TABLE II. Characteristics of Study Patients According to the Genetic Polymorphisms Near the *IL28B* Gene

	Patients with TT genotype of rs8099917 (n = 207)	Patients with TG/GG genotype of rs8099917 (n = 65)	*P*-value
Age (years)	56.5 ± 10.4	54.4 ± 12.4	0.4112
Sex (female/male)	107 (51.7)/100 (48.3)	32 (49.2)/33 (50.8)	0.8384
Body weight (kg)	57.8 ± 10.9	57.8 ± 9.4	0.8361
Alanine aminotransferase (IU/L)	65.1 ± 53.3	62.8 ± 65.6	0.2548
Aspartate aminotransferase (IU/L)	53.6 ± 34.8	54.7 ± 62.0	0.3339
Gamma-glutamyl transpeptidase (IU)	44.2 ± 37.1	62.3 ± 59.0	0.0003
Alkaline phosphatase (IU/L)	263.1 ± 90.3	282.8 ± 129.9	0.3875
Albumin (g/dl)	4.04 ± 0.36	4.05 ± 0.43	0.8020
Total bilirubin (mg/dl)	0.79 ± 0.30	0.76 ± 0.32	0.3010
White blood cell count (/μl)	4826 ± 1333	5100 ± 1320	0.1608
Hemoglobin (g/dl)	13.9 ± 1.3	14.1 ± 1.4	0.3339
Platelet count ($\times 10^3/\mu l$)	161 ± 49	169 ± 57	0.3871
Liver histology-activity (A0/A1/A2/A3)*	2 (1.1)/98 (52.4)/ 74 (39.6)/13 (6.9)	1 (1.7)/38 (64.4)/ 18 (30.5)/2 (3.4)	0.3241
Liver histology-fibrosis (F0/F1/F2/F3)*	21 (11.2)/83 (44.4)/ 57 (30.5)/26 (13.9)	6 (10.2)/31 (52.5)/ 13 (22.0)/9 (15.3)	0.6401
Pretreatment HCV RNA concentration ($\log_{10}$ IU/ml)	6.37 ± 0.85	6.29 ± 0.55	0.0582
Reduction in the peginterferon dose	61 (29.5)	20 (30.8)	0.9644
Reduction in the ribavirin dose	101 (48.8)	29 (44.6)	0.5565
Final outcomes (sustained virologic response /relapse/ no response)	106 (51.2)/ 69 (33.3)/32 (15.5)	12 (18.4)/15 (23.1)/ 38 (58.5)	<0.0001

HCV, hepatitis C virus.
Percentages are shown in parentheses.
*Liver biopsy was not performed in 26 patients.

$\log_{10}$ to 5.71 $\log_{10}$ (mean, 3.12 $\log_{10}$). The reduction in serum HCV RNA levels was ≥3 $\log_{10}$ in 98 patients (47.3%), <3 $\log_{10}$ and ≥2 $\log_{10}$ in 52 patients (25.1%), <2 $\log_{10}$ and ≥1 $\log_{10}$ in 23 patients (11.1%), and <1 $\log_{10}$ in 15 patients (7.3%). Figure 1A shows the rate of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy in patients with the TT genotype. The rates were higher significantly in patients who achieved a rapid virologic response or had a ≥3 $\log_{10}$ decrease in

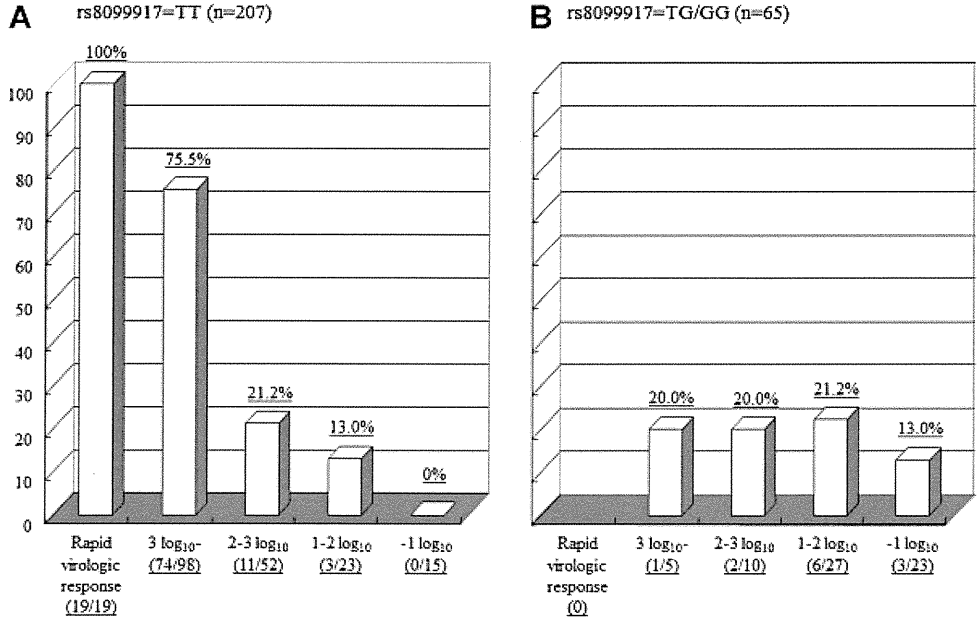

Fig. 1. The rate of sustained virologic responses (%) based on the reduction in serum HCV RNA levels at 4 weeks after starting therapy. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

serum HCV RNA levels at 4 weeks compared to those with a <3 $\log_{10}$ decrease in serum HCV RNA levels ($P < 0.0001$). When a 3 $\log_{10}$ decrease in serum HCV RNA levels was defined as the cut-off point, 56.5% of patients were considered to have a ≥3 $\log_{10}$ decrease in serum HCV RNA levels. The sensitivity, specificity, positive predictive value, and negative predictive value for a sustained virologic response were 86.8, 75.2, 78.6, and 84.4%, respectively.

Among the 65 patients who had the TG/GG genotype, no patient achieved a rapid virologic response at 4 weeks after initiating therapy. The decrease in serum HCV RNA levels at 4 weeks after starting therapy ranged from 0.11 $\log_{10}$ to 4.75 $\log_{10}$ (mean, 1.66 $\log_{10}$). The reduction in serum HCV RNA levels at 4 weeks after starting the therapy were smaller in patients with the TG/GG genotype than those with the TT genotype (1.66 ± 1.02 $\log_{10}$ in patients with the TG/GG genotype vs. 3.12 ± 1.37 $\log_{10}$ in patients with TT genotype excluding RVR, $P < 0.0001$). The reduction in serum HCV RNA levels was ≥3 $\log_{10}$ in five patients (7.7%), <3 $\log_{10}$ and ≥2 $\log_{10}$ in 10 patients (15.4%), <2 $\log_{10}$ and ≥1 $\log_{10}$ in 27 patients (41.5%), and <1 $\log_{10}$ in 23 patients (35.4%). Figure 1B shows the rates of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy in patients with the TG/GG genotype. There were no differences in the rate of a sustained virologic response based on the reduction in HCV RNA levels at 4 weeks after starting therapy; the rate of a sustained virologic response remained at 20% approximately regardless of the reduction in HCV RNA levels in 42 patients with a ≥1 $\log_{10}$ reduction in serum HCV RNA levels.

Association Between an Early Virologic Response at 12 Weeks and Treatment Outcome Based on Genetic Polymorphisms Near the *IL28B* Gene

Figure 2 shows the rate of patients with the TT genotype or TG/GG genotype for rs8099917 who achieved a complete early virologic response, a partial early virologic response, and those who did not achieve early virologic response at 12 weeks after starting therapy based on the reduction in serum HCV RNA level at 4 weeks after initiating therapy. Nearly 75% of patients with the TT genotype whose HCV RNA levels were reduced by ≥3 $\log_{10}$ at 4 weeks after starting the therapy achieved a complete early virologic response. In contrast, 80% of patients with the TG/GG genotype whose HCV RNA levels were reduced by ≥3 $\log_{10}$ at 4 weeks after starting the therapy showed a partial early virologic response. The majority of patients with the TT or TG/GG genotypes achieved a partial early virologic response when their reduction in HCV RNA levels was <3 $\log_{10}$ and ≥2 $\log_{10}$ or <2 $\log_{10}$ and ≥1 $\log_{10}$.

Figure 3 shows the rates of a sustained virologic response according to the type of early virologic response in patients with the TT genotype (Fig. 3A) and TG/GG genotype (Fig. 3B). Among patients with the TT genotype, the rate of sustained virologic response was significantly higher in patients with a complete early virologic response than in those with a partial early virologic response ($P < 0.0001$). In contrast, there was no difference in the rate of a sustained virologic response between patients with a complete early virologic response and those with a partial early virologic response ($P = 0.8917$) among patients with

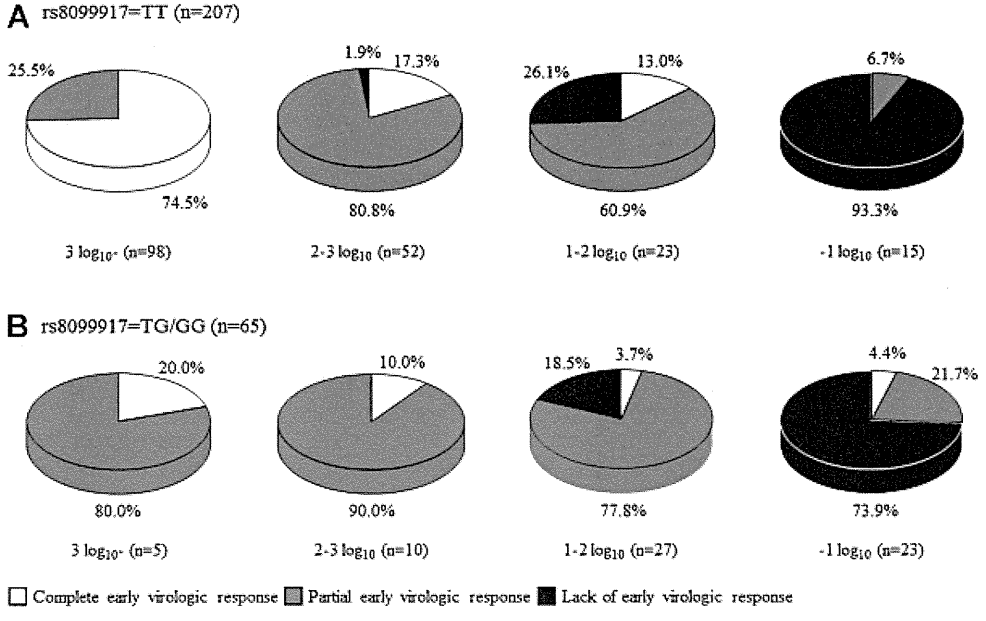

Fig. 2. The association between the virologic responses at 12 weeks after starting therapy and the reduction in serum HCV RNA levels at 4 weeks after starting therapy. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

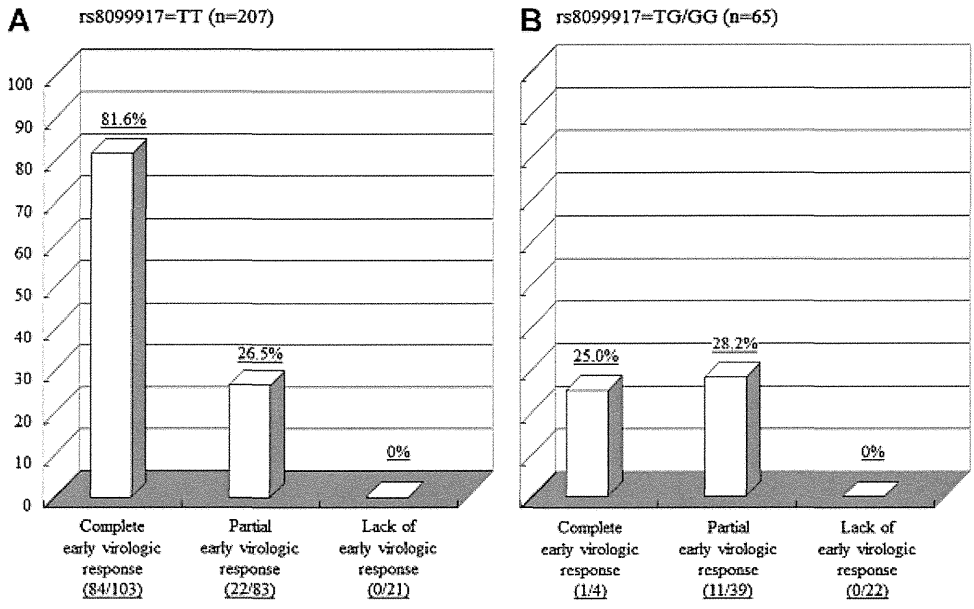

Fig. 3. The rate of sustained virologic responses based on the type of early virologic response. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

the TG/GG genotype. None of the patients with the TT genotype or TG/GG genotype who yielded a lack of an early virologic response reached a sustained virologic response.

Univariate and Multivariate Analyses for Factors Associated With a Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy in Patients With the TT and the TG/GG Genotype for the rs8099917

Univariate and multivariate analyses were conducted for factors associated with a sustained virologic response based on different genetic polymorphisms near the *IL28B* gene. In patients with the TT genotype, the factors that were associated with a sustained virologic response included serum alkaline phosphatase levels, serum albumin, platelet counts, hepatitis activity grade, liver fibrosis grade, reduction in HCV RNA levels at 4 weeks after starting therapy, and a complete early virologic response based on a univariate analysis (Table IIIA). In a multivariate analysis, the serum albumin levels, reduction in HCV RNA levels 4 weeks after starting therapy, and a complete early virologic response were independent factors that were significantly associated with a sustained virologic response (Table IIIB). A reduction in HCV RNA levels 4 weeks after starting therapy was the strongest factor that affected a sustained virologic response. In patients with the TG/GG genotype, the factors that were associated with a sustained virologic response included patient age, platelet counts, and pretreatment HCV RNA levels based on a univariate analysis (Table IIIA). A reduction in the HCV RNA levels at 4 weeks after starting therapy was not associated with a sustained virologic response. In a multivariate analysis, patient age and pretreatment HCV RNA levels were independent factors that were significantly associated with a sustained virologic response (Table IIIC).

Characteristics of Patients who Achieved a Sustained Virologic Response to the Combination Therapy Despite the Unfavorable TG/GG Genotype Near the *IL28B* Gene

Table IV shows the characteristics of 12 patients who achieved a sustained virologic response despite having the unfavorable TG/GG genotype for rs8099917 near the *IL28B* gene. All but one patient was under 60 years old and had liver fibrosis not more than grade 2 (one patient did not undergo a liver biopsy). Except for one patient, the reduction in the serum HCV RNA levels at 4 weeks after starting therapy was less than 3 $\log_{10}$ and all but one patient showed a partial early virologic response at 12 weeks after starting the therapy. In all 11 patients with a partial early virologic response, the serum HCV RNA was undetectable up to 24 weeks after starting the therapy. All but one patient extended the treatment duration from 48 to 72 weeks (two patients discontinued therapy at 60 weeks during the extended treatment period). When the characteristics of patients who achieved a sustained virologic response were compared between those with the unfavorable TG/GG genotype and those with the favorable TT genotype, patients with the TG/GG genotype were younger (41.8 ± 14.4 years vs. 55.1 ± 10.4 years, $P = 0.0023$) and had lower pretreatment HCV RNA levels (5.91 ± 0.44 $\log_{10}$ IU/ml vs. 6.21 ± 1.05 $\log_{10}$ IU/ml, $P = 0.0199$).

TABLE III. Univariate and Multivariate Analyses for Factors Associated With a Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy in Patients With the TT and the TG/GG Genotype for the rs8099917

(A) Univariate analyses	*P*-value	
	Patients with TT genotype of rs8099917 (n = 207)	Patients with TG/GG genotype of rs8099917 (n = 65)
Age (years)	0.0505	0.0007
Sex (female/male)	0.1830	0.2296
Body weight (kg)	0.6891	0.2456
Alanine aminotransferase (IU/L)	0.7988	0.4032
Aspartate aminotransferase (IU/L)	0.5021	0.1705
Gamma-glutamyl transpeptidase (IU)	0.6340	0.6648
Alkaline phosphatase (IU/L)	0.0315	0.0599
Albumin (g/dl)	0.0002	0.6594
Total bilirubin (mg/dl)	0.2929	0.7130
White blood cell count (/μl)	0.2508	0.5549
Hemoglobin (g/dl)	0.0847	0.2289
Platelet count ($\times 10^3$/μl)	0.0454	0.0411
Liver histology-activity (A0–1/A2–3)	0.0445	0.1117
Liver histology-fibrosis (F0–1/F2–3)	0.0002	0.2283
Pretreatment HCV RNA concentration (≥ 6.5 $\log_{10}$ vs. <6.5 $\log_{10}$)	0.5279	0.0379
Reduction in the peginterferon dose	0.4316	0.5563
Reduction in the ribavirin dose	0.1823	0.4272
Reduction in HCV RNA levels at 4 weeks after starting the therapy (≥ 3 $\log_{10}$ vs. < 3 $\log_{10}$)	<0.0001	0.9265
Early virologic response (complete vs. partial)	<0.0001	0.9777
Early virologic response (partial vs. non)	0.8632	0.0686

(B) Multivariate analyses: Patients with TT genotype of rs8099917	*P*-value	Odds ratio (95% confidence interval)
Alkaline phosphatase (IU/L)	0.2617	
Albumin (g/dl)	0.0365	28.287 (1.4107–755.41)
Platelet count ($\times 10^3$/μl)	0.2599	
Liver histology-activity (A0–1/A2–3)	0.6678	
Liver histology-fibrosis (F0–1/F2–3)	0.2307	
Reduction in HCV RNA levels at 4 weeks after starting the therapy (≥ 3 $\log_{10}$ vs. <3 $\log_{10}$)	<0.0001	16.029 (6.8593–40.406)
Early virologic response (complete vs. partial)	0.0224	0.3685 (0.1557–0.8749)

(C) Multivariate analyses: Patients with TG/GG genotype of rs8099917	*P*-value	Odds ratio (95% confidence interval)
Age (years)	0.0022	0.0034 (0.0000–0.0840)
Platelet count ($\times 10^3$/μl)	0.3344	
Pretreatment HCV RNA concentration (≥ 6.5 $\log_{10}$ vs. <6.5 $\log_{10}$)	0.0304	0.0548 (0.0020–0.4950)

HCV, hepatitis C virus.

DISCUSSION

Several previous studies reported that patients who achieved a rapid virologic response, in which serum HCV RNA become undetectable at 4 weeks after starting therapy, had a high likelihood of achieving a sustained virologic response [Martinez-Bauer et al., 2006; Poordad et al., 2008; de Segadas-Soares et al., 2009; Martinot-Peignoux et al., 2009]. In addition, several recent studies reported the predictive value of the degree of reduction in serum HCV RNA levels at 4 weeks after starting therapy [Yu et al., 2007; Huang et al., 2010; Toyoda et al., 2011]. Therefore, the viral dynamics of HCV at 4 as well as 12 weeks after starting therapy is important for response-guided therapy.

Genetic polymorphisms near the *IL28B* gene have emerged as the strongest predictive factor of a sustained virologic response in patients infected with HCV genotype 1 [Hayes et al., 2011; Kurosaki et al., 2011]. In addition, Thompson et al. [2010 reported that genetic polymorphisms near the *IL28B* gene were associated strongly with early viral dynamics during PEG-IFN and ribavirin combination therapy. These findings raised an important issue of whether response-guided therapy, based on the reduction in serum HCV RNA levels at 4 or 12 weeks after starting

TABLE IV. Patients who Achieved a Sustained Virologic Response Despite the TG/GG Genotype for the rs8099917

	Age (years)	Sex	Liver histology	Pretreatment HCV RNA level (log_{10} IU/ml)	HCV RNA reduction at 4 weeks	Response at 12 weeks	HCV RNA became undetectable (weeks)	Treatment duration (weeks)
1.	31	Female	A1/F1	6.13	2.19	partial EVR	20	48
2.	55	Male	A1/F1	5.80	1.77	partial EVR	16	72
3.	57	Female	A1/F1	5.58	3.01	partial EVR	16	72
4.	57	Female	A1/F1	6.21	1.81	partial EVR	20	72
5.	62	Male	N.D.	6.23	1.13	partial EVR	24	72
6.	21	Male	A1/F2	6.04	1.83	partial EVR	24	72
7.	42	Male	A1/F1	6.27	0.57	partial EVR	24	72
8.	29	Female	A1/F2	5.83	1.83	partial EVR	20	60
9.	52	Male	A1/F0	5.91	2.12	complete EVR	12	48
10.	40	Male	A2/F1	5.84	1.34	partial EVR	20	72
11.	27	Male	N.D.	5.63	0.42	partial EVR	24	72
12.	28	Male	A1/F0	6.59	0.76	partial EVR	20	60

N.D., not done; HCV, hepatitis C virus; EVR, early virologic response.

therapy, retains a predictive value when considering genetic polymorphisms near the *IL28B* gene.

In the present study, the predictive value of the decrease in serum HCV RNA levels was evaluated at 4 and 12 weeks after starting therapy in Japanese patients infected with HCV genotype 1b based on genetic polymorphisms near the *IL28B* gene. Consistent with previous reports, patients with the TG/GG genotype for rs8099917 had a smaller reduction in serum HCV RNA levels at 4 weeks after starting treatment ($P < 0.0001$), which indicates an unfavorable response to the combination therapy. Patients with the TT genotype for rs8099917, which is associated with a favorable response to the combination therapy, exhibited a significant difference in the rate of a sustained virologic response based on the reduction in serum HCV RNA levels at 4 weeks after initiating the therapy. Patients with a rapid virologic response or with a ≥ 3 log_{10} reduction in HCV RNA levels had a higher likelihood of achieving a sustained virologic response.

In contrast, these factors did not have any predictive value in patients with the TG/GG genotype. Only 18.5% of patients achieved a sustained virologic response (12 of 65 patients), and it was difficult to identify these patients based on the reduction in HCV RNA levels at 4 weeks or the type of an early virologic response at 12 weeks after starting therapy. Patients who achieved a sustained virologic response, despite the TG/GG genotype for rs8099917, were identified among those with a <2 log_{10} and ≥ 1 log_{10} or even <1 log_{10} reduction in HCV RNA levels at 4 weeks after starting therapy. Interestingly and paradoxically, the possibility of a sustained virologic response can be expected in patients with a <1 log_{10} reduction in HCV RNA levels at 4 weeks after starting therapy only when they have the unfavorable TG/GG genotype.

In the evaluation at 12 weeks after starting therapy, patients with the TT genotype who achieved a complete early virologic response had a higher rate of a sustained virologic response significantly than patients who achieved a partial early virologic response, whereas this difference was not found in patients with the TG/GG genotype. No patients who failed to achieve an early virologic response achieved a sustained virologic response regardless of the genetic polymorphisms near the *IL28B* gene. Thus, the lack of an early virologic response retained a strong predictive value for the failure of achieving a sustained virologic response. This result supports the recommendation in the AASLD guidelines, in which treatment may be discontinued in patients without an early virologic response at 12 weeks of treatment.

The characteristics of patients who achieved a sustained virologic response despite the unfavorable TG/GG genotype were younger in age and lower pretreatment HCV RNA levels. Most patients with the TG/GG genotype who achieved a sustained virologic response showed a partial early virologic response and extended the treatment duration. It was difficult to identify these patients according to viral dynamics at 4 or 12 weeks after starting therapy.

There are several limitations in this study. Some patients with a slow virologic response did not have their treatment period extended from 48 to 72 weeks. This is because the effectiveness of a 72-week combination therapy regimen in patients with HCV genotype 1 with a slow virologic response [Berg et al., 2006; Pearlman et al., 2007] had not been established in Japan in the earlier part of this study. This fact might have influenced the treatment outcome especially in patients with the unfavorable TG/GG genotype. Another limitation is a smaller sample size of patients with the TG/GG genotype in comparison to that of patients with the TT genotype. This sample size could have caused the lack of statistical significance in the rate of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy or according to the type of an early virologic response in patients with the TG/GG genotype. In addition, the data were based on Japanese patients infected with HCV genotype 1b. Therefore, these results should be confirmed in other ethnicities and patients infected with HCV genotype 1a.

In conclusion, among patients infected with HCV genotype 1b with the TT genotype for rs8099917, a rapid virologic response or a ≥ 3 $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy, or a complete early virologic response indicate strongly that these patients will achieve a sustained virologic response as a final outcome for PEG-IFN and ribavirin combination therapy. Early viral dynamics retain the predictive value in this patient subpopulation. A reduction in HCV RNA levels at 4 weeks after starting therapy or the type of an early virologic response does not predict the likelihood that patients with the TG/GG genotype will achieve a sustained virologic response. In contrast, the lack of an early virologic response retains a strong predictive value for the failure to achieve a sustained virologic response regardless of *IL28B* polymorphisms, which remains useful as a factor to stop therapy.

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RESEARCH ARTICLE

Higher hepatic gene expression and serum levels of matrix metalloproteinase-2 are associated with steatohepatitis in non-alcoholic fatty liver diseases

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Abstract

We investigated the gene expression of tissue inhibitor metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) and serum levels of TIMPs, MMPs, and hyaluronic acid that are associated with liver fibrosis in 64 patients with nonalcoholic fatty liver diseases (NAFLD). Whereas, no differences were found between patients with and without nonalcoholic steatohepatitis (NASH) in serum levels of hyaluronic acid when excluding NASH patients with advanced fibrosis, the quantity of MMP2 mRNA in liver tissue and serum MMP2 levels were significantly higher in patients with NASH than those without, even focusing on patients with less advanced fibrosis, indicating the initiation of liver fibrosis.

Keywords: Non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, matrix metalloproteinases, tissue inhibitors of metalloproteinases, hyaluronic acid, gene expression, serum levels

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases in both Western and Asian countries (Angulo 2002; Clark et al. 2003; Kojima et al. 2003; Chitturi et al. 2007; Torres & Harrison 2008), affecting 30% of the general Western adult population (Musso et al. 2010). NAFLD encompasses a histological spectrum that ranges from simple steatosis to nonalcoholic steatohepatitis (NASH). Whereas simple steatosis is usually benign, patients with NASH can progress to cirrhosis and end-stage liver disease (Angulo 2002; Fassio et al. 2004; Hashimoto et al. 2005). Therefore, it is important to differentiate patients with NASH from patients with more benign forms of NAFLD.

Liver fibrosis accumulates with the progression of NASH toward cirrhosis, as is reported in the case of viral hepatitis. Changes in many proteins associated with fibrosis have been reported during the course of viral hepatitis. Matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) are reportedly associated with the progression of liver fibrosis (Hemmann et al. 2007). It is unclear whether changes in the gene expression of fibrosis-associated proteins occur in the liver of patients with NASH, as they do in patients with viral hepatitis, and whether there are differences in the gene expression patterns and serum levels of these proteins between NASH and simple steatosis. In the present study, we investigated the gene expression patterns of several fibrosis-associated proteins in the livers of patients with NAFLD, comparing patients with and without NASH. Serum levels of fibrosis-associated proteins were also investigated.

Patients and methods

Patients

The study population consisted of 64 patients (36 males and 28 females with a mean age of 51.0 ± 15.0

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years) who underwent ultrasound-guided liver biopsy between 2008 and 2010 for the diagnosis of NAFLD. They were patients who agreed with liver biopsy among 268 patients who had admitted to our clinics and had been advised to receive liver biopsy because of the clinical diagnosis of NAFLD during the study period. Liver biopsy was performed to examine the presence of NASH and to confirm the diagnosis. Patients were clinically diagnosed with NAFLD prior to biopsy based on the following criteria: (i) persistent abnormal liver function tests for more than 3 months, (ii) ultrasonographic images showing steatosis, (iii) no evidence of alcohol abuse, and (iv) exclusion of other liver diseases and other known causes of steatosis based on the results of specific clinical, biochemical, or imaging studies. The ultrasonographic findings of steatosis were based on established criteria such as hepatorenal echo contrast, liver brightness, deep attenuation, and vascular blurring (Hamaguchi et al. 2007). The first two criteria were used as definitive criteria, while the latter two criteria were taken into account as needed. All patients were confirmed not to have chronic viral hepatitis with negative results for hepatitis B virus (HBV) surface antigen, HBV DNA, hepatitis C virus (HCV) antibody, and HCV RNA. No patients were diagnosed as having autoimmune hepatitis, primary biliary cirrhosis, or other liver diseases.

All patients underwent ultrasonography-guided fine needle liver biopsy using a 17G biopsy needle. NAFLD was pathologically diagnosed based on pathologic findings in the biopsied liver specimens. The liver biopsy specimens were stained with hematoxylin and eosin, Masson's trichrome, and periodic-acid Schiff stains and then examined by experienced pathologists. The liver specimens were categorized into types 1–4 pathologically based on Matteoni classification (Matteoni et al. 1999), and types 3 and 4 were defined as NASH. Patologic evaluations were performed by two pathologists independently.

The study protocol was in compliance with the Helsinki Declaration and was approved by the institutional review board of Ogaki Municipal Hospital. All patients provided written informed consent for the use of their clinical data and the analyses of biopsy specimens and serum samples.

RNA extraction and real-time PCR for gene expression analyses

Liver biopsy specimens were stored in Ambion RNAlater solution (Life Technologies, Carlsbad, CA, USA) at –80°C until RNA extraction. Total RNA was extracted using the mirVana miRNA isolatidon kit (Life Technologies) according to the manufacturer's instructions.

cDNA was synthesized using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). Total RNA (2 mg) in 10.4 μL of nuclease-free water was added to 1 mL of 50 mM random hexamer. The denaturing reaction was performed for 10 min at 65°C. The denatured RNA mixture was added to 4 mL of 5× reverse transcriptase buffer, 2 mL of 10 mM dNTP, 0.5 mL of 40 U/mL RNase inhibitor, and 1.1 mL of reverse transcriptase (FastStart Universal SYBR Green Master, Roche) in a total volume of 20 mL. The reaction ran for 30 min at 50°C (cDNA synthesis), and 5 min at 85°C (enzyme denaturation). All reactions were run in triplicate. The Chromo4 detector (Bio-Rad, Hercules, CA, USA) was used to detect mRNA expression. The primer sequences are follows: TIMP1: 5′-cttggcttctgcactgatgg-3′ (sense), 5′-acgctggtataaggtggtct-3′ (antisense); TIMP2: 5′-agtggactctggaaacgaca-3′ (sense); 5′-tctctgtgacccagtccatc-3′ (antisense); MMP2: 5′- aacgccgatggggagtactg-3′ (sense); 5′-cagggctgtccttcagcgtt-3′ (antisense); MMP13: 5′-gaggctccgagaaatgcagt-3′ (sense); 5′-atgccatcgtgaagtctggt-3′ (antisense); and β-actin: 5′-ccactggcatcgtgatggac-3′ (sense), 5′-tcattgccaatggtgatgacct-3′ (antisense). Assays were performed in triplicate, and the expression levels of target genes were normalized to the expression of the β-actin gene as quantified using real-time quantitative PCR as an internal control.

Measurement of serum levels of fibrosis-associated proteins

Serum levels of TIMP1, MMP2, and hyaluronic acid were measured in stored fasting serum samples that had been obtained at the time of liver biopsy. Serum TIMP1 levels were measured by enzyme immunoassay (hTIMP-1 kit, Daiichi Fine Chemical, Toyama, Japan). Serum MMP2 levels were measured by enzyme immunoassay (hMMP-2 Activity Assay System, GE Healthcare Japan, Tokyo, Japan). Serum hyaluronic acid was measured by the latex agglutination method (Hyaluronic acid LT, Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis

Quantitative values are expressed as means ± SD. Between-group differences were analyzed by the chi-square test. Differences in quantitative values between two groups were analyzed by the Mann–Whitney *U* test. Correlation between liver tissue mRNA and serum levels of TIMP1 and MMP2 were evaluated with Spearman's test. Multivariate analysis was performed using logistic regression models. All p values were 2-tailed, and $p < 0.05$ was considered to indicate statistical significance.

Results

Background characteristics of study patients

Table 1 summarizes the characteristics of the study patients. Pathologic examination revealed that all patients had steatosis involving at least 10% of the hepatocytes, and NASH was diagnosed in 43 patients (67.2%). Among 43 patients diagnosed with NASH, 13 patients (30.2%) were diagnosed with stage 3 or 4 fibrosis according to the Brunt classification (Brunt et al. 1999). No significant differences were found between patients with and without NASH with respect to age, sex, body weight,

laboratory data, and degree of steatosis on pathologic evaluation (Supplemental Table 1).

Expression of TIMP1, TIMP2, MMP2, and MMP13 mRNA in liver tissue in patients with NAFLD

Figure 1 compares the gene expression levels of TIMP1, TIMP2, MMP2, and MMP13 based on the quantification of mRNA in the liver tissue of patients with and without NASH. The quantity of MMP2 mRNA was significantly higher in patients with NASH (2.69±1.40, relative expression level) than those without (1.50±0.57; $p < 0.0001$). No significant differences were found in the quantity of TIMP1, TIMP2, and MMP13 mRNA. There were no differences in the quantity of TIMP1, TIMP2, MMP2, and MMP13 mRNA according to the degree of steatosis (data not shown).

Table 1. Characteristics of study patients ($n = 64$).

Age (years)	51.0±15.0
Sex (male/female)	36 (56.3)/28 (43.7)
Body weight (kg)	70.3±11.5
Body mass index (kg/m^2)	27.1±3.5
Alanine aminotransferase (IU/L)	88.5±76.4
Aspartate aminotransferase (IU/L)	54.3±32.5
Gamma-glutamyl transpeptidase (IU)	87.1±64.6
Total bilirubin (mg/dL)	0.71±0.54
Albumin (g/dL)	4.22±0.51
Glucose (mg/dL)*	131.6±61.3
Total cholesterol (mg/dL)*	197.8±39.4
Triglyceride (mg/dL)*	162.1±84.3
Hemoglobin A_1-c (%)	6.07±1.55
Hemoglobin (g/dL)	14.7±1.6
Platelet count (×10^3/μL)	238±70
Ferritin (ng/mL)	231.0±190.7
Steatosis (<30%/30-50%/50-70%/70%≤)**	17 (26.6)/18 (28.1)/19 (29.7)/10 (15.6)
Diagnosis (NASH/simple steatosis)	43 (67.2)/21 (32.8)
Fibrosis (grade 1/2/3/4)**,#	20 (46.5)/10 (23.3)/10 (23.3)/3 (6.9)

NASH, non-alcoholic steatohepatitis.
*Measured under fasting conditions.
**Based on pathologic examination.
#Only in patients with NASH by Brunt classification (Brunt 1999).

Serum levels of TIMP1, MMP2, and hyaluronic acid in patients with NAFLD

Figure 2 compares the levels of TIMP1, MMP2, and hyaluronic acid in serum samples obtained at the time of liver biopsy between patients with and without NASH. Serum levels of TIMP1 and MMP2 showed a significant correlation with liver tissue mRNA levels, respectively, although the correlation was not strong ($p = 0.0003$ and $\rho = 0.472$ for TIMP1, and $p < 0.0001$ and $\rho = 0.534$ for MMP2, Supplemental Figure 1). The serum levels of MMP2 and hyaluronic acid were significantly higher in patients with NASH than in patients without (MMP2, $p = 0.0198$, and hyaluronic acid, $p = 0.0042$). No significant differences were found in the serum levels of TIMP1 between patients with and without NASH.

Univariate and multivariate analyses were performed for factors associated with NASH (Table 2). In the univariate analysis, serum aspartate aminotransferase (AST), MMP2, and hyaluronic acid levels were associated with NASH. In multivariate analysis, serum AST, MMP2, and hyaluronic acid levels were independently associated with NASH.

Expression of MMP2 mRNA in liver tissue and serum levels of MMP2, and hyaluronic acid in NASH patients with mild fibrosis

When excluding patients with NASH and advanced fibrosis (Brunt's fibrosis stage [Brunt et al. 1999] 3 or 4), the quantity of hepatic MMP2 mRNA remained significantly higher in patients with NASH than those without ($p = 0.0010$, Figure 3). Serum levels of MMP2 were also significantly higher in patients with NASH than those without ($p = 0.0020$, Figure 3). No significant differences in serum levels of hyaluronic acid were observed ($p = 0.1296$, Figure 3). In both univariate and multivariate analyses, only serum AST and MMP2 levels were associated with NASH (Table 3).

The predictive value of serum levels of MMP2 and hyaluronic acid were analyzed with receiver-operating characteristic (ROC) analysis. In all patients, serum MMP2 and hyaluronic acid levels had comparable ability for predicting NASH among patients with NAFLD with

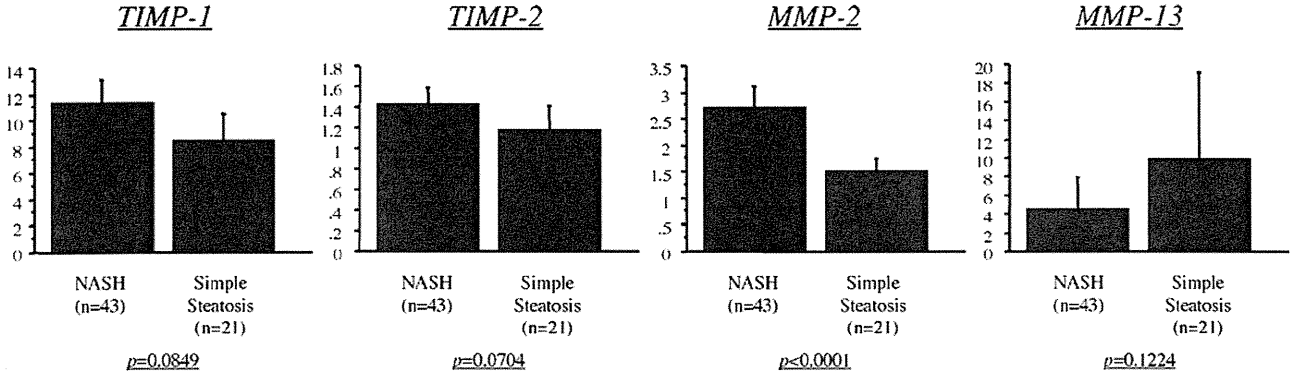

Figure 1. Relative mRNA expression levels of tissue inhibitor metalloproteinase-1 and -2 (TIMP1 and TIMP2), and matrix metalloproteinase 2 and 13 (MMP2 and MMP13) in the liver tissue of patients with nonalcoholic steatohepatitis (NASH) versus simple steatosis.

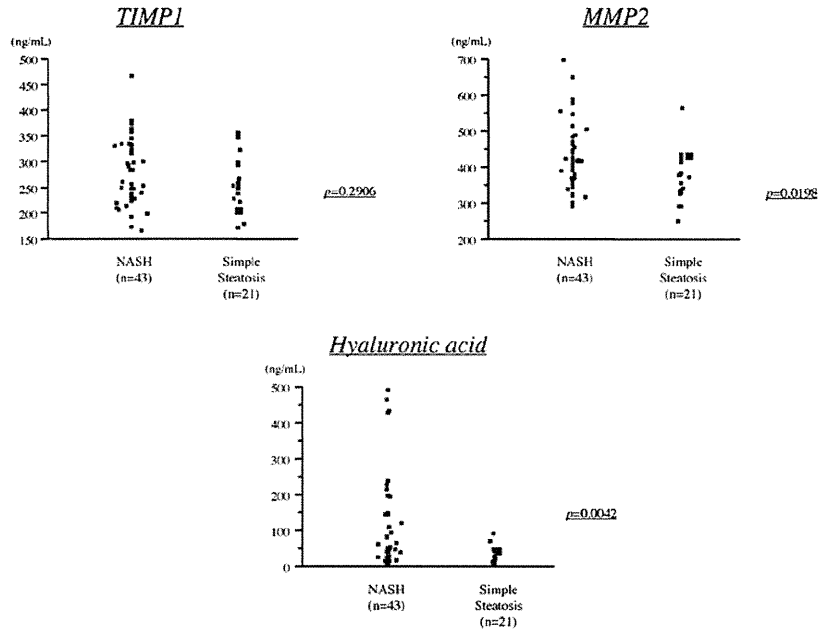

Figure 2. Serum levels of tissue inhibitor metalloproteinase-1 (TIMP1), matrix metalloproteinase-2 (MMP2), and hyaluronic acid in all patients with nonalcoholic steatohepatitis (NASH) versus simple steatosis. TIMP1, 277.4 ± 64.6 ng/mL with NASH vs. 256.5 ± 55.6 ng/mL with simple steatosis; $p = 0.2906$, MMP2, 427.8 ± 95.2 ng/mL with NASH vs. 353.6 ± 55.1 ng/mL with simple steatosis; $p = 0.0198$, and hyaluronic acid, 111.0 ± 131.5 ng/mL with NASH vs. 29.3 ± 21.2 ng/mL with simple steatosis; $p = 0.0042$.

Table 2. Univariate and multivariate analyses for distinguishing between patients with NASH and simple steatosis ($n = 64$).

	Univariate analysis (p value)	Multivariate analysis (p value)	Odds ratio (95% confidence interval)
Age (years)	0.4523	-	
Sex (male/female)	0.9199	-	
Body weight (kg)	0.4524	-	
Body mass index (kg/m^2)	0.2999	-	
Alanine aminotransferase (IU/L)	0.2061	-	
Aspartate aminotransferase (IU/L)	0.0146	0.0258	938.371 (4.9097–922101.2)
Gamma-glutamyl transpeptidase (IU)	0.5724	-	
Total bilirubin (mg/dL)	0.1126	-	
Albumin (g/dL)	0.1959	-	
Glucose (mg/dL)*	0.1898	-	
Total cholesterol (mg/dL)*	0.2338	-	
Triglyceride (mg/dL)*	0.1696	-	
Hemoglobin A_1-c (%)	0.9370	-	
Hemoglobin (g/dL)	0.5974	-	
Platelet count (×10^3/μL)	0.0613	-	
Ferritin (ng/mL)	0.5443	-	
TIMP1 (ng/mL)	0.2224	-	
MMP2 (ng/mL)	0.0058	0.0275	364.171 (3.9968–174225.9)
Hyaluronic acid (ng/mL)	0.0228	0.0351	23346.68 (32.5694–298598)
Steatosis (<30%/30–50%/50–70%/70%≤)**	0.8713	-	

NASH, non-alcoholic steatohepatitis; TIMP1, tissue inhibitor metalloproteinase-1; MMP2, matrix metalloproteinase-2.
*Measured during fasting conditions.
**Based on pathologic examination.

similar area under the ROC curves (AUROC) (MMP2, 0.73 and hyaluronic acid, 0.77, Supplemental Figure 2). When NASH patients with advanced fibrosis were excluded, the ability of serum hyaluronic acid levels to predict NASH decreased (AUROC, 0.63), whereas the predictive ability of serum MMP2 levels remained similar (AUROC, 0.74, Supplemental Figure 3).

Discussion

In the present study, we observed enhanced gene expression of MMP2 in liver tissue, along with elevated serum levels of MMP2, both of which showed the correlation, in patients with NASH compared to those with simple steatosis. MMP2 expression reportedly increases during the

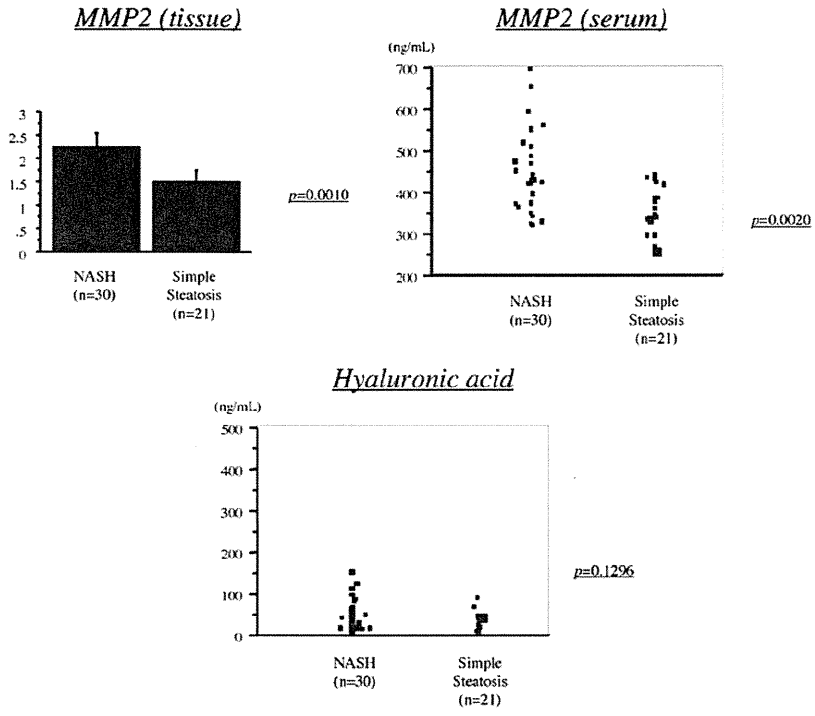

Figure 3. Quantity of matrix metalloproteinase-2 (MMP2) mRNA in liver tissue and serum levels of MMP2, and hyaluronic acid in nonalcoholic steatohepatitis (NASH) patients without advanced fibrosis versus patients with simple steatosis. MMP2 mRNA, 2.23±0.84 with NASH vs. 1.50±0.57 with simple steatosis; p = 0.0010, serum levels of MMP2, 441.8±99.6 ng/mL with NASH vs. 353.6±55.1 ng/mL with simple steatosis; p = 0.0020, and serum levels of hyaluronic acid, 143.1±31.1 ng/mL with NASH vs. 130.3±63.1 ng/mL with simple steatosis; p = 0.1296.

Table 3. Univariate and multivariate analyses for distinguishing patients with NASH and simple steatosis, excluding NASH patients with advanced fibrosis (n = 51).

	Univariate analysis (p value)	Multivariate analysis (p value)	Odds ratio (95% confidence interval)
Age (years)	0.7699	-	
Sex (male/female)	0.9730	-	
Body weight (kg)	0.3069	-	
Body mass index (kg/m^2)	0.2221	-	
Alanine aminotransferase (IU/L)	0.1769	-	
Aspartate aminotransferase (IU/L)	0.0309	0.0251	240.057 (3.5678–72252.5)
Gamma-glutamyl transpeptidase (IU)	0.7185	-	
Total bilirubin (mg/dL)	0.1344	-	
Albumin (g/dL)	0.3718	-	
Glucose (mg/dL)*	0.5584	-	
Total cholesterol (mg/dL)*	0.5173	-	
Triglyceride (mg/dL)*	0.1327	-	
Hemoglobin A_1-c (%)	0.7368	-	
Hemoglobin (g/dL)	0.8608	-	
Platelet count (×10^3/μL)	0.5635	-	
Ferritin (ng/mL)	0.8109	-	
TIMP1 (ng/mL)	0.2302	-	
MMP2 (ng/mL)	0.0047	0.0068	2759.72 (19.7697–2163013)
Hyaluronic acid (ng/mL)	0.1007	-	
Steatosis (<30%/30–50%/50–70%/70%≤)**	0.2877	-	

NASH, non-alcoholic steatohepatitis; TIMP1, tissue inhibitor metalloproteinase-1; MMP2, matrix metalloproteinase-2.
*Measured during fasting conditions.
**Based on pathologic examination.

progression of liver fibrosis (Ebata et al. 1997; Hemmann et al. 2007), and our results indicated that liver fibrosis is proceeding in patients with NASH.

NASH is usually diagnosed by histological evaluation of specimens obtained by liver biopsy and this is currently the only reliable and accepted method for the evaluation of liver fibrosis. However, liver biopsy is invasive and carries the risk of intraperitoneal bleeding. Therefore, noninvasive indicators of NASH in NAFLD patients would be important. Several biomarkers have been studied as indicators of NASH in patients with NAFLD (Malik et al. 2009; Miele et al. 2009). The serum level of hyaluronic acid is an important marker for the identification of patients with NASH. Because serum hyaluronic acid levels increase with the progression of liver fibrosis (Adams 2011) and the increase can simply reflect accumulated fibrosis in the liver as a result of the progression of NASH, it may not be an indicator of NASH but with mild fibrosis (i.e. early stage of NASH). Although serum hyaluronic acid levels were significantly higher in patients with NASH than those without NASH in the present study, we failed to find significant differences in serum hyaluronic acid levels between NASH patients with mild fibrosis and those without NASH. Whereas the AUROC for serum hyaluronic acid level in predicting NASH was more than 0.7 in patients including advanced fibrosis in the study by Malik et al. and in the present study, it decreased from 0.77 to 0.63 when focusing on patients with mild fibrosis in the present study. Serum hyaluronic acid levels, therefore, do not appear to be useful for distinguishing NASH patients with mild fibrosis from patients without NASH. In contrast, the AUROC for serum MMP2 levels in predicting NASH remains greater than 0.73 even in the subpopulation with mild fibrosis. In clinical practice, it will be important to identify NASH patients with mild fibrosis, before the progression of liver fibrosis caused by NASH, from NAFLD patients. Serum MMP2 levels may be useful for this purpose.

Conclusion

In patients with NASH, gene expression of MMP2, a protein associated with liver fibrosis, was enhanced in the liver tissue and serum levels of MMP2 were increased, indicating the initiation of liver fibrosis in this subpopulation. These results were also observed when NASH patients with advanced fibrosis were excluded. MMP2 may be a noninvasive indicator of early stage of NASH in patients with NAFLD.

Declaration of interest

The authors declare no conflicts of interest.

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Research Article

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p53/p66Shc-mediated signaling contributes to the progression of non-alcoholic steatohepatitis in humans and mice

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Background & Aims: The tumor suppressor p53 is a primary sensor of stressful stimuli, controlling a number of biologic processes. The aim of our study was to examine the roles of p53 in non-alcoholic steatohepatitis (NASH).

Methods: Male wild type and p53-deficient mice were fed a methionine- and choline-deficient diet for 8 weeks to induce nutritional steatohepatitis. mRNA expression profiles in normal liver samples and liver samples from patients with non-alcoholic liver disease (NAFLD) were also evaluated.

Results: Hepatic p53 and p66Shc signaling was enhanced in the mouse NASH model. p53 deficiency suppressed the enhanced p66Shc signaling, decreased hepatic lipid peroxidation and the number of apoptotic hepatocytes, and ameliorated progression of nutritional steatohepatitis. In primary cultured hepatocytes, transforming growth factor (TGF)-β treatment increased p53 and p66Shc signaling, leading to exaggerated reactive oxygen species (ROS) accumulation and apoptosis. Deficient p53 signaling inhibited TGF-β-induced p66Shc signaling, ROS accumulation, and hepatocyte apoptosis. Furthermore, expression levels of p53, p21, and p66Shc were significantly elevated in human NAFLD liver samples, compared with results obtained with normal liver samples. Among NAFLD patients, those with NASH had significantly higher hepatic expression levels of p53, p21, and p66Shc compared with the group with simple steatosis. A significant correlation between expression levels of p53 and p66Shc was observed.

Conclusions: p53 in hepatocytes regulates steatohepatitis progression by controlling p66Shc signaling, ROS levels, and apoptosis, all of which may be regulated by TGF-β. Moreover, p53/p66Shc signaling in the liver appears to be a promising target for the treatment of NASH.

Keywords: P53; Non-alcoholic steatohepatitis; P66Shc; Reactive oxygen species; Transforming growth factor-β.

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ROS, reactive oxygen species; MCD, methionine-deficient and choline-deficient; ALT, alanine aminotransferase; HE, haematoxylin-eosin; HNE, hydroxynonenal; TUNEL, terminal deoxy-nucleotidyl transferase-mediated nick end-labeling; PCR, polymerase chain reaction; TGF-β, transforming growth factor-β; PFT, pifithrin; Col11, 1 (I) collagen; Col12, 2 (I) collagen; SMA, smooth muscle actin; MDA, malondialdehyde.

Introduction

Non-alcoholic fatty liver disease (NAFLD) afflicts as much as 20% of the US adult population [1]. Non-alcoholic steatohepatitis (NASH)—part of the spectrum of NAFLD—is the most prevalent liver disease in the US, affecting approximately 3–4% of the population [1].

NAFLD and NASH are often co-morbid with disorders characterized by insulin resistance, such as diabetes and obesity. Thus, these liver diseases can be considered hepatic manifestations of metabolic syndrome. Given the growing number of patients with metabolic syndrome, the incidences of NAFLD and NASH are expected to increase further, particularly in North America, Europe, Asia, and countries in the Western Pacific.

NASH is a progressive disease. In a study that followed NASH patients for ten years, the disease progressed to cirrhosis in 20% of the patients and led to fatal liver disease in 8% of the cases [2]. A population-based cohort study demonstrated that

Table 1. Histological characteristics of patients.

	NASH (n = 57)	Simple steatosis (n = 13)
Steatosis		
1	31	10
2	18	3
3	8	0
Inflammatory activity		
0	0	11
1	9	2
2	43	0
3	5	0
Fibrosis stage		
0	0	13
1	35	0
2	14	0
3	8	0
4	0	0

approximately 3% of the patients diagnosed with NAFLD developed cirrhosis or a liver-related complication [3]. The progressive nature and serious consequences of NASH highlight the need for effective therapies. The pathologic mechanisms underlying NASH, however, have not yet been clarified.

Recently, a number of diagnostic tests that incorporate clinical markers, including age, have been reported for NAFLD. Indeed, advanced age is a major risk factor for the progression of NASH [4]. On the other hand, the tumor suppressor p53—a master sensor of stressful conditions—controls many biological processes, including aging [5]. Reactive oxygen species (ROS), which are thought to make major contributions to aging, stimulate p53 stabilization and subsequent induction of apoptosis via a feed-forward regulatory loop [6]. Hepatic p53 expression is elevated in patients with NASH [7]. A recent report has also shown that hepatic p53 expression and hepatocyte apoptosis significantly increase in a mouse model of NASH [8]. These results suggest that p53 plays a role in the pathophysiology of NASH.

In the present study, we examined the role of p53 signaling during NASH using p53-deficient mice and a mouse model of NASH. We found that hepatic p53 signaling markedly contributes to the pathogenesis of NASH. Our findings suggest hepatic p53 signaling as a promising target for new modalities in the treatment of NASH.

Materials and methods

Please refer to the Supplementary Materials and methods section for more detailed descriptions.

Animal studies

Eight-week-old male C57BL/6J mice were purchased from CLEA Japan Inc. $p53^{+/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). $p53^{+/+}$ and $p53^{-/-}$ mouse littermates were obtained from crosses of $p53^{+/-}$ mice with the C57BL/6J background.

In experiments for nutritional steatohepatitis, 8-week-old male $p53^{+/+}$ mice or $p53^{-/-}$ mouse littermates were fed a methionine- and choline-deficient (MCD) diet (cat No. 960439; ICN, Aurora, Ohio) or a standard chow (CE-2; CLEA Japan Inc.) for 8 weeks. In order to make a time-course analysis of nutritional steatohepatitis, 8-week-old male C57BL/6J mice were fed an MCD diet for 3 or 8 weeks.

All animals received humane care in compliance with the National Research Council's criteria outlined in the "Guide for the Care and Use of Laboratory Animals", prepared by the US National Academy of Sciences and published by the US National Institutes of Health.

Human liver tissue samples

Liver tissues were obtained from 70 patients undergoing ultrasound-guided liver biopsy for suspected NASH and from 10 patients undergoing surgical operation at the Keio University School of Medicine and National Defense Medical College. Patient characteristics are shown in Table 1. Written informed consent was obtained from all patients. This study protocol was approved by the Ethical Committee of the Keio University Hospital and National Defense Medical College Hospital, and followed the ethical guidelines of the Declaration of Helsinki.

Statistical analysis

Data are expressed as means (SEM) or median and interquartile range. Statistical analyses were performed using unpaired Student's *t* test, one-way analysis of variance (ANOVA), or Mann–Whitney's U test for univariate comparison, as appropriate. Correlations were assessed using the Pearson product-moment correlation coefficient. Differences were considered statistically significant at p values less than 0.05. All of the computations were performed with a commercial statistical package (SPSS version 12, Chicago, USA).

Results

p53 deficiency ameliorates the progression of nutritional steatohepatitis in mice

Following administration of an MCD diet, mice rapidly and consistently develop a severe form of steatohepatitis with the characteristic pathology of steatosis, mixed cell inflammatory infiltrate, hepatocyte death, and pericellular fibrosis, which resembles human NASH [9]. Therefore, to examine the effects of p53 signaling on the progression of NASH, we fed wild type and p53-deficient mice an MCD diet for 8 weeks. After 8 weeks of MCD diet, the p53-deficient mice exhibited significantly fewer lipid droplets in their hepatocytes, and reduced infiltration of inflammatory cells into their livers, compared with those observed in the wild type group (Fig. 1A). The MCD diet also produced a higher increase of serum alanine aminotransferase (ALT) levels in wild type mice than in p53-deficient mice (Fig. 1B). After 8 weeks of MCD diet, the livers of wild type mice exhibited a significant increase in lipid droplets in hepatocytes and elevated hepatic TG concentrations compared with p53-deficient mice (Fig. 1C).

Moreover, the livers of wild type mice showed increased collagen deposition, whereas fibrosis was markedly reduced in the livers of p53-deficient mice (Fig. 1A). Quantification of Masson trichrome staining and liver hydroxyproline levels confirmed the histologic results (Fig. 1D). Those levels were significantly lower in p53-deficient mice compared with wild type mice (Fig. 1D). Real-time PCR analyses of whole liver homogenates from mice fed the MCD diet revealed significantly increased mRNA levels of collagen type I α1 (*Col1α1*), collagen type I α2 (*Col1α2*), and transforming growth factor *(TGF)-β*, compared with those of mice fed the control diet. Compared with the wild type

livers, the livers of p53-deficient mice showed significantly decreased mRNA expression of *Col1α1*, *Col1α2*, and *TGF-β* after the mice were fed the MCD diet (Fig. 1E). The MCD diet significantly increased hepatic mRNA expression of *α-SMA* (smooth muscle actin), a marker of hepatic stellate cell activation; p53-deficient mice showed significantly lower levels of expression compared to wild type mice (Fig. 1E).

p53 signaling regulates hepatic ROS accumulation in nutritional steatohepatitis

Hepatic ROS accumulation is thought to play a role in the pathogenesis of NASH [10–12]. We immunostained samples for 4-hydroxynonenal (4-HNE) protein adducts as products of lipid peroxidation. Lipid peroxidation in the liver was significantly enhanced in mice with nutritional steatohepatitis compared with mice fed the control diet (Fig. 1F). Compared with p53-deficient mice, wild type mice showed more intense staining of hepatocytes after being fed the MCD diet (Fig. 1F). Levels of malondialdehyde (MDA)—another product of lipid peroxidation—in liver homogenates also showed that inhibition of p53 signaling significantly decreased hepatic lipid peroxidation in nutritional steatohepatitis (Fig. 1G).

p53 deficiency decreases hepatocyte susceptibility to damage in a mouse model of nutritional steatohepatitis

Because liver injury in NASH is associated with increased hepatocyte apoptosis [10], we performed terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) assays to evaluate apoptosis in mice with nutritional steatohepatitis. The percentage of TUNEL-positive apoptotic hepatocytes was significantly larger in the group fed the MCD diet than in the group fed the control diet, while p53 deficiency significantly ameliorated this effect (Fig. 1H and I).

Fig. 1. Effect of p53 deficiency on nutritional steatohepatitis, ROS accumulation, and hepatocyte injury, induced by MCD dietary feeding. (A) Effect of hepatic p53 deficiency on nutritional steatohepatitis. Haematoxylin-eosin (H&E)-stained and Masson trichrome-stained sections of liver samples are representative of the indicated groups, which received the MCD or control diet for 8 weeks (magnification, 100×). (B) Serum ALT activity in the indicated groups. (C) The percentage of hepatocytes involved in steatosis (left) and hepatic TG levels (right). (D) Quantification of Masson trichrome staining (left) and liver hydroxyproline concentrations (right). Values are means ± SEM (n = 6–9 mice/group). KO, p53-deficient mice; WT, wild type mice. *p <0.05 compared with wild type mice fed the control diet. **p <0.05 compared with wild type mice fed the MCD diet. (E) Real-time PCR analysis was used to quantitate hepatic mRNA levels of *Col1α1*, *Col1α2*, *TGF-β*, and *α-SMA* (n = 6–9 mice/group). *p <0.05 compared with wild type mice fed the control diet. **p <0.05 compared with wild type mice fed the MCD diet. (F) The effect of p53 deficiency on 4-HNE expression. Representative 4-HNE-stained sections (left). Quantification of 4-HNE staining (right). (G) Liver MDA levels in the indicated groups. Values are means ± SEM (n = 5 mice/group). *p <0.05 compared with wild type mice fed the control diet. **p <0.05 compared with wild type mice fed the MCD diet. (H) The effect of p53 deficiency on hepatocyte apoptosis. Representative TUNEL-stained sections. (I) Percentages of TUNEL-positive hepatocytes (n = 6–8 mice/group). *p <0.05 compared with wild type mice fed the control diet, and **p <0.05 compared with wild type mice fed the MCD diet. (This figures appears in color on the web).

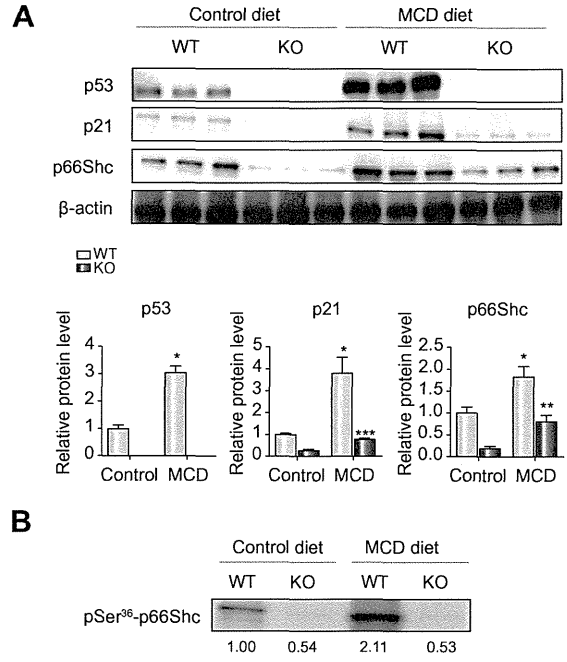

Fig. 2. p53 deficiency suppresses the enhancement of hepatic p66Shc signaling in nutritional steatohepatitis. (A) Western blot analysis of hepatic p53, p21, and p66Shc protein levels (upper panels), and quantification of the expression levels (lower panels) in the indicated groups, which were fed the MCD or control diet for 8 weeks. $^{*}p$ <0.05 compared with wild type mice fed the control diet, $^{**}p$ <0.05 compared with wild type mice fed the MCD diet, and $^{***}p$ <0.05 compared with the other groups. (B) Immunoprecipitation analysis of hepatic p66Shc phosphorylated at Ser^{36} in the indicated groups. Relative protein levels were used to quantify changes relative to results from wild type mice fed the control diet, and are shown below each blot. KO, p53-deficient mice; WT, wild type mice.

p53 deficiency suppresses the enhancement of hepatic p66Shc signaling in nutritional steatohepatitis

We next examined hepatic p53 signaling in mice with nutritional steatohepatitis. Hepatic protein expression levels of p53 and p21 (a protein downstream of p53) significantly increased in mice with nutritional steatohepatitis compared with mice fed the control diet (Fig. 2A). Because p66Shc is associated with aging and oxidative stress [13] and functions downstream of p53 [14], we next examined hepatic p66Shc signaling. The levels of total p66Shc and p66Shc phosphorylated at Ser 36 in the liver were significantly enhanced in the mouse NASH model (Fig. 2A and B). After 8 weeks of MCD diet, a lack of p53 reduced the enhanced expression to the levels observed in wild type mice fed the control diet or lower (Fig. 2A and B). These results suggest that hepatic p21 and p66Shc signaling in the mouse model of NASH is mainly regulated by p53 signaling.

Deficiency of p53 reverses TGF-β-induced enhancement of p66Shc signaling and suppresses TGF-β-induced ROS accumulation in hepatocytes

Hepatic levels of p53 and p66Shc proteins in the MCD diet-fed mice significantly increased 3 weeks after treatment, and continued to rise until 8 weeks after treatment (Supplementary Fig. 1A). At the same time, hepatic TGF-β levels rose until 8 weeks after treatment in the MCD diet-fed mice compared with those in mice fed the control diet (Supplementary Fig. 1B). Lipid peroxidation was also significantly enhanced in the liver of mice with nutritional steatohepatitis after 8 weeks of MCD diet (Supplementary Fig. 1B). Besides TGF-β and ROS, TNFα is reportedly involved in the pathogenesis of NASH [15,16]; however, hepatic TNFα expression in mice fed the MCD diet peaked 3 weeks after treatment, and then decreased until 8 weeks after treatment (Supplementary Fig. 1B).

Additionally, TGF-β significantly increased p53 protein levels in primary cultured hepatocytes (Fig. 3A and B), whereas treatment with H_2O_2 or TNFα did not produce notable effects (Supplementary Fig. 1C and D). These results suggest that TGF-β contributes to the upregulation of p53 signaling in MCD diet-induced steatohepatitis. Furthermore, previous reports have shown that ROS, TGF-β, and the feedback between these signals play key roles in the pathogenesis of NASH [11,12]. Therefore, we detailed the relationships among TGF-β, ROS, and p53 and p66Shc signaling in hepatocytes.

TGF-β significantly enhanced the levels of p21, p66Shc, and p66Shc phosphorylated at Ser36 in hepatocytes (Fig. 3A and B). p53 deficiency or inactivation of p53 with the reversible p53 inhibitor pifithrin (PFT)-α reversed the TGF-β-induced changes in the levels of these proteins in hepatocytes (Fig. 3A and B). We also confirmed that PFT-α significantly lowered nuclear levels of p53, resulting in decreased p53 signaling (Fig. 3B).

Measures of protein carbonyls showed that TGF-β significantly increased ROS levels in primary cultured hepatocytes, whereas p53 deficiency prevented this effect (Fig. 3C). Inactivation of p53 with PFT-α also significantly suppressed TGF-β-induced ROS accumulation in hepatocytes (Fig. 3C). Similarly, quantification of MDA levels in hepatocyte cultures showed that blocking p53 signaling significantly decreased TGF-β-induced lipid peroxidation in hepatocytes (Fig. 3D).

p53 deficiency inhibits TGF-β-induced apoptosis in hepatocytes

Because TGF-β induces hepatocyte apoptosis via ROS generation [17,18], we performed *in vitro* TUNEL analysis using an enzyme-linked immunosorbent assay kit. The present result shows that TGF-β enhances apoptosis in primary cultured hepatocytes, while p53 deficiency or co-treatment with PFT-α ameliorates this effect (Fig. 3E and F). TGF-β treatment also significantly enhances caspase-3 activity in primary cultured hepatocytes. Deficiency or inactivation of p53 signaling results in significant inhibition of TGF-β-induced caspase-3 activity (Fig. 3E and F).

Increased hepatic expression of p53 and p21 in patients with NASH correlates with increased p66Shc expression

We next examined hepatic p53 and p66Shc signaling in human NASH. Using mRNA samples and paraffin-embedded tissue sections prepared from human liver biopsies performed to diagnose non-alcoholic fatty liver (NAFLD) or human non-tumorous normal liver samples obtained during surgery for colorectal liver metastases, we examined hepatic *p21* and *p66Shc* mRNA levels by real-time PCRs and hepatic p53 and p66Shc protein levels by immunohistochemistry. The mRNA expression levels of *p21* and *p66Shc* (Fig. 4A), and the protein expression levels of p53 and p66Shc (Fig. 4B and C) were significantly elevated in NAFLD liver

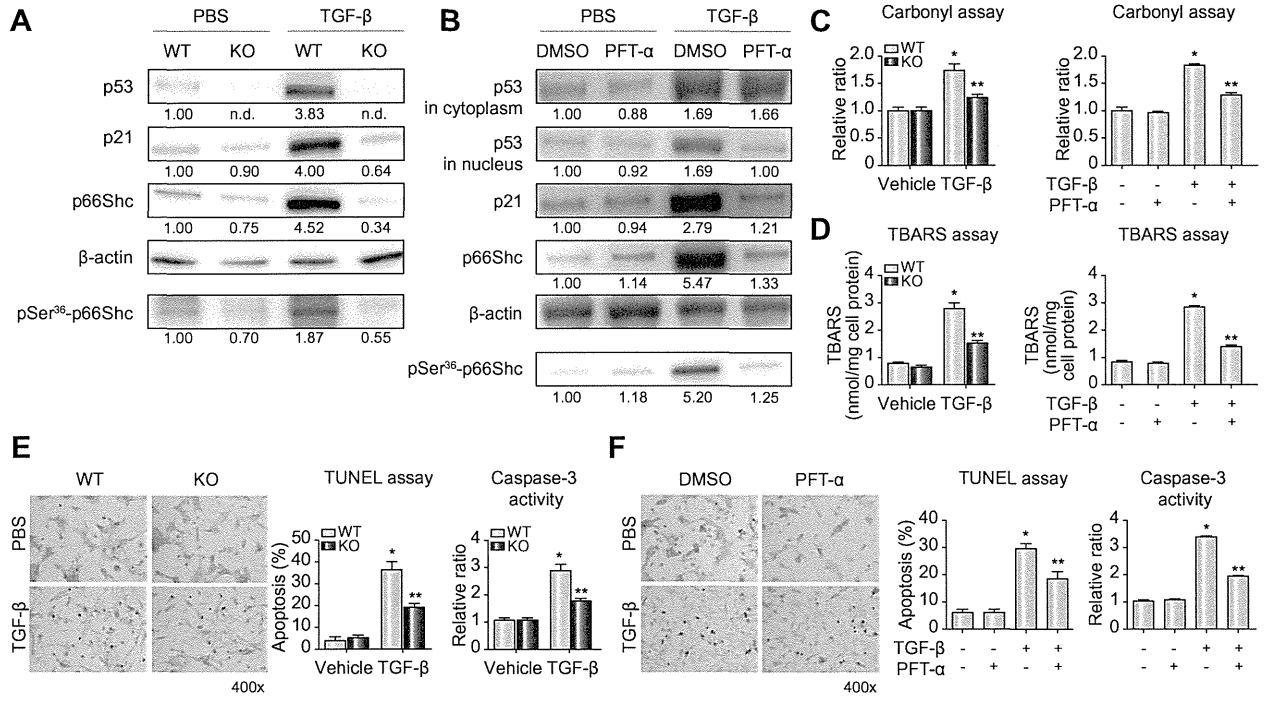

Fig. 3. Interrupting p53 signaling inhibits TGF-β-induced p66Shc signaling, ROS accumulation, and hepatocyte apoptosis. We examined hepatocyte cultures as previously described [12,17,18]. Protein levels of p53, p21, p66Shc, and phosphorylated p66Shc at Ser 36 (A) in primary cultured wild type (WT) or p53-deficient (KO) hepatocytes, exposed to TGF-β or not, and (B) in primary cultured WT hepatocytes, exposed to TGF-β with or without PFT-α. Relative protein levels (other than p66Shc phosphorylated at Ser 36, all protein levels were normalized to β-actin levels) were used to quantify changes relative to results from WT hepatocytes not treated with TGF-β or PFT-α, and are shown below each blot. Protein carbonyl levels (C) and MDA levels (D) in primary cultured WT or KO hepatocytes with or without TGF-β treatment (left panel), and in primary cultured WT hepatocytes exposed to TGF-β with or without PFT-α (right panel). The levels of protein carbonyls were normalized to results from WT hepatocytes without TGF-β or PFT-α. Means ± SEM from four individual experiments are shown. *p <0.05 compared with control levels. **p <0.05 compared with levels in WT hepatocytes after incubation with TGF-β but not PFT-α. (E, F) Representative images of TUNEL assays, apoptotic index values, and caspase-3 activity were obtained (E) for primary cultured WT or KO hepatocytes with or without TGF-β treatment, and (F) for primary cultured WT hepatocytes exposed to TGF-β with or without PFT-α. Caspase-3 activity was normalized to results from WT hepatocytes cultures without TGF-β or PFT-α. Means ± SEM from four individual experiments are shown. *p <0.05 compared with control group. **p <0.05 compared with results from WT hepatocytes after incubation with TGF-β but not PFT-α. (This figures appears in color on the web).

samples, in comparison with those in normal liver samples. Among NAFLD patients, the NASH group showed significantly higher hepatic mRNA expression levels of *p21* and *p66Shc* (Fig. 4D), and significantly higher hepatic p53 and p66Shc protein levels (Fig. 4E) compared with the simple steatosis group. When we analyzed the relationship between hepatic expression levels of p53, p21, and p66Shc and the histological stage of fibrosis in NAFLD patients, we observed a stepwise increase with the increasing severity of hepatic fibrosis ($p = 0.0014$ for *p21* mRNA, $p = 0.0255$ for *p66Shc* mRNA, $p = 0.0005$ for p53 protein, $p < 0.0001$ for p66Shc protein by Kruskal–Wallis test) (Fig. 4F). There were significant differences in hepatic mRNA expression of *p21* between F0 and F1, F0 and F2, F0 and F3 ($p = 0.0191$, 0.0016, and 0.0005, respectively) (Fig. 4F). There were also significant differences in hepatic mRNA expression of *p66Shc* between F0 and F1, F0 and F2, F0 and F3 ($p = 0.0043$, 0.0102, and 0.0077, respectively) (Fig. 4F). Furthermore, we detected significant differences in hepatic p53 protein levels between F0 and F3 samples, and between F1–2 and F3 samples ($p = 0.0007$ and 0.0010, respectively). We also detected significant differences in hepatic protein expression of p66Shc between F0 and F1–2, F0 and F3, F1–2 and F3 samples ($p = 0.0018$, 0.0008, and 0.0010, respectively) (Fig. 4F). In addition, we detected significant correlations between *p21* and *p66Shc* mRNA levels, and between p53 and p66Shc protein levels (Fig. 4G).

Discussion

The tumor suppressor p53 mediates responses to stress and induces the expression of proteins involved in cell cycle arrest or apoptosis. Recent studies have shown that, in addition to controlling cell-fate decisions and suppressing tumor development, p53 contributes to implantation, metabolism, and aging [5]. Some reports have examined the roles of p53 in liver diseases. For instance, inhibition of p53-dependent apoptosis reduced LPS-induced liver injury [19]. A recent report showed that p53 activation correlated with susceptibility to ethanol-induced liver damage, a pathologic condition that is thought to mechanistically resemble NASH [20]. In addition, hepatic p53 expression and hepatocyte apoptosis increase in patients with NASH and a mouse model of NASH [7,8]. These results suggest that p53 plays a role in the pathophysiology of NASH, although the precise contributions have not been fully elucidated.

In the present study, we examine p53 signaling in a mouse model of NASH induced by an MCD diet. In the nutritional

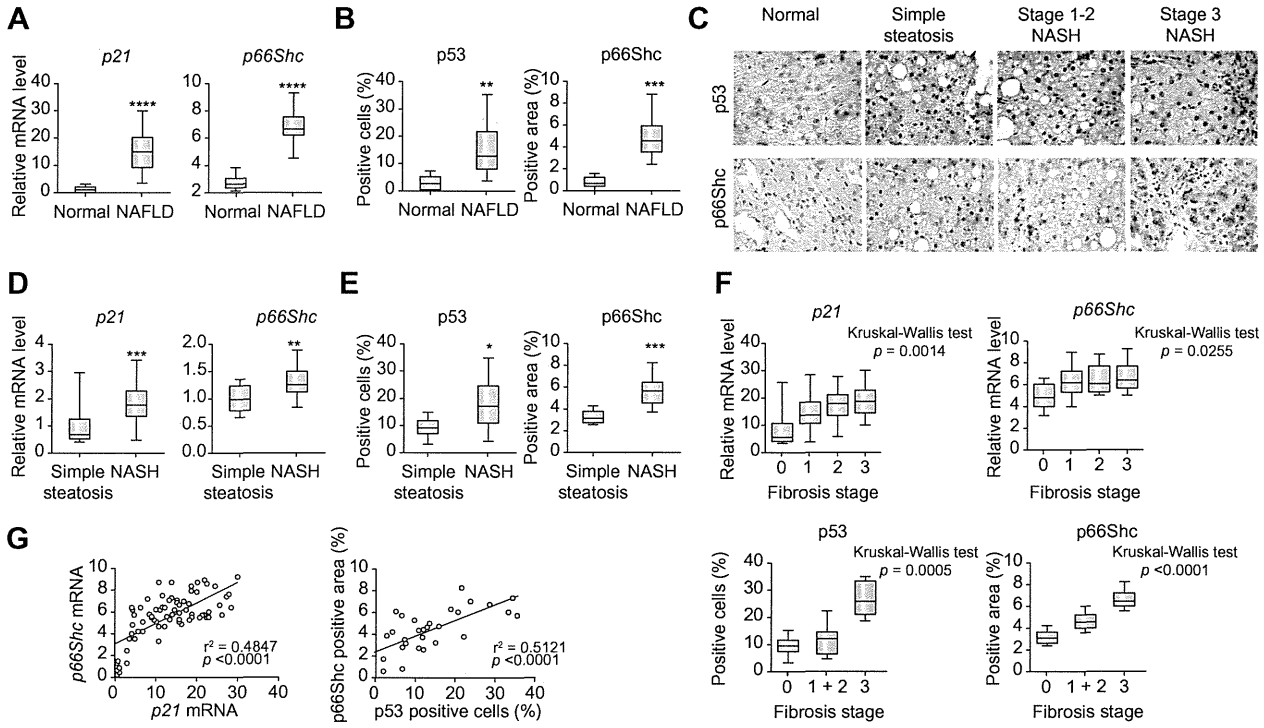

Fig. 4. Increased hepatic expression of p53 and p21 in patients with NASH correlates with increased p66Shc expression. (A) Hepatic mRNA levels of *p21* and *p66Shc* in 70 patients with NAFLD and 10 control subjects. ****p <0.0001 compared with the control group. (B) Hepatic p53 and p66Shc protein levels were immunohistochemically quantified in a subset of patients, including 5 control subjects, 8 patients with simple steatosis, 10 patients with stage 1–2 NASH, and 8 patients with stage 3 NASH. **p <0.01 and ***p <0.001 compared with the control group. (C) Immunohistochemically stained liver samples are representative of the indicated groups (400×). (D) Hepatic mRNA levels of *p21* and *p66Shc* in simple steatosis patients and NASH patients. **p <0.01 and ***p <0.001 compared with the simple steatosis group. (E) Hepatic protein levels of p53 and p66Shc in simple steatosis patients and NASH patients. *p <0.05 and ***p <0.001 compared with the simple steatosis group. (F) Hepatic *p21* and *p66Shc* mRNA levels, and hepatic p53 and p66Shc protein levels of each stage of liver fibrosis in NAFLD patients. Box plots demonstrate the interquartile range (box) as well as median and range. (G) Analysis of correlations between *p21* and *p66Shc* mRNA levels, and between p53 and p66Shc protein levels. (This figures appears in color on the web).

steatohepatitis, hepatic p53 signaling is enhanced and inhibition of p53 expression significantly ameliorates the pathologic manifestations of NASH. We use p53-deficient mice and PFT-α to analyze the roles of p53 in NASH. PFT-α selectively inhibits p53 transcriptional activity in various mouse cell lines, and prevents DNA damage-induced apoptosis in these cells [21].

ROS and TGF-β signaling in the liver are thought to play key roles in the pathogenesis of non-alcoholic steatohepatitis [11]. Our previous report also showed that ROS production and TGF-β expression in the mouse liver were significantly enhanced at every stage of nutritional steatohepatitis [12]. In addition, our present results show that TGF-β could play a central role in the upregulation of p53 signaling pathway in a mouse model of NASH, while p53 deficiency significantly inhibits ROS accumulation and reduces TGF-β expression in this NASH model. ROS in the liver activate hepatic non-parenchymal cells, including hepatic stellate cells, Kupffer cells, and endothelial cells [22]. Activated non-parenchymal cells release TGF-β, a profibrotic factor that has been implicated in autocrine or paracrine activation of hepatic stellate cells [23,24]. Hepatic stellate cells contribute to liver fibrosis; activated by stimuli as ROS and TGF-β, these cells produce collagen and additional TGF-β, leading to further activation of hepatic stellate cells via paracrine or autocrine mechanisms [25].

In a mouse model of NASH, hepatic p53 and p66Shc signaling was enhanced, and p53 deficiency suppressed the increase in p66Shc signaling in the liver. A previous study reported that p66Shc contributed to the regulation of cellular ROS levels and apoptosis as a downstream of p53 [14]. In addition, our *in vitro* experiments showed that TGF-β administration enhanced ROS levels and p66Shc signaling in hepatocytes. We also demonstrated that interrupting p53 signaling inhibited p66Shc activity and suppressed TGF-β-induced ROS accumulation in hepatocytes. Therefore, we hypothesized that ROS accumulation in hepatocytes was regulated by p53/p66Shc signaling to play a key role in NASH pathogenesis.

In the present study, we also showed that p53 deficiency ameliorated hepatocyte apoptosis in a mouse model of NASH. *In vitro* analysis also demonstrated that p53 deficiency inhibited TGF-β-induced apoptosis in primary cultured hepatocytes. These results implicate TGF-β-induced, p53-dependent apoptosis in the pathology of nutritional steatohepatitis.

In recent years, the signaling adapter protein p66Shc has received significant attention as a major determinant of cell resistance to oxidative stress and oxidant-induced cell damage and death [13,26]. p66Shc was also shown to play a pivotal role in impaired liver regeneration in older mice by inducing oxidative stress and apoptosis immediately after hepatectomy [13]. Our study showed that TGF-β enhanced p66Shc activity in hepato-

cytes via activation of p53 signaling. Taken together, these results suggest that TGF-β-induced hepatocyte injury in NASH may result from a cycle of p53 activation, enhanced p66Shc activities, and ROS accumulation, leading to apoptosis. In addition, our analysis of human samples demonstrated that enhanced p53/p66Shc signaling plays an important role in the progression of human NAFLD.

A previous study reported that IL-6/STAT3 pathway was positively correlated with hepatic expression of p21 [27]. In addition, TGF-β induced *p21* promoter activation in human hepatoma cells [28]. These reports suggest the possibility that the significant correlation between hepatic expression levels of p21 and p66Shc in our human samples might be in part due to increased expression of IL-6 and TGF-β during hepatic inflammation. Further studies using human samples are needed to fully elucidate the mechanisms.

In summary, we demonstrated that disrupted p53 signaling in hepatocytes ameliorated the progression of nutritional steatohepatitis. p53 signaling plays a pivotal role in the pathology-regulating mechanism, which is initiated by ROS that accumulate due to exaggeration of p66Shc activity in hepatocytes. Because p53 may regulate the susceptibility to NASH, future genetic and proteomic analyses may provide important insights.

There are no established therapeutic strategies for NASH, and effective treatments are urgently needed. Suppression of the p53/p66Shc signaling in the liver provides a promising target for the treatment of NASH.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to declare regarding funding from industry or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.05. 013.

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