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HEPATOLOGY

Clinical features and outcomes of cirrhosis due to non-alcoholic steatohepatitis compared with cirrhosis caused by chronic hepatitis C

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Abstract

Background and Aim: Ethnic differences in non-alcoholic steatohepatitis (NASH) are well-documented, but there has been no study on the prognosis of Japanese NASH patients with cirrhosis. Accordingly, we compared cirrhotic NASH with liver cirrhosis caused by chronic hepatitis C (LC-C) to clarify its clinical features and define the risk factors for death.

Methods: A prospective evaluation of the outcomes of NASH patients with severe fibrosis was started in 1990. Data on age- and sex-matched patients with biopsy-proven LC-C were collected retrospectively and used as the control.

Results: There were 68 patients with cirrhotic NASH and 69 with LC-C. The Child–Turcotte–Pugh (CTP) class was similar in these two groups. Although the outcome of the NASH group was better than that of the LC-C group, cirrhotic NASH followed a similar course to that of LC-C; that is, complications of cirrhosis developed, including hepatocellular carcinoma (HCC; the 5-year HCC rate was 11.3% for NASH and 30.5% for HCV) and death (the 5-year survival rates were 75.2% and 73.8%, respectively). HCC was the leading cause of death in both groups (NASH, 47%; HCV, 68%). The occurrence of HCC and the CTP class were significant risk factors for mortality in NASH patients according to a multivariate analysis (HCC: hazard ratio [HR] 7.96, 95% confidence interval [CI] 2.45–25.88, CTP class A: HR 0.17, 95% CI 0.06–0.50).

Conclusion: In conclusion, the present study confirmed that cirrhotic NASH has a similar course to LC-C. The occurrence of HCC was the strongest predictor of mortality in the NASH groups. These findings may be helpful when deciding on therapeutic interventions for NASH and also for the daily management of these patients.

Introduction

Recently, lifestyle-related diseases and metabolic syndromes (diabetes, hypertension, and hyperlipidemia) have become leading public health problems because of the dramatic increase of these diseases in both Western countries and Asia. Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of the metabolic syndrome, so NAFLD and non-alcoholic steatohepatitis (NASH) have now become major liver diseases in these countries.[1–3]

Annual health checks have resulted in 20–35% of Japanese adults being diagnosed with NAFLD by ultrasonography (US).[4,5]

Based on this high prevalence of NAFLD, the prevalence of NASH is estimated to be 1–2% among Japanese adults. It has been reported that NASH progresses to cirrhosis in up to 20% of patients.[6,7] In NASH patients, old age, obesity, insulin resistance, hypertension, and diabetes mellitus are all associated with a higher risk of cirrhosis.[8–11] We previously reported a case series of NASH patients with hepatocellular carcinoma (HCC) development,[12] but there have not been many studies about NASH and HCC.[12–18] NASH shows a wide range of severity, from minimal fibrosis to cirrhosis, so it is important to clarify the natural history of each stage (especially cirrhotic NASH) in order to determine how to manage these patients. A prospective study of the natural history of NASH patients with severe fibrosis was started at Tokyo Women's Medical University Hospital (Tokyo, Japan) in 1990. We have reported on the natural history of NASH, showing liver failure and HCC as the major causes of death in NASH patients with advanced fibrosis.[15] Although several studies have provided data about the natural history of NASH, there have been few reports on cirrhotic NASH.[19,20] Accordingly, the present study was performed to clarify the clinical features of cirrhotic NASH, by comparing the clinical features of liver cirrhosis due to NASH with those of liver cirrhosis caused by hepatitis C virus (HCV) infection, as well as to

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define the risk factors for the development of HCC and mortality in cirrhotic NASH patients.

Methods

Patients

From 1990 to December 2006, 412 Japanese patients were diagnosed with biopsy-proven NAFLD at Tokyo Women's Medical University. Among them, 70 patients had cirrhosis. An evaluation of the natural history of NASH patients with severe fibrosis was started in 1990. All patients gave informed consent to participate in a study examining the natural history of their disease, and their clinical data were collected prospectively. Data on age- and sex-matched patients with biopsy-proven cirrhosis due to HCV who were concurrently managed at our hospital were also collected retrospectively and used as the control.

We excluded two patients because of a lack of informed consent, so 68 cirrhotic NASH patients (39 women and 29 men) were included in this study. A total of 69 age- and sex-matched patients with liver cirrhosis due to chronic hepatitis C (LC-C) formed the control group.

Definitions

Follow up was started from the time of liver biopsy, which was performed in all of the patients. Although some patients had ascites at baseline, it was resolved after treatment with diuretics, and a liver biopsy was performed following the disappearance of ascites. A diagnosis of NASH was based on the following criteria: (i) detection of steatohepatitis by liver biopsy; (ii) intake of less than 100 g ethanol per week; and (iii) appropriate exclusion of other liver diseases.[21–23] Cirrhosis was diagnosed by histological examination. The HCV group had histologically-proven cirrhosis and the patients were positive for HCV viral RNA by a quantitative polymerase chain reaction assay. They had either not been treated with interferon or were virological non-responders to interferon therapy.

Obesity was defined as a body mass index (BMI) of more than 25 according to the Japanese Obesity Association criteria. The diagnosis of type II diabetes mellitus was based on the Japanese criteria. Hyperlipidemia was diagnosed if the patient was being treated with lipid-lowering medications or had elevated levels of total cholesterol (> 220 mg/dL) and/or triglycerides (> 150 mg/dL) on at least three occasions. Hypertension was diagnosed if the patient was on antihypertensive therapy or had blood pressure greater than 140/90 mmHg on at least three occasions.

All liver biopsy specimens of the cirrhotic NASH patients were examined for fibrosis, and the NAFLD activity score was calculated.[21–25] Liver biopsy specimens from the HCV patients with cirrhosis were evaluated according to the criteria of Desmet *et al.*[26]

HCC was diagnosed histologically or by the detection of consistent findings using at least two imaging techniques from among US, computed tomography (CT), magnetic resonance imaging (MRI), and selective hepatic arteriography.[27]

Patient management

A complete history was obtained and a physical examination was performed in all of the patients. The following laboratory parameters were measured: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, alkaline phosphatase, γ-glutamyltranspeptidase (γGTP), albumin, platelet count, prothrombin time, hepaplastin test, and Child–Turcotte–Pugh (CTP) class. Screening for HCC was performed in the cirrhotic NASH and HCV patients at least three times a year by measuring serum α-fetoprotein and/or prothrombin induced by vitamin K absence-II and by US. Other imaging studies (CT, MRI, and/or selective hepatic angiography) were performed in patients with suspected HCC.

In obese patients, weight was mainly controlled by diet and exercise. Thirty-five patients received drug treatment for NASH (ursodeoxycholic acid in 24 and/or vitamin E plus vitamin C in 22 patients). None of the patients underwent bariatric surgery, and liver transplantation was only performed in one patient. None of the patients were on hormone replacement therapy for menopause.

Statistical analysis

Analysis was performed with SPSS software (SPSS, Chicago, IL, USA). The Mann–Whitney test or the χ^2-test was used to compare baseline variables between the NASH and HCV groups. The starting date for the analysis was the date of liver biopsy in all patients. Patients in both groups were followed up until they died and were censored at the time of their last clinic visit. The patient who underwent liver transplantation was followed up until the day of transplantation, which was handled in the same way as the day of death. The primary outcomes were the occurrence of complications of cirrhosis (gastrointestinal varices and variceal hemorrhage, ascites, and encephalopathy), the development of HCC, overall survival, and liver-related mortality. The time frame for a particular outcome was defined as the interval from liver biopsy until the occurrence of the relevant event. The time-to-failure analysis (Kaplan–Meier) was performed, and the log–rank test was used for comparisons between the NASH and HCV groups. To clarify the risk factors for the development of HCC and mortality among cirrhotic NASH patients, the Cox proportional hazards analysis was used. Age, BMI, diabetes, hyperlipidemia, hypertension, bilirubin, albumin, AST, ALT, γGTP, platelet count, prothrombin time, gastrointestinal varices, ascites, encephalopathy, HCC, and CTP class A were included in the model. All parameters that had a *P*-value of less than 0.1 according to univariate analysis were selected for the multivariate analysis with the Cox proportional hazards model. A *P*-value of 0.05 or less was considered statistically significant.

Results

Baseline data

Baseline demographic, clinical, and laboratory data from the patients with cirrhotic NASH or cirrhotic HCV are shown in Table 1. The mean age of the patients with cirrhosis due to NASH was 62.7 years, with a range of 16 to 89 years (two pediatric patients). The mean age of the patients with cirrhosis due to HCV was 61.3 years (range: 53 to 75 years).

The NASH patients had a higher prevalence of obesity and lifestyle-related diseases, and the between-group differences in the prevalence of obesity, diabetes mellitus, and hyperlipidemia were

Table 1 Baseline demographic, clinical features, and laboratory findings

	NASH $n = 68$ mean ± SD or %	HCV $n = 69$ *n*/mean% or ± SD	*P*-value
Follow-up period (months)	41.1 ± 39.3	74.5 ± 52.4	0.001
Age (years)	62.7 ± 13.2	61.3 ± 5.8	N.S.
Sex (female)	57%	57%	N.S.
BMI (kg/m^2)	27.8 ± 5.4	24.0 ± 3.0	> 0.001
Obesity (BMI ≥ 25 kg/m^2)	66%	32% (/65)	> 0.001
Severe obesity (BMI ≥ 30 kg/m^2)	24%	3% (/65)	0.002
Diabetes	68%	33% (/67)	> 0.001
Hyperlipidemia	34%	13%	0.004
Hypertension	47%	43%	N.S.
Alb (g/dL)	3.6 ± 0.7	3.6 ± 0.6	N.S.
T-bil (g/dL)	1.1 ± 0.9	0.9 ± 0.5	N.S.
AST (IU/L)	60.9 ± 42.2	92.2 ± 45.4	> 0.001
ALT (IU/L)	54.9 ± 41.3	81.9 ± 42.1	> 0.001
ALP (IU/L)	332.3 ± 202.2	287.6 ± 132.0	N.S.
γGTP (IU/L)	116.7 ± 90.1	58.0 ± 36.5	> 0.001
Plt (× 10^4/μL)	11.8 ± 4.3	9.5 ± 3.4	0.001
Prothrombin time (%)	77.6 ± 15.6	69.0 ± 18.1	0.006
HPT (%)	75.9 ± 21.1	68.6 ± 18.3	0.034
Child–Turcotte–Pugh score	6.1 ± 1.4	6.1 ± 1.4	N.S.

Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, γ-glutamyltranspeptidase; HCV, hepatitis C virus; HPT, hepaplastin test; NASH, non-alcoholic steatohepatitis; NS, not significant; Plt, platelet count; T-bil, total bilirubin.

Table 2 Complications of cirrhosis

	NASH ($n = 68$)			HCV ($n = 69$)		
	At baseline (*n*)	During the follow-up period (*n*)	At the end of the follow-up period (*n*)	At baseline (*n*)	During the follow-up period (*n*)	At the end of the follow-up period (*n*)
Ascites	9% (6)	10% (7)	19% (13)	6% (4)	36% (25)	42% (29)
GI varices[†]	44% (27)	15% (9)	58% (36)	42% (27)	26% (17)	68% (44)
Encephalopathy	3% (2)	10% (7)	13% (9)	1% (1)	12% (8)	13% (9)
HCC	21% (14)	10% (7)	31% (21)	13% (9)	35% (24)	48% (33)

[†]Sixty-two patients underwent endoscopy in the non-alcoholic steatohepatitis (NASH) group, and 65 patients in the hepatitis C virus (HCV) group. GI, gastrointestinal; HCC, hepatocellular carcinoma.

significant. Transaminases were significantly higher in the HCV group, while the γGTP level was significantly higher in the NASH group. The platelet count was significantly higher, and the prothrombin time was significantly shorter in the cirrhotic NASH group. However, 67% of NASH patients had a CTP score of less than 7 at the time of entry, as did 62% of HCV patients. Histologically, steatosis and ballooning degeneration were detected in all of the NASH patients, while necroinflammatory changes were variable.

Complications of cirrhosis

Ascites was present in six NASH patients and four HCV patients at the time of the initial evaluation. Of the remaining patients, seven with NASH and 25 with HCV developed ascites during follow up (Table 2). The 5-year occurrence rate of ascites was 19.1% in the NASH group versus 29.6% in the HCV group. The Kaplan–Meier analysis showed that HCV patients developed ascites more frequently after 100 months of follow up, but there was no significant difference between the two groups ($P = 0.175$).

At entry into the study, 44% of cirrhotic NASH patients (62 patients underwent endoscopy) and 42% of cirrhotic HCV patients (65 patients underwent endoscopy) had evidence of esophageal varices. During the follow-up period, nine additional patients with NASH and 17 with HCV were diagnosed as having esophageal varices by endoscopy. During follow up, 15 patients with NASH versus nine with HCV suffered from variceal hemorrhage, and one and five patients died of bleeding, respectively (Table 2). The 5-year occurrence rate of varices was 28.2% in the NASH group versus 34.5% in the HCV group, and the Kaplan–Meier analysis showed a similar incidence in both groups ($P = 0.789$).

At enrollment, two patients with NASH and one patient with HCV had a history of encephalopathy. Over time, seven other patients with NASH and eight patients with HCV developed encephalopathy (Table 2). The 5-year occurrence rate of ascites was 16.1% in the NASH group versus 9.1% in the HCV group.

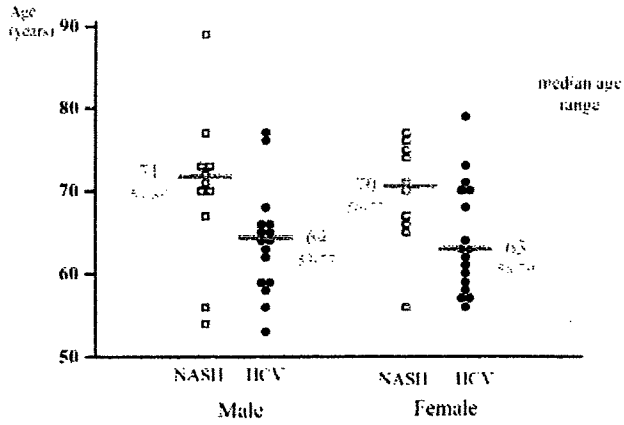


Figure 1 Age distribution at diagnosis of hepatocellular carcinoma. HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis.

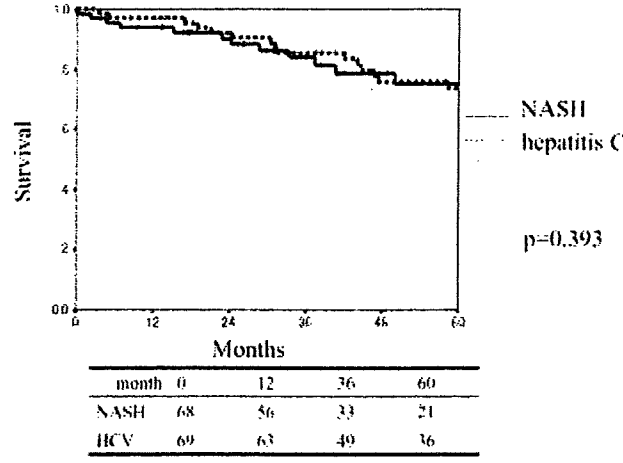


Figure 2 Survival curves. (a) Non-alcoholic steatohepatitis (NASH; $n = 68$); (b) hepatitis C virus ($n = 69$). $P = 0.393$. —, NASH; ...., hepatitis C.

Although NASH patients showed a higher incidence of encephalopathy, according to the Kaplan–Meier analysis there was no significant difference between the groups ($P = 0.253$).

Fourteen patients with NASH and nine patients with HCV had HCC at enrollment. During the follow-up period, seven patients with NASH developed HCC, as did 24 patients with HCV (Table 2). There were 11 males and 10 females with HCC in the cirrhotic NASH group and the median age was 71 for males and 70 for females. There were 16 males and 17 females with HCC in the cirrhotic HCV group and the median age was 64 for males and 63 for females. The median ages of the males and females in each group are shown in Figure 1. The median ages of the male and female NASH patients were significantly higher than those of the HCV patients. The 5-year occurrence rate of HCC was 11.3% in the NASH group versus 30.5% in the HCV group. The development of HCC showed a higher rate in the latter group, but the difference was not significant ($P = 0.185$).

A total of 19 patients with NASH (including one patient who underwent liver transplantation) and 28 patients with HCV died. Survival curves are shown in Figure 2. Survival was slightly better for cirrhotic NASH patients (the 5-year survival rate was 75.2% in the NASH group and 73.8% in the HCV group), but the difference was not significant. The causes of death are shown in Figure 3. HCC was the leading cause of death in both groups (nine deaths in the NASH group and 19 in the HCV group), followed by liver failure (six and seven deaths, respectively). Among the patients with liver failure, one NASH patient and five HCV patients died of variceal bleeding. There were more non-liver-related deaths in the NASH group, but the difference was not significant. The causes of non-liver related death in the NASH group were endometrial cancer in one patient, cholangitis in one patient, interstitial pneumonia in one patient, and cerebral infarction in one patient. Only two patients suffered from non-liver-related death in the HCV group (interstitial pneumonia and cerebral hemorrhage).

There were no differences in the baseline characteristics between the HCV patients with and without prior anti-HCV therapy, and the long-term outcome was similar for these two subgroups.

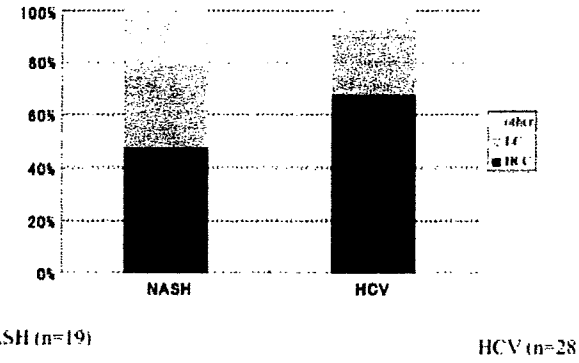


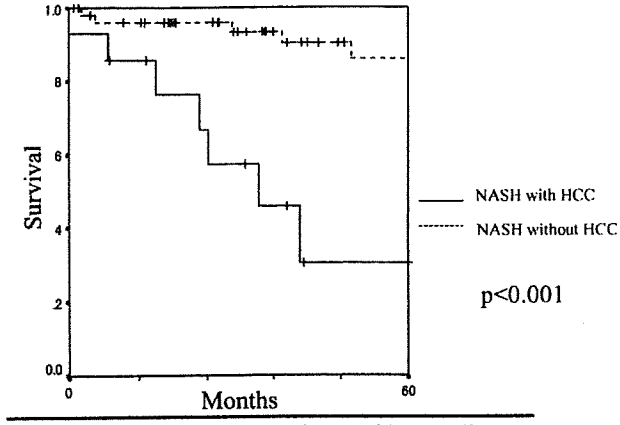
Figure 3 Causes of death (%). In cirrhotic non-alcoholic steatohepatitis patients, non-liver-related death accounted for 21% of all deaths, while in the case of cirrhotic hepatitis C patients, it only accounted for 7%. LC, liver cirrhosis.

Table 3 Cox proportional hazards analysis of factors related to mortality in non-alcoholic steatohepatitis patients with cirrhosis

	P-value	HR	95% CI
Hepatocellular carcinoma	0.001	7.957	2.447–25.877
Child–Turcotte–Pugh score grade A	0.001	0.170	0.058–0.502

CI, confidence interval.

The Cox proportional hazards analysis did not identify any risk factors for the development of HCC, although we also investigated the metabolic factors. The univariate analysis showed that HCC, ascites, albumin, bilirubin, and CTP class A were significant risk factors for mortality, but diabetes was not. According to the multivariate analysis, HCC and CTP class A were significant risk factors for mortality (Table 3). Figure 4 shows the survival of cirrhotic NASH patients with or without HCC, which was a significant risk factor that led to the death of these patients.



month	0	12	36	60
NASH without HCC	54	47	29	20
NASH with HCC	14	10	4	1

Figure 4 Survival curves of cirrhotic non-alcoholic steatohepatitis (NASH) patients with or without hepatocellular carcinoma (HCC). $P < 0.001$. —, NASH with HCC; ....., NASH without HCC.

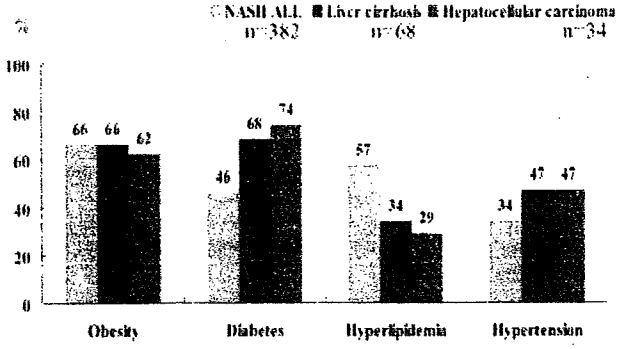


Figure 5 Prevalence of lifestyle-related diseases in all non-alcoholic steatohepatitis (NASH), cirrhotic NASH patients, and NASH patients with hepatocellular carcinoma. , NASH All; , Liver cirrhosis; , Hepatocellular carcinoma.

Figure 5 shows the prevalence of lifestyle-related diseases in all of the NASH patients, cirrhotic NASH patients, and NASH patients with HCC. The prevalence of diabetes was significantly higher among the NASH patients with HCC, but the prevalence of obesity and hyperlipidemia was lower in the NASH patients with cirrhosis or HCC.

Discussion

Ethnic differences with respect to the prevalence and features of NAFLD are well documented,[28] but there has been no information available on the prognosis of Japanese NASH patients with cirrhosis. The present report provides data from the first Japanese prospective study on the natural history of NASH patients with cirrhosis who underwent follow up using a predefined screening protocol for HCC at a single tertiary care hospital. All of the patients were Japanese, and follow up was started at the time of liver biopsy. Several therapeutic approaches have been tested for the treatment of NASH, but there is no pharmacological therapy that has conclusively proven to be effective.[29,30] In the present series, none of the patients underwent bariatric surgery, and liver transplantation was only performed in one patient. Furthermore, only two patients were lost to follow up. Therefore, our patients were suitable for assessing the natural history of Japanese NASH patients with cirrhosis, despite the existence of referral hospital bias. The main limitation of this study is that the control group was investigated retrospectively, but a predefined screening protocol for HCC was also employed in the HCV group. Furthermore, there were no differences in the baseline characteristics between patients from the HCV group with and without prior anti-HCV therapy. The long-term outcome was similar for these two subgroups, as was found in a recent large-scale nationwide study.[31]

When we compared the outcome of cirrhotic NASH with that of cirrhosis due to HCV, our study confirmed that the morbidity and mortality rates of the NASH group were better than those of the HCV group. However, cirrhosis due to NASH still followed a similar course to cirrhosis caused by LC-C; that is, the development of complications, occurrence of HCC, and then death.

Interestingly, baseline data showed significant differences between the NASH and HCV groups. As expected, there was a significantly higher prevalence of obesity, diabetes, and hyperlipidemia in the NASH group. In addition, the NASH patients with cirrhosis had significantly lower transaminase levels and higher γGTP levels than the HCV patients with cirrhosis. Although there was no significant difference of the CTP class between the two groups, the NASH patients had a significantly higher platelet count and prothrombin time than the HCV patients. It is unlikely that this was because of the earlier detection of cirrhotic NASH, because NASH is still not well known in Japan, while HCV tends to be diagnosed early because of screening for this disease by government heath services. The reasons for these differences in the laboratory data are unknown. It has been reported that a low normal ALT value does not guarantee the absence of underlying steatohepatitis with advanced fibrosis,[32] so the differences that we detected might be related to the characteristics of cirrhotic NASH.

Hui *et al.* reported on the long-term morbidity and mortality (median follow up: 60 months) of 23 patients with NASH-associated cirrhosis.[19] The outcomes were compared with those of 46 age- and sex-matched patients who had cirrhosis related to LC-C. Liver failure was the main cause of morbidity and mortality in patients with NASH-associated cirrhosis, and the prognosis was either similar or less severe than that of HCV-related cirrhosis. These findings were confirmed by Sanyal *et al.*,[20] who reported on the largest prospective study of patients with cirrhosis due to NASH or HCV. Their study compared 152 patients with cirrhosis due to NASH and 150 matched patients with cirrhosis caused by LC-C who were followed up for 10 years. They reported that the outcome for NASH patients with CTP class A cirrhosis was significantly better than that for patients with HCV-related cirrhosis, while there were no significant differences of mortality for patients with CTP class B or C cirrhosis. They also found that death was most commonly due to sepsis and multiple organ failure associated with ascites, while the risk of developing ascites was significantly lower in NASH patients than in patients with HCV. Our series was small, but we found that CTP class A was a significant negative

risk factor for mortality. The reasons for such a difference in the complication of cirrhosis need to be studied further.

Another important finding was that no patient died of heart disease among the cirrhotic NASH and HCV groups, although several reports have indicated that NAFLD or NASH is a risk factor for heart disease.[20,33] As seen in Figure 4, the lipid levels, platelet count, and prothrombin time were not high in NASH patients with advanced fibrosis, which might be part of the reason for the lack of cardiac-related death.[10] It is also possible that patients with symptomatic heart disease were not referred to our tertiary liver center.

Evidence of progression from NASH to HCC in prospective studies is minimal. However, with the exception of Hui *et al.*'s report, previous studies have shown that some patients develop HCC within several years after the diagnosis of cirrhotic NASH. Hui *et al.* did not find any NASH patients who developed HCC during follow up. The most important reason for this difference was probably the ages of the patients (a mean of 52.6 ± 13.6 years in Hui *et al.*'s study *vs* a median of 64 years for cirrhotic NASH and 70 years for NASH with HCC in our study). Therefore, Hui *et al.*'s patients were relatively young and unlikely to develop HCC. In NASH, as in other liver diseases, the severity of fibrosis and age are important risk factors for HCC.

The highest rate of HCC was reported by Ratziu *et al.*[34] They assessed survival, occurrence of HCC, and complications of hepatic insufficiency in patients with obesity-related cryptogenic cirrhosis, which was suggested to be burnt-out NASH, and compared the results with those for cirrhosis due to other causes. HCC was detected in eight of 27 (27%) cryptogenic cirrhosis patients versus 21% of the matched HCV-infected controls of similar age, suggesting a comparable carcinogenic potential. This is reasonable because burnt-out NASH is the most advanced stage of cirrhotic NASH.[13,35] In the present study, we found that HCC was a significant independent risk factor for mortality, as was the CTP class (Table 3).

In conclusion, the present study confirmed that, although the morbidity and mortality of NASH cirrhosis were lower than those of cirrhosis due to LC-C, both followed a similar course with respect to the onset of complications, development of HCC, and eventual death. The occurrence of HCC was the strongest predictor of mortality in both groups. These findings are likely to be helpful when devising therapeutic interventions for NASH and also for the daily management of these patients.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Steatosis and hepatic expression of genes regulating lipid metabolism in Japanese patients infected with hepatitis C virus

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Abstract
Purpose Steatosis is a histological finding associated with the progression of chronic hepatitis C. The aims of this study were to elucidate risk factors associated with steatosis and to evaluate the association between steatosis and hepatic expression of genes regulating lipid metabolism.
Methods We analyzed 297 Japanese patients infected with hepatitis C virus and a subgroup of 100 patients who lack metabolic factors for steatosis. We determined intrahepatic mRNA levels of 18 genes regulating lipid metabolism in these 100 patients using real-time reverse transcription-polymerase chain reaction. Levels of peroxisome proliferator-activated receptor α and sterol regulatory element-binding protein 1 proteins were assessed by immunohistochemistry.
Results Steatosis was present in 171 (57%) of 297 patients. The presence of steatosis was independently associated with a higher body mass index, higher levels of γ-glutamyl transpeptidase and triglyceride, and a higher fibrosis stage. Steatosis was present in 43 (43%) of 100 patients lacking metabolic factors. Levels of mRNA and protein of peroxisome proliferator-activated receptor α, which regulates β-oxidation of fatty acid, were lower in patients with steatosis than in patients without steatosis.
Conclusions These findings indicate that impaired degradation of lipid may contribute to the development of hepatitis C virus-related steatosis.

Keywords Steatosis · Hepatitis C virus · Fibrosis · Gene expression · Peroxisome proliferator-activated receptor α

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Introduction

The prevalence of hepatic steatosis ranges from 40 to 86% (mean ~55%) in patients infected with hepatitis C virus (HCV) [1]. This range is higher than in the general population of adults in the Western world (20–30%) [2]. Steatosis appears to be associated with a more rapid progression of liver fibrosis and a lower response to interferon-α-based therapy [3–5].

Patients with HCV infection may have metabolic cofactors, such as obesity, diabetes, and alcohol abuse that contribute to the development of fatty liver. It is likely that two types of steatosis, viral and metabolic, coexist in patients with chronic hepatitis C [1, 3]. Known risk factors associated with steatosis include HCV genotype 3, a higher body mass index (BMI), diabetes, hyperlipidemia, ongoing alcohol abuse, older age, the presence of fibrosis, and

hepatic inflammation [1, 5]. However, different populations may have different risk factors for steatosis, and the distribution of HCV genotype differs from region to region. For example, HCV genotype 3, which is thought to be directly responsible for steatosis [6–8], is far less frequent in Japan than in Europe [7] or the United States [9].

Although the mechanisms of HCV-related steatosis are not well known, several viral and host factors appear to be involved [3]. In vitro studies [10] and a transgenic mouse models [11] have shown that HCV core protein can induce steatosis. HCV core protein, in turn, inhibits the activity of microsomal triglyceride transfer protein, which is essential for the assembly and secretion of very low density lipoproteins [12]. The intrahepatic levels of microsomal triglyceride transfer protein mRNA show an inverse correlation with the degree of steatosis in patients with chronic hepatitis C [13]. HCV infection and HCV core protein upregulates the expression of sterol regulatory element-binding protein 1 (SREBP1), a key transcriptional factor that activates the expression of genes involved in lipid synthesis [14, 15]. In addition, HCV core protein binds to retinoid X receptor α, a transcriptional regulator that controls many cellular functions including lipid metabolism [16]. HCV core protein also down-regulates the expression of peroxisome proliferator-activated receptor α (PPARα) and carnitine palmitoyl transferase 1 (CPT1) [17, 18], and the mRNA levels of PPARα and CPT1 are found to be reduced in patients with chronic HCV infection [19].

In the present study, we investigated the risk factors associated with steatosis in Japanese patients with chronic HCV infection. To elucidate the molecular mechanisms underlying HCV-related (i.e., viral) steatosis, we also systematically measured the intrahepatic expression levels of genes that regulate lipid degradation, secretion, synthesis, and uptake in patients who lack metabolic factors for steatosis.

Methods

Patients

The study included a total of 297 Japanese patients with chronic HCV infection who underwent liver biopsy between April 2004 and June 2006 at the Hospital of Kyoto Prefectural University of Medicine, Kyoto, Japan. To eliminate selection biases, the patients were recruited consecutively. Inclusion criteria were as follows: patients older than 18 years, positive for anti-HCV (third-generation enzyme immunoassay; Chiron, Emeryville, CA), and positive for serum HCV-RNA (Amplicor HCV assay; Roche Diagnostic Systems, Tokyo, Japan). Exclusion criteria were as follows: positive for hepatitis B virus surface antigen (radioimmunoassay; Dainabot, Tokyo, Japan); other types of liver diseases, including primary biliary cirrhosis, autoimmune hepatitis, alcoholic liver disease, Wilson's disease, or hemochromatosis; coinfection with human immunodeficiency virus; treated with antiviral or immunosuppressive agents within 6 months of enrollment; treated with drugs known to produce hepatic steatosis, including corticosteroids, high dose estrogen, methotrexate, or amiodarone within 6 months of enrollment; a history of gastrointestinal bypass surgery.

BMI was calculated using the following formula: weight in kilograms/(height in meters)^2. Obesity was defined as a BMI $\geq$25, according to the criteria of the Japan Society for the Study of Obesity [20]. Diabetes was defined as a fasting glucose level $\geq$126 mg/dl or by the use of insulin or oral hypoglycemic agents to control blood glucose. The ongoing alcohol intake per week recorded and converted to average grams per day. Significant alcohol intake was defined as consumption of >20 g/day.

The Ethics Committee of the Kyoto Prefectural University of Medicine approved this study. Informed consent was obtained from each patient in accordance with the Helsinki declaration.

Laboratory tests

Venous blood samples were taken in the morning after a 12-h overnight fast. The laboratory evaluation included a blood cell count and the measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), total cholesterol, triglyceride, and fasting plasma glucose. These parameters were measured using the standard clinical chemistry techniques. The HCV genotype was determined according to the classification of Simmonds et al. [21]. The serum HCV-RNA level was quantified by Amplicor HCV monitor assay (version 2.0; Roche). These clinical and laboratory data were collected at the time of liver biopsy.

Histopathological examination

Liver biopsy specimens were obtained percutaneously from all patients for diagnostic purposes and divided into two parts. One part was fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin, Masson's trichrome, and silver impregnation. The sections were analyzed by an experienced hepatologist (T.O.) who was blinded to the laboratory parameters and clinical data. The degrees of inflammation and fibrosis were evaluated according to the criteria proposed by Desmet et al. [22]. Steatosis was graded based on percent of hepatocytes in the biopsy involved: none (0%), mild (<33%), moderate (33–66%), or severe (>66%) [23, 24]. The other part of the liver

biopsy was frozen immediately in liquid nitrogen and stored at –80°C for mRNA analysis.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

We quantified mRNA by real-time fluorescence detection. Total RNA was obtained using an RNeasy Kit (Qiagen, Tokyo, Japan). Residual genomic DNA was removed and single-stranded complementary DNA was generated using a Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Real-time quantitative RT-PCR experiments were performed with the LightCycler system using Faststart DNA Master Plus SYBR Green I (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol. The 18 genes chosen for the current study, their protein products, and the primer sequences for amplifying them are listed in Table 1. The primers were designed using Primer3 version 0.4 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) on the basis of sequence data obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). *ACTB* (β-actin gene) was used as an endogenous control.

Immunohistochemistry

Immunohistochemical staining for PPARα and SREBP1 was performed on formalin-fixed, paraffin-embedded sections from 100 liver biopsy specimens using rabbit polyclonal antibodies against human PPARα (clone H-98; Santa Cruz Biotechnology, Santa Cruz, CA) and SREBP1 (clone K-10; Santa Cruz Biotechnology), respectively. Deparaffinized sections were microwaved in a citrate buffer (pH 6.0) for 20 min. After blocking the endogenous peroxidase, the sections were incubated for 90 min at room temperature with 1:100 anti-PPARα or anti-SREBP1 antibodies. The sections were then incubated for 30 min at room temperature with peroxidase-labeled polymer-conjugated goat anti-rabbit immunoglobulin (Histofine Simple Stain Max-Po (Multi); Nichirei, Tokyo, Japan), followed by 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. The sections were then lightly counterstained with hematoxylin. Negative controls were evaluated by substituting the primary antibody with nonimmunized rabbit serum. Immunoreactivity was scored according to the intensity of staining as follows: 1+, weak or absent; 2+, moderate; 3+, strong.

Table 1 Genes and primer sequences used for reverse transcription-polymerase chain reaction assays

Function/gene symbol	Alternate symbol	Protein product	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Nuclear receptor				
PPARA	PPARα	Peroxisome proliferator-activative receptor α	ggaaagcccactctgcccct	agtcaccgaggaggggctcga
PPARG	PPARγ	Peroxisome proliferator-activative receptor γ	cattctggcccaccaactttgg	tggagatgcaggctccactttg
NR1H3	LXRα	Liver X receptor α	cgggcttccactacaatgtt	tcaggcggatctgttcttct
RXRA	RXRα	Retinoid X receptor α	tccttctcccaccgctccatc	cagctccgtcttgtccatctg
Fatty acid oxidation				
CPT1A	CPT1	Carnitine palmitoyltransferase 1	catcatcactggcgtgtacc	ttggcgtacatcgttgtcat
ACADS	SCAD	Short chain acyl-CoA dehydrogenase	ctcacgttggggaagaaaga	tgcgacagtcctcaaagatg
ACADM	MCAD	Medium chain acyl-CoA dehydrogenase	ttgagttcaccgaacagcag	aggggactggatattcacc
ACADL	LCAD	Long-chain acyl-CoA dehydrogenase	ttggcaaaacagttgctcac	ctcccacatgtatccccaac
ACADVL	VLCAD	Very long-chain acyl-CoA dehydrogenase	agccgtgaaggagaagatca	tgtgtttgaagccttgatgc
EHHADH	LBP	Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	cttcagccctggatgttgat	aaaagaagtgggtgccaatg
HADHA	LCHAD	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit	cacctctctgcctgttcctc	ggcaaagatgctgacacaga
ACOX1	AOX	Acly-CoA oxidase	tgatgcgaatgagtttctgc	agtgccacagctgagaggtt
CYP2E1	CYP2E	Cytochrome P450 CYP2E	cccaaaggatatcgacctca	agggtgtcctccacacactc
Intake of fatty acid				
SLC27A5	FATP5	Fatty acid transporor protein 5	acacactcggtgtccctttc	ctacagggcccactgtcatt
Transfer of trigyceride				
MTP	MTP	Microsomal triglyceride transfer protein	catctggcgaccctatcagt	ggccagctttcacaaaagag
Biosynthesis of fatty acid				
SREBF1	SREBP1	Sterol regulatory element-binding protein 1	tgcattttctgacacgcttc	ccaagctgtacaggctctcc
ACACA	ACC	Acetyl CoA carboxylase	gagaactgccctttctgcac	ccaagctccaggcttcatag
FASN	FAS	Fatty acid synthase	ttccgagattccatcctacg	tgtcatcaaaggtgctctcg

Table 2 Patient characteristics

Characteristic	
n	297
Age[a]	58 (20–78)
Male gender (%)	131 (44.9%)
BMI[a]	22.7 (15.6–35.1)
Obesity (%)	76 (25.6%)
Alcohol intake (%)	67 (22.6%)
Diabetes (%)	9 (3.0%)
HCV genotype (%)	
1	212 (71.4%)
2	76 (25.6%)
3	2 (0.7%)
Unknown	7 (2.3%)
HCV-RNA level (KIU/ml)[a]	1100 (5–9400)
Platelet count ($\times 10^4/\mu L$)[a]	17.6 (5.3–37.4)
AST (IU/L)[a]	47 (14–413)
ALT (IU/L)[a]	59 (9–537)
γ-GTP (IU/L)[a]	39 (10–490)
Fasting glucose (mg/dL)[a]	96 (68–223)
Total cholesterol (mg/dL)[a]	173 (19–318)
Triglyceride (mg/dL)[a]	91 (26–930)
Histological activity (%)	
0	3 (1.0%)
1	127 (42.8%)
2	120 (40.4%)
3	47 (15.8%)
Fibrosis (%)	
0	4 (1.3%)
1	100 (33.7%)
2	120 (40.4%)
3	62 (20.9%)
4	11 (3.7%)
Steatosis (%)	
None	126 (42.4%)
Mild (<33%)	163 (54.9%)
Moderate (33–66%)	7 (2.4%)
Severe (>66%)	1 (0.3%)

[a] Median (range)

Table 3 Univariate analysis of factors associated with steatosis

Factors	No steatosis ($n = 126$)	Steatosis ($n = 171$)	*P*
Age[a]	56 (20–78)	59 (27–75)	0.019
Male gender (%)	44 (34.9%)	87 (50.9%)	0.007
BMI[a]	21.8 (16.5–30.7)	23.9 (15.6–35.1)	<0.0001
Alcohol intake (%)	29 (23.0%)	38 (22.2%)	0.89
Diabetes (%)	4 (3.2%)	5 (2.9%)	1.00
HCV genotype (%)			
1	91 (72.2%)	121 (70.8%)	
2	31 (24.6%)	45 (26.3%)	
3	1 (0.8%)	1 (0.9%)	
Unknown	3 (2.4%)	4 (2.4%)	0.78
HCV-RNA level (KIU/ml)[a]	1257 (5–7030)	1063 (5–9400)	0.14
Platelet count ($\times 10^4/\mu L$)[a]	18.4 (5.9–32.7)	17.4 (5.3–37.4)	0.19
AST (IU/L)[a]	36 (15–413)	58 (14–339)	<0.0001
ALT (IU/L)[a]	40 (9–537)	73 (12–509)	<0.0001
γ-GTP (IU/L)[a]	25 (10–298)	56 (12–490)	<0.0001
Fasting glucose (mg/dL)[a]	95 (68–207)	97 (77–223)	0.002
Total cholesterol (mg/dL)[a]	179 (109–285)	171 (104–318)	0.13
Triglyceride (mg/dL)[a]	83 (26–214)	96 (32–930)	<0.0001
Histological activity (%)			
0	2 (1.6%)	1 (0.6%)	
1	72 (57.1%)	55 (32.2%)	
2	42 (33.3%)	78 (45.6%)	
3	10 (7.9%)	37 (21.6%)	<0.0001
Fibrosis (%)			
0	3 (2.4%)	1 (0.6%)	
1	62 (49.2%)	38 (22.2%)	
2	47 (37.3%)	73 (42.7%)	
3	11 (8.7%)	51 (29.8%)	
4	3 (2.4%)	8 (4.7%)	0.001

[a] Median (range)

Statistical analysis

Results are presented as numbers with percentages in parenthesis for qualitative data or as the medians and ranges for quantitative data. Univariate comparisons were made using a chi-square test for qualitative factors or a Mann–Whitney *U* test on ranks for quantitative factors with non-equal variance. Logistic regression analysis was used for multivariate analysis. *P* values below 0.05 by two-sided test were considered to be significant. Variables that achieved statistical significance on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors for steatosis. All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

Results

The characteristics of the 297 patients are summarized in Table 2. Steatosis was present in 171 (57.6%) patients. The grade of steatosis was mild in 163 (54.9%) patients, moderate in 7 (2.4%), and severe in 1 (0.3%).

Table 4 Multivariate analysis of factors independently associated with steatosis

Factors	Odds ratio	95% confidence interval	*P*
Age	1.02	1.00–1.05	0.05
Male gender	0.99	0.51–1.93	0.99
BMI	1.19	1.06–1.33	0.002
AST	1.00	0.98–1.02	0.54
ALT	0.99	0.98–1.00	0.37
γ-GTP	1.01	1.00–1.01	0.005
Fasting glucose	0.99	0.97–1.01	0.37
Triglyceride	1.01	1.00–1.01	0.007
Activity grade A2 or A3	1.81	0.94–3.51	0.07
Fibrosis stage F3 or F4	2.59	1.11–6.02	0.02

Data are from a total of 297 patients

Univariate correlations between variables and steatosis are shown in Table 3. Patients with steatosis, as compared to patients without steatosis, were older, more often male, had a higher BMI, higher AST, ALT, γ-GTP, fasting glucose, and triglyceride levels, a higher histological activity grade, and a higher fibrosis stage. Multivariate analysis revealed that the BMI, levels of γ-GTP and triglyceride, and fibrosis stage correlated independently with the presence of steatosis (Table 4).

To determine whether HCV has a direct effect on steatosis, we next analyzed a subgroup of patients lacking known metabolic causes of steatosis. Patients with obesity, diabetes, or ongoing alcohol intake were excluded. From the remaining 173 patients, we selected 100 patients whose liver RNA was available for gene expression analyses. There was no difference in clinicopathological characteristics between these 100 patients and the remaining 73 patients whose liver RNA was not available (data not shown). Steatosis was present in 43 (43%) of these 100 patients (Table 5). The presence of steatosis was associated with higher levels of AST, ALT, and γ-GTP, higher fasting glucose levels, and a higher fibrosis stage (Table 5).

To investigate the molecular mechanisms underlying HCV-related steatosis, we examined the expression of 18 genes regulating lipid metabolism in the liver (Table 1) using liver tissues derived from the 100 patients without obesity, diabetes, or ongoing alcohol intake. Real-time quantitative RT-PCR revealed that the expression of 10 genes (*PPARA*, *NR1H3*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, *ACOX1*, *CYP2E1*, *SLC27A5*, and *ACACA*) were significantly lower in patients with steatosis than in patients without steatosis (Fig. 1). There was no difference in the expression of the other 8 genes, including *SREBF1*, between the two groups.

To determine whether the protein levels corresponded with the mRNA levels, we performed immunohistochemistry

Table 5 Univariate analysis of factors associated with steatosis in patients without obesity, diabetes, or alcohol intake

Factors	No steatosis ($n = 57$)	Steatosis ($n = 43$)	*P*
Age[a]	56 (30–77)	60 (27–73)	0.12
Male gender (%)	15 (26.3%)	12 (27.9%)	0.86
BMI[a]	21.4 (17.0–24.8)	22.0 (17.8–24.9)	0.34
HCV genotype (%)			
1	39 (68.4%)	30 (69.8%)	
2	18 (31.6%)	13 (30.2%)	
3	0 (0%)	0 (0%)	
Unknown	0 (0%)	0 (0%)	0.89
HCV-RNA level (KIU/mL)[a]	1510 (5–7030)	1110 (5–5100)	0.60
Platelet count ($\times 10^4/\mu L$)[a]	19.8 (9.8–31.1)	17.3 (5.9–32.7)	0.06
AST (IU/L)[a]	31 (15–138)	61 (15–131)	<0.0001
ALT (IU/L)[a]	32 (12–175)	73 (14–290)	<0.0001
γ-GTP (IU/L)[a]	22 (10–137)	47 (12–151)	<0.0001
Fasting glucose (mg/dL)[a]	95 (75–112)	99 (79–121)	0.029
Total cholesterol (mg/dL)[a]	180 (120–281)	171 (119–300)	0.76
Triglyceride (mg/dL)[a]	86 (26–209)	88 (44–178)	0.23
Histological activity (%)			
0	1 (1.7%)	1 (2.3%)	
1	33 (58.0%)	14 (32.6%)	
2	19 (33.3%)	20 (46.5%)	
3	4 (7.0%)	8 (18.6%)	0.06
Fibrosis (%)			
0	1 (1.8%)	1 (2.3%)	
1	30 (52.6%)	10 (23.3%)	
2	20 (35.1%)	18 (41.9%)	
3	6 (10.5%)	13 (30.2%)	
4	0 (0%)	1 (2.3%)	0.018

[a] Median (range)

for PPARα (encoded by *PPARA*) and SREBP1 (*SREBF1*) proteins in liver biopsy tissues from the same 100 patients. We chose these two proteins because they are key regulators of lipid degradation and lipid synthesis, respectively. The results are summarized in Table 6, and representative images are shown in Fig. 2a. PPARα was expressed in hepatocytes. Its expression was mainly observed in the nuclei. SREBP1 was expressed in the cytoplasm of hepatocytes. Levels of PPARα and SREBP1 proteins tended to correlate with levels of *PPARA* and *SREBF1* mRNA, respectively (Fig. 2b). As shown in Table 6, the expression of the PPARα protein was significantly lower in patients with steatosis than in patients without steatosis

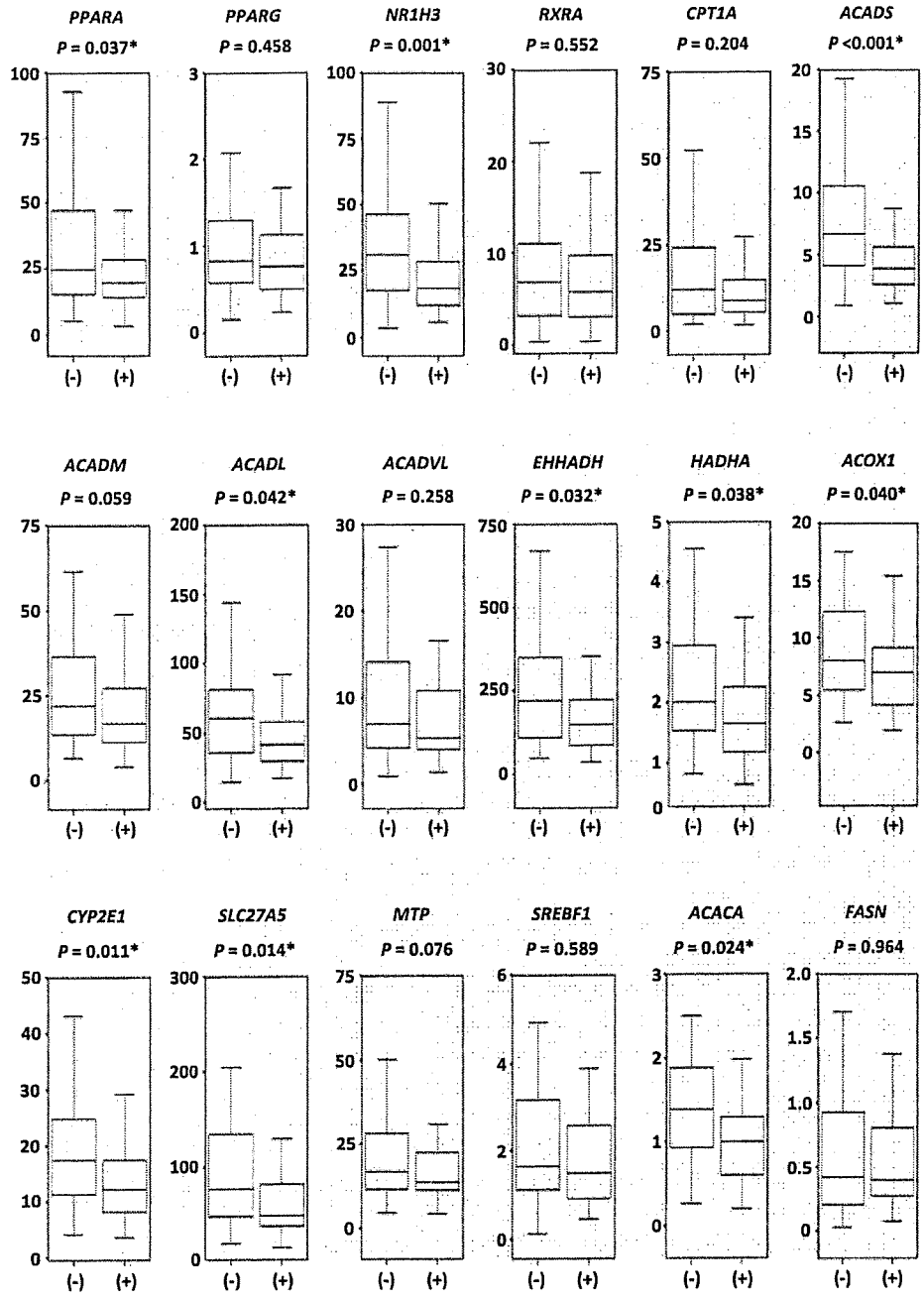

Fig. 1 Relative expression levels of 18 genes (see Table 1) in liver tissues from 57 patients without steatosis (−) and 43 patients with steatosis (+). Gene expression was evaluated by real-time quantitative RT-PCR. Results are presented relative to the expression of a reference gene (*ACTB*) to correct for variation in the amount of RNA in the RT-PCR. The box contains the values between the 25th and 75th percentiles, and the horizontal line is the median; the error bars stretch from the 10th to 90th percentiles. Differences between groups were analyzed using the Mann–Whitney *U* test. Asterisks indicate that the differences were statistically significant

($P = 0.017$). On the other hand, the presence of the SREPB1 protein was not associated with the steatosis. These findings agree with those from our analyses of *PPARA* and *SREBF1* mRNA levels. We also examined the relationship between the levels of PPARα and SREBP1 proteins and the degree of fibrosis (Table 6). The level of the PPARα protein was not associated with the degree of fibrosis. The expression of the SREBP1 protein tended to be higher in patients who had a higher fibrosis stage, although the association was not statistically significant.

Discussion

Our results demonstrated a high prevalence (57.6%) of steatosis among patients with chronic HCV infection in Japan, which confirms previous reports in Europe and the United States [1, 25–28]. The prevalence of steatosis was high (43.0%) even when known factors of steatosis, such as obesity, diabetes, or ongoing alcohol intake, were excluded. Consistent with previous reports [1, 29], the grade of steatosis was mild in most cases.

Table 6 Relationship between the presence of steatosis or the degree of fibrosis and levels of PPARα and SREBP1 proteins in liver tissues from patients without obesity, diabetes, or alcohol intake

	Steatosis			Fibrosis		
	Absent ($n = 57$)	Present ($n = 43$)	P	F1/F2 ($n = 80$)	F3/F4 ($n = 20$)	P
PPARα protein expression						
1+; mild or absent	9	17		20	6	
2+; moderate	38	23		49	12	
3+; strong	10	3	0.017	11	2	0.85
SREBP1 protein expression						
1+; mild or absent	16	6		17	5	
2+; moderate	31	29		52	8	
3+; strong	10	8	0.23	11	7	0.055

Multivariate analysis on the 297 patients with steatosis, including those with metabolic cofactors, revealed that a higher BMI, higher levels of γ-GTP and triglyceride, and a higher fibrosis stage correlate independently with steatosis. Previous studies have also observed an association between these clinicopathological factors and steatosis [1]. A recent meta-analysis of patients with chronic HCV infection in Europe, Australia, and the United States showed that steatosis is associated independently with HCV genotype 3, the presence of fibrosis, diabetes, hepatic inflammation, ongoing alcohol intake, a higher BMI, and an older age [5]. Although several studies have shown a significant and independent association between HCV genotype 3 and the presence of steatosis [1], we did not observe this association. This is due to the much lower prevalence of genotype 3 in Japan (<1%) than in Europe (24%) [7] and the United States (14%) [9]. There is some controversy with regard to the influence of steatosis on the progression of fibrosis [1, 3]. Some investigators suggest that steatosis accelerates fibrosis only in genotype 3-infected patients [7, 29, 30], whereas others suggest that there is an association in patients infected with genotype 1 [5, 31]. An analysis using paired liver biopsies revealed that steatosis was the only independent factor predictive of progression of fibrosis [32]. In agreement with a previous study [33], we also found that patients with steatosis had a higher γ-GTP. An increase in serum γ-GTP is associated with hepatic steatosis, central obesity and insulin resistance, and is a marker of metabolic and cardiovascular risk [34–36]. Elevated values of γ-GTP are caused by damage to cellular membranes, cellular regeneration or by enhanced synthesis as a result of induction of the biotransformation enzyme system. However, the mechanisms that explain the contribution of γ-GTP to steatosis have not been fully elucidated.

We analyzed the intrahepatic expression of genes that regulate (i) lipid degradation, (ii) lipid secretion, (iii) lipid synthesis, and (iv) lipid uptake. We then investigated the relationship between these levels and the presence of steatosis. Our experiments included more candidate genes than previous studies [13, 18, 19, 37]. The expression of *PPARA*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, *ACOX1*, and *CYP2E1* were lower in patients with steatosis. Immunohistochemistry confirmed that the expression of the PPARα protein was significantly lower in patients with steatosis than in patients without steatosis. PPARα, one of the proteins involved in lipid degradation, is a nuclear receptor that controls fatty acid metabolism by regulating the expression of genes encoding enzymes involved in mitochondrial and peroxisomal β-oxidation of fatty acids [38]. Short chain acyl-CoA dehydrogenase (encoded by *ACADS*), long-chain acyl-CoA dehydrogenase (*ACADL*), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (*EHHADH*), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit (*HADHA*), and acyl-CoA oxidase (*ACOX1*) are involved in fatty acid β-oxidation. *CYP2E1* encodes a member of the cytochrome P450 superfamily of enzymes that is involved in microsomal ω-oxidation. Acyl-CoA oxidase is the rate-limiting enzymes of peroxisomal β-oxidation. Also, *EHHADH*, *HADHA* and *ACOX1* are known to be a direct transcriptional target of PPARα [38]. The reduced expression of *PPARA*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, and *ACOX1* may lead to steatosis through down-regulation of fatty acid β-oxidation. However, not all of the genes regulating β-oxidation were down-regulated in patients with steatosis. For example, carnitine palmitoyl transferase 1 (encoded by *CPT1A*) is the rate-limiting enzymes of mitochondrial β-oxidation, and although *CPT1A* is a transcriptional target of PPARα [38], their expression was not significantly reduced in patients with steatosis.

In agreement with a previous study [13], we also found that the expression of *MTP*, a gene involved in lipid secretion, tended to be lower in patients with steatosis, although the association was not statistically significant. *MTP* is a transcriptional target of PPARα [38]. Because

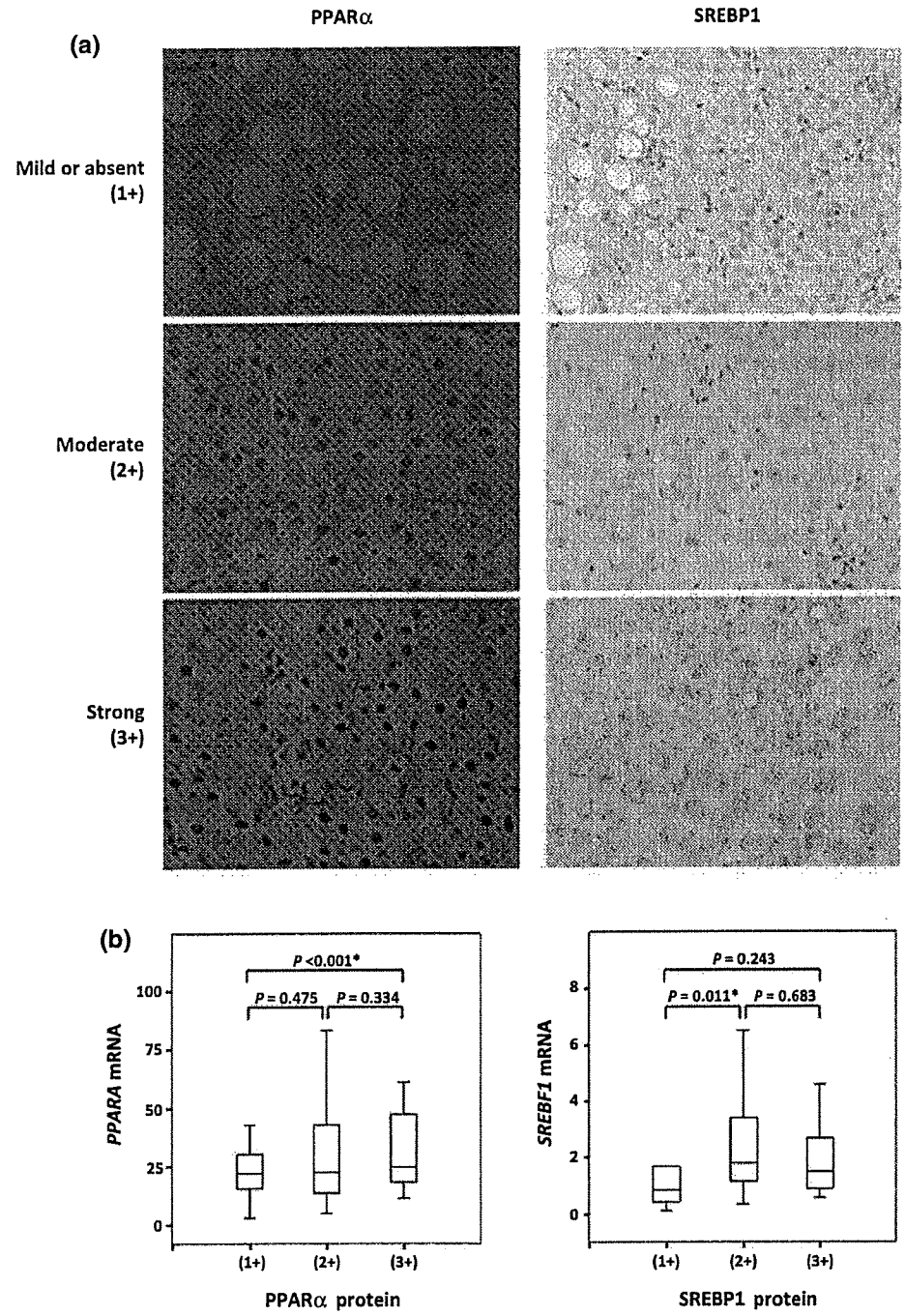

Fig. 2 Immunohistochemistry for PPARα and SREBP1 proteins. **a** Representative images from immunostaining for PPARα and SREBP1 proteins in liver tissues from patients with chronic hepatitis C. Shown are weak or absent staining (1+), moderate staining (2+), and strong staining (3+). Original magnification, ×400. **b** Relationship between relative levels of *PPARA* and *SREBF1* mRNA and proteins. *PPARA* and *SREBF1* mRNA levels were determined as described in Fig. 1. Levels of PPARα and SREBP1 proteins were evaluated as described in **a**. Differences between groups were analyzed using the Mann–Whitney *U* test. Asterisks indicate that the differences were statistically significant

microsomal triglyceride transfer protein plays a pivotal role in assembly and secretion of very low density lipoproteins, its reduced expression is expected to result in the increased accumulation of triglycerides (i.e., steatosis).

The nuclear receptor liver X receptor α (encoded by *NR1H3*) is known to promote hepatic lipogenesis by activating SREBP1. SREBP1 increases the transcription of genes involved in hepatic fatty acid synthesis, such as *FASN* (encoding fatty acid synthase) and *ACACA* (acetyl CoA carboxylase), and induces steatosis through increased accumulation of triglyceride. Unexpectedly, the levels of both *SREBF1* mRNA and protein and of *FASN* mRNA were not up-regulated in patients with steatosis. In addition, the expression of *NR1H3* and *ACACA* were lower in patients with steatosis. These findings contradict the idea that the increased expression of genes involved in synthesis of fatty acids leads to steatosis. One possible explanation is that the decreased expression of *NR1H3* and

ACACA compensates for the increased accumulation of triglycerides.

Of the genes involved in lipid uptake, fatty acid transporter protein 5, a liver-specific member of the fatty acid transporter protein family, mediates the uptake of long-chain fatty acids. Unexpectedly, the expression of *SLC27A5* (encoding fatty acid transporter protein 5) was not up-regulated but rather down-regulated in patients with steatosis. Again, this expression could be a compensatory response to increased accumulation of triglyceride.

Further studies are needed to determine the importance of the products of these genes because the limited size of biopsy samples prevented measurement of the enzyme activities. Changes in enzymatic activities of their products are more important for the development of steatosis than changes in their transcriptional levels. Moreover, in vitro studies and mouse models have shown that HCV proteins cause mitochondrial injury, leading to oxidative stress [39–43]. Oxidative stress may inhibit enzymes involved in lipid metabolism, and reactive oxygen species may cause peroxidation of membrane lipids and structural proteins, such as those involved in trafficking and secretion of lipids. Oxidative stress perturbs lipid metabolism, thus contributing to steatosis. It is possible that, instead of a direct effect of HCV proteins on the transcription of genes regulating lipid metabolism, nonspecific inhibition of lipid metabolism through oxidative stress leads to HCV-related steatosis.

In conclusion, a higher BMI, higher levels of γ-GTP and triglyceride, and a higher fibrosis stage correlate independently with steatosis in HCV-infected Japanese patients. Thus, the down-regulation of genes involved in fatty acid oxidation may contribute to the development of steatosis in these patients.

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Epigenetic silencing of *RELN* in gastric cancer

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Abstract. RELN (Reelin) is an extracellular glycoprotein that plays a critical role in neuronal migration. Here we show that the *RELN* gene is frequently silenced in gastric cancers (GCs) by aberrant promoter hypermethylation. Although RELN was strongly expressed in non-tumor gastric epithelia, its expression was weak, or absent, in GC cell lines and primary GC tumors. Absence of RELN expression significantly correlated with a more advanced stage of GC. Methylation of the *RELN* promoter was frequently found in GC cell lines and in primary GC tumors. These findings suggest that disruption of the RELN pathway may be involved in gastric carcinogenesis.

Introduction

Gastric cancer (GC) is the second most common cause of cancer-associated death worldwide (1). The molecular basis of GC involves several genetic changes including oncogenic activation of *β-catenin* and *KRAS*, amplification of *ERBB2* and *MET*, inactivation of tumor suppressor genes, such as *p53, APC, CDH1 (E-cadherin)* and *CDKN2A (p16)*, and microsatellite instability.

Epigenetic alterations, as well as genetic alterations, are involved in the development and progression of cancer. DNA methylation of CpG islands in the 5' region of tumor suppressor genes is known to inhibit transcriptional initiation and thereby silence these genes (2). Several tumor suppressor genes, including *CDKN2A, CDH1, hMLH1* and *RUNX3*, have been reported to be inactivated by promoter methylation in GC (3).

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Key words: RELN, Reelin, gastric cancer, methylation, epigenetic silencing

RELN (Reelin) is an extracellular 420-kDa glycoprotein that plays a critical role in the regulation of neuronal migration during brain development (4,5). The *Reln* gene was isolated from *reeler* mice that have an autosomal recessive mutation in the *Reln* gene, which results in widespread disruption of laminated regions of the brain (4). Secreted RELN binds to two cell surface receptors termed the very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor (ApoER2), which transmit the extracellular RELN signal to intracellular signaling processes through Disabled-1 (DAB1), an intra-cellular adaptor protein that activates the tyrosine kinase (6,7). These signaling components are essential for RELN signaling since knockout mice lacking *Vldlr, ApoER2* or *Dab1* mimic the phenotype of mice lacking *Reln* (7,8).

The recent observation that the *VLDLR* gene is frequently silenced by promoter hypermethylation in GC suggested that disruption of the RELN pathway may be involved in gastric carcinogenesis (9). The *RELN* gene harbors a long CpG-rich promoter region (10) and expression of *RELN* is regulated by the methylation status of the promoter (11). Thus, the *RELN* promoter is hypermethylated in schizophrenia (12,13) and *RELN* is silenced in pancreatic adenocarcinomas by aberrant promoter hypermethylation (14). These studies prompted us to investigate the methylation and expression status of *RELN* in GC.

Materials and methods

Cell lines and primary tumors. Nine human GC cell lines were used in this study: MKN1, MKN28, MKN45, MKN74 (15,16), TMK1 (17), NUGC3 (18), SNU16 (19), KATO-III (20) and AZ-521 (21). All the cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin/100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. For immunohistochemistry, primary GC samples were obtained from 25 patients who underwent surgery at the Hospital of Kyoto Prefectural University of Medicine (Kyoto, Japan). Surgical specimens were fixed in formalin and embedded in paraffin using standard procedures. For methylation analysis, paired GC tissues and non-tumor gastric epithelial tissues were obtained during upper gastrointestinal endoscopic inspection from an additional 15 patients who underwent biopsy for

Table I. Primer sequences and PCR conditions.

Method	Gene	Forward primer	Reverse primer	Annealing temp. (°C)
RT-PCR	*RELN*	5'-ACCAGTGGGCAGTCGATGACATCAT-3'	5'-CTTCATTAGCCAACATCAACCACAC-3'	55
	GAPDH	5'-CGGAGTCAACGGATTTGGTCGTAT-3'	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'	67
COBRA and bisulfite-sequencing		5'-GGTTTTAAGAAGGTGTGGAG-3'	5'-TCCCCATCCCCTTCCAAC-3'	63

diagnostic purposes at the Hospital of Kyoto Prefectural University of Medicine. Normal gastric epithelial tissues were obtained from three *Helicobacter pylori*-negative healthy volunteers who underwent endoscopy. All biopsy specimens were immediately frozen in liquid nitrogen and stored at -80°C until required. *H. pylori* infection status was examined with a rapid urease test (PyloriTek Test kit; Serim Research Corp., Elkhart, IN, USA), with hematoxylin-eosin staining or with a serum IgG antibody test (SBS, Kanagawa, Japan). A patient was defined as *H. pylori*-positive if one or more of these tests gave a positive result. Atrophic gastritis was diagnosed by endoscopy (22). None of the patients had undergone radiation therapy, chemotherapy or immunotherapy prior to the operation. Genomic DNA and total RNA were isolated from the GC cell lines and primary GC tumors using the DNeasy Tissue kit (Qiagen, Minneapolis, MN), the RNeasy Mini kit (Qiagen) or the AllPrep DNA/RNA Mini kit (Qiagen). Prior to the study, informed consent was obtained and the study was approved by the ethics committee.

Reverse transcription-polymerase chain reaction (RT-PCR). Single-stranded cDNAs were generated from total cellular RNA using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. Conventional PCR of *RELN* was performed using the Ex Taq DNA polymerase (Takara, Otsu, Japan). The PCR products were separated on 3% agarose gels and stained with ethidium bromide. Quantitative real-time PCR experiments were performed with the LightCycler system using FastStart DNA Master Plus SYBR Green I (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol. The primer sequences and PCR conditions are shown in Table I. *GAPDH* was used as an internal control. Human stomach total RNA (Clontech Laboratories, Mountain View, CA) was used as a control for RT-PCR.

Methylation analysis. The methylation status of the 5' CpG island of *RELN* was examined by methylation-specific PCR (MSP) as described previously (14). Methylation of *RELN* was further analyzed by bisulfite PCR followed by restriction enzyme digestion [combined bisulfite and restriction analysis (COBRA)] (23) and bisulfite sequencing analysis. For COBRA, genomic DNA (2 μg) was treated with sodium bisulfite using an EZ DNA Methylation kit (Zymo Research, Orange, CA) and subjected to PCR using primers (Table I) designed to amplify a region from -178 to +311 bp relative to the transcription start site of *RELN*. The PCR products were digested with *Afl*III, which recognizes sequences unique to the methylated alleles but cannot recognize unmethylated alleles, and the digested products were electrophoresed on 3% agarose gels and stained with ethidium bromide. The gel images were saved as TIFF files. Methylation levels were calculated as the ratio of the gray scale value of the methylated band to that of the combined methylated and unmethylated bands. The gray scale value was obtained by scanning the gel with Adobe Photoshop CS3 Extended software (Adobe Systems Incorporated, San Jose, CA, USA). For bisulfite-sequencing, the PCR products were cloned using the TOPO XL PCR Cloning kit (Invitrogen, Carlsbad, CA) and then sequenced. DNA derived from normal peripheral blood lymphocytes and CpGenome universal methylated DNA (Chemicon, Billerica, MA) served as controls for unmethylated and methylated DNA, respectively.

Drug treatment. Cells were treated with 1 or 5 μM of 5-aza-2'-deoxycytidine (5-aza-dCyd; Sigma-Aldrich, St. Louis, MO) for 4 days or with 50 ng/ml of trichostatin A (TSA; Wako, Osaka, Japan) for 1 day. For assay of drug synergy, the cells were cultured in the presence of 1 or 5 μM of 5-aza-dCyd for 4 days, and were then treated for an additional 24 h with 50 ng/ml of TSA.

Immunohistochemistry. Immunohistochemical staining of the RELN protein was performed on formalin-fixed, paraffin-embedded sections from 25 primary GCs, consisting of paired tumor and surrounding non-tumor tissues, using a mouse monoclonal antibody against RELN (clone E-5; Santa Cruz Biotechnology, Santa Cruz, CA). Deparaffinized sections were microwaved in 10 mM citrate buffer (pH 6.0) for 20 min. After blocking of endogenous peroxidase with 3% hydrogen peroxide, the sections were incubated overnight at 4°C with the anti-RELN antibody (1:50). The sections were then incubated for 20 min at room temperature with peroxidase-labeled polymer-conjugated goat anti-mouse immunoglobulin [Histofine Simple Stain Max-Po (Multi); Nichirei, Tokyo, Japan], followed by 3,3'-diaminobenzidine tetrahydrochloride as the chromogen. The sections were then lightly counterstained with hematoxylin. Negative controls were evaluated in the absence of the primary antibody. Immunoreactivity was scored according to the intensity of staining as follows: 0, absent; 1+, weak; 2+, strong. GCs were classified into intestinal or diffuse type according to

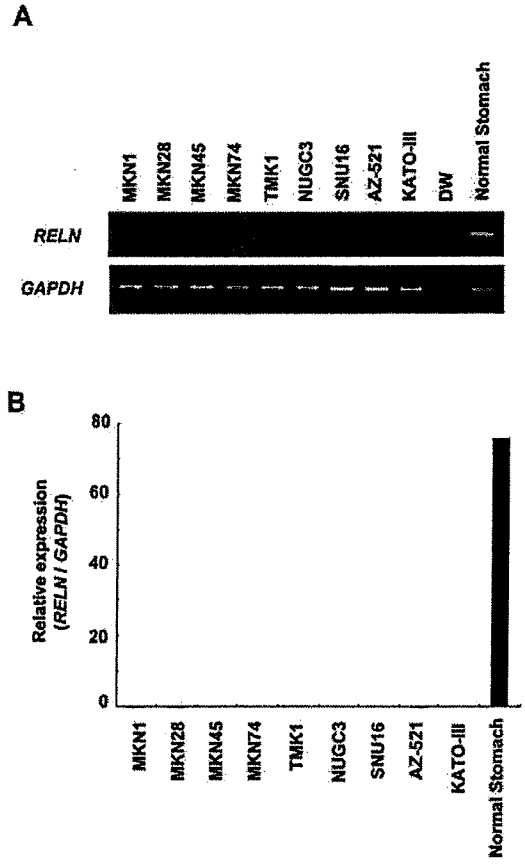

Figure 1. Expression of *RELN* mRNA in nine GC cell lines and in normal stomach. *RELN* mRNA expression was examined in the indicated GC cell lines and in normal stomach by conventional RT-PCR (A) and by quantitative real-time RT-PCR (B). *GAPDH* was used as an internal control. DW in (A) is a deionized water control.

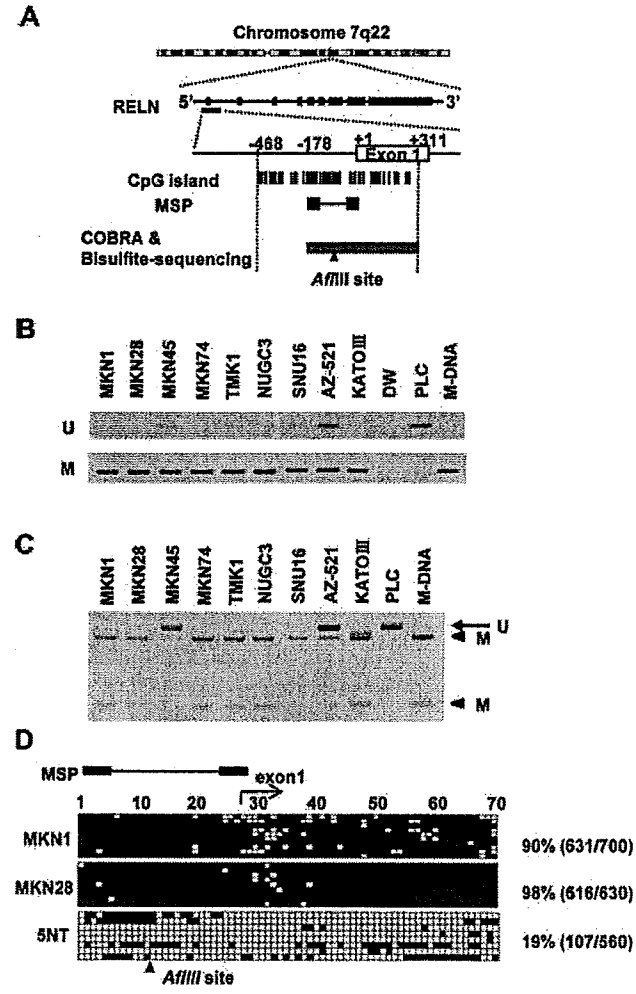

Figure 2. Analysis of *RELN* methylation. (A) Schematic map of the CpG island extending into exon 1 of *RELN*. Exon 1 is indicated by an open box, and the transcription start site is marked at +1. CpG sites are indicated by vertical ticks. The regions selected for MSP and for COBRA and bisulfite-sequencing are indicated. The restriction site for *Afl*III is indicated by the black arrowhead. (B) MSP analysis of *RELN* in the nine indicated GC cell lines and in normal peripheral lymphocytes (PLC). Parallel amplification reactions were performed using primers specific for unmethylated (U) or methylated (M) DNA. M-DNA indicates CpGenome universal methylated DNA. PLC and M-DNA were used as controls for unmethylated and methylated DNA, respectively. DW is a deionized water control. (C) COBRA of *RELN* in the nine GC cell lines. The arrow and arrowheads indicate undigested products (U, unmethylated DNA) and digested fragments (M, methylated DNA), respectively. (D) Bisulfite-sequencing of two GC cell lines (MKN1 and MKN28) and a non-tumor gastric epithelial tissue (5NT). All 70 CpG sites were sequenced. Each square indicates CpG dinucleotides: open squares, unmethylated; solid squares, methylated. Percentages indicate the fraction of methylated CpG dinucleotides. The regions selected for MSP and the restriction site for *Afl*III are indicated.

Lauren's histological classification (24). Tumor stages were classified according to the TNM (tumor-node-metastasis) classification of the Japanese Classification of Gastric Cancer (25).

Statistical analyses. The χ^2 test, Fisher's exact probability test, and the Wilcoxon signed-rank test were performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL). P-values of <0.05 were considered significant.

Results

Loss of expression of RELN mRNA in GC cell lines. To determine the potential role of *RELN* in GC, we first analyzed the expression of *RELN* mRNA in nine human GC cell lines by conventional RT-PCR (Fig. 1A) and by quantitative real-time RT-PCR (Fig. 1B). *RELN* expression was not be detected in any of the nine cell lines, whereas its expression was detected in normal stomach.

Methylation of the RELN promoter in GC cell lines. Aberrant methylation of DNA in 5' regulatory regions harboring CpG-rich regions (CpG islands) is strongly associated with transcriptional silencing (2). We therefore examined whether the lack of expression of the *RELN* gene in the nine GC cell lines might be due to aberrant methylation of the *RELN* promoter. For analysis of the methylation status of the *RELN* promoter, we identified a CpG island within a 799-bp sequence, that stretches from -468 to +311 bp relative to the transcription start site and extends into exon 1 of *RELN*, by means of the genome database of the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/cpgplot/) (Fig. 2A). This region corresponds to a part of the promoter of *RELN* (11).