also increase as a result of primary virus infections such as Epstein–Barr virus or parvovirus B19.[12] Thus, it remains unclear whether and how chronic viral infections, especially HCV, affect serum granulysin levels. In the present study, we compared serum granulysin levels between healthy volunteers and chronic hepatitis C patients; the results show that chronic HCV infection was not associated with serum granulysin levels (Fig. 1).

Chung *et al.* have reported that granulysin is the most highly expressed cytotoxic molecule in blisters of SJS/TEN and that massive keratinocyte death was induced by granulysin.[11] Fujita *et al.* reported that serum granulysin levels increased in the early stage of SJS/TEN caused by drugs including carbamazepine, imatinib and phenytoin.[14] Taken together with our results, we speculate that granulysin may be involved in the pathogenesis of early stage telaprevir-mediated dermatological adverse reactions possibly through induction of keratinocyte death.

Of five patients with grade 3 reactions, two patients without severe systemic manifestations did not have elevated serum granulysin of more than 10 ng/mL or did not have elevated levels before symptoms worsened. On the contrary, three patients with severe systemic manifestations had peak serum granulysin levels exceeding 10 ng/mL, and the symptoms of two patients with serum granulysin levels already exceeding 8 ng/mL at onset and within 6 days worsened. Therefore, serum granulysin tests may predict grade 3 dermatological adverse reaction with systemic manifestations. Furthermore, if serum granulysin levels elevate more than 8 ng/mL, more attention should be paid.

In Western countries, the prevalence of dermatological reactions in patients treated with telaprevir-based and

PEG IFN/RBV therapy are reported to be approximately 55% and 33%, respectively;[9,23] meanwhile, in Japanese patients, the respective rates are 74.9% and 58.7%. Moreover, approximately 4% and 9% of patients in Western and Japanese patients develop grade 3 reactions, respectively;[10] this is almost the same as that in the present study (10%). The difference may be due to genetic or ethnic variation. Therefore, genome-wide association studies may have identified a gene locus associated with telaprevir-induced severe dermatological reactions.

A limitation of this study is that the number of patients with grade 3 dermatological reactions is relatively small. However, the serum granulysin levels of patients with grade 3 dermatological reactions were significantly higher than those of other patients. Also, in two of the three patients with severe dermatological reactions, the serum granulysin level elevated before symptoms worsened, which are novel findings. Further study is required.

Triple therapy with the second-generation protease inhibitor simeprevir is reported to result in a similar prevalence of adverse reactions as PEG IFN and RBV combination therapy.[24,25] However, simeprevir is not approved worldwide. Although simeprevir-based triple therapy is effective, only 36–53% of prior non-responders achieve SVR.[24] Shimada *et al.* recently reported that by extending PEG IFN and RBV therapy from 24 to 48 weeks, telaprevir-based triple therapy improves the SVR to up to 68% in prior null responders.[26] Thus, telaprevir is a therapeutic option for prior null responders.

In conclusion, the present study suggests that male sex is a significant risk factor for severe telaprevir-induced dermatological reactions. In addition, serum granulysin levels are significantly associated with the severity of dermatological reactions and thus may be a good predictor of severe dermatological reactions with systemic manifestations in patients treated with telaprevir-based triple therapy.

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