Table 5
Differential distribution of IL-1B genotypes in HBV carriers

Locus	Genotype	Non-LC ($n = 129$)	LC ($n = 108$)	OR (95% CI)	*P*
IL-1B −31	T/C, T/T	93 (72.1%)	93 (86.1%)	2.40 (1.23–4.68)	0.0089
	C/C	36 (27.9%)	15 (13.9%)		

IL-1B −31 T carrier (*IL-1B* −31 T/T or T/C) was significantly higher in HBV carriers with LC compared to those without LC (LC; 86.1% vs non-LC; 72.1%, $P = 0.009$). When the LC patients were divided using Child–Pugh classification, there was no significant difference in *IL-1B* gene genotype frequencies between LC patients with Child–Pugh classification A and Child–Pugh classification B or C (Table 6A). In *IL-1RN* genotypes, *IL-1RN* 1/1 genotype was the most common genotype and 1/2 genotype was increased in LC patients with Child–Pugh classification A. However, there was no significant difference in genotype of *IL-1RN* among these LC patient groups (Table 6B). Furthermore, we examined the relationship between *IL-1B* gene genotype and the stage of hepatic fibrosis (Table 7). The frequency of the C/C genotype was reduced in patients with fibrosis stage F3–F4 (11.8%) compared to those with F0–F2 (28.6%), however, the number of patients who had undergone liver biopsy was limited and statistically significant difference was observed.

4. Discussion

Liver fibrogenesis is initiated by hepatocyte damage and the subsequent recruitment and activation of inflammatory cells [19]. These inflammatory cells produce fibrogenic cytokines and growth factors that activate hepatic satellite cells (HSC) [20]. The role of cytokine gene polymorphism in the progression of liver fibrosis or development of cirrhosis in patients with chronic liver diseases has been investigated extensively. Yee et al. indicated that TNF2 allele (−238A) and TNF3 allele (−308A) are more frequently found in patients with cirrhosis in chronic HCV infection [21]. Polymorphisms of TGF-β gene are thought to be one of the determinants of fibrosis progression in viral hepatitis [11]. Therefore, cytokine polymorphism could be involved in fibrosis progression in HBV infection. Recent studies have indicated that HSC play an important role in hepatic fibrogenesis and that IL-1 is a potent cytokine that induces the myofibroblastic activation of HSC [3]. Our data indicate that the frequency *IL-1B* −31 T carrier (*IL-1B* −31 T/T or *IL1B* −31 T/C) was significantly higher in HBV carriers with LC compared to in those without LC. These results suggest that the *IL-1B* genotype may influence fibrotic progression in HBV-related hepatitis. On the other hand, the frequency of the *IL-1RN* (intron 2, VNTR)* A2 allele was extremely uncommon in the study subjects and was not significantly different between HBV carriers with or without liver cirrhosis.

In our study, *IL-1B* −511 was in a complete linkage disequilibrium (LD) with IL-1B −31 in the Japanese population. Therefore, the effect of the *IL-1B* −511 C/T may be due to LD with *IL-1B* −31 T/C. There are some confusions regarding the 1*L-1B* (−31) alleles in earlier literatures. El-Omar et al. reported that −31C alleles increased the risk of gastric cancer [5,6]. They considered the *IL-1B* −31C allele but not the T allele as a pro-inflammatory gene. The *IL-1B* −31 T/C polymorphism is situated on a TATA box in the promoter region. However, the mutation of T to C in the TATA box in the *IL-1B* gene promoter (−31) will result in down regulation of the *IL-1B* gene in electrophoretic mobility-shift assay [5,6]. Xuan et al. found that *IL-1B* polymorphisms (*IL-1B* −511 C/C and −31T/T) enhanced IL-1β production in the gastric body of Japanese patients [22]. Similarly, the *IL-1B* −31 T/T genotype has been shown to be associated with an increased risk for HCC in Japanese patients with HCV infection [23]. Therefore, it is possible that the *IL-1B* −31T/T allele could be implicated in inflammatory processes. Our finding that HBV carriers harboring an *IL-1B* −31C/C genotype were less frequent in LC patients

Table 6A
Distribution of IL-1B genotype and Child–Pugh classification in LC patients

Locus	Genotype	Child–Pugh classification		
		A ($n = 79$) (%)	B ($n = 20$) (%)	C ($n = 9$) (%)
IL-1B −31	C/C	10 (12.7)	4 (20.0)	1 (11.1)
	C/T	44 (55.7)	12 (60.0)	5 (55.6)
	T/T	25 (31.6)	4 (20.0)	3 (33.3)

Table 6B
Distribution of IL-1RN genotype and Child–Pugh classification in LC patients

Variables	Child–Pugh classification		
	A ($n = 79$) (%)	B ($n = 20$) (%)	C ($n = 9$) (%)
IL-1RN			
1/1	72 (91.1)	19 (95.0)	8 (88.9)
1/2	6 (7.6)	0	0
1/3	0	0	0
1/4	1 (1.3)	1 (5.0)	0
2/2	0	0	1 (11.1)
2/4	0	0	0

Table 7
Distribution of IL-1B genotypes and METAVIR score in HBV carriers

Locus	Genotype	Stage of fibrosis		OR (95% CI)	*P*
		F0–F2 ($n = 28$) (%)	F3–F4 ($n = 51$) (%)		
IL-1B −31	T/C, T/T	20 (71.4)	45 (88.2)	3.0 (0.92–9.8)	0.061
	C/C	8 (28.6)	6 (11.8)		

than non-LC patients is in accord with these findings. IL-1 is a proinflammatory cytokine which is involved in the fibrotic response. IL-1 causes tissue injury, which induces the fibrotic response, by producing chemotactic molecules, such as chemokines [24]. IL-1 is also implicated in the proliferation of HSC [25] and the regulation of the expression of various matrix metalloproteinases, which play a key role in the turnover and the deposition of extracellular matrix (ECM) [3]. Therefore, it is possible that genetic polymorphism of *IL-1B* gene may influence the progression of hepatic fibrosis by affecting the hepatic expression of IL-1 during the process of liver injury. Since this is the first report of the association between *IL-1B* polymorphism and hepatic fibrosis in HBV carriers, further investigation is required to confirm and extend our findings.

Several studies reported that *IL-1B* −31T is a risk haplotype for the development of cancer. Hirankarn et al. reported that IL-1B- 511C (−31T) allele is a genetic marker for the development of HCC in chronic hepatitis B patients in Thai population [26]. More recently, Chen et al. reported that in the presence of the *IL-1RN**2 allele, a ~5-fold increased risk of HCC was found for HBV carriers harboring the *IL-1B* −31 T/T or IL-1B −511 C/C genotype compared with those harboring the IL-1B −31 C/C or *IL-1B* −511 T/T genotype [27]. The frequencies of the *IL-1B* −31 genotype between HCC and non-HCC patients were insignificant in our study. However, our study included only 49 HCC patients and could not assess the interaction between polymorphisms of the *IL-1RN* and *IL-1B* genes due to the infrequency of the *IL-1RA**2 allele among Japanese subjects. Further investigations using large-scale case-control studies are needed to elucidate the relationship between the HCC risk and the *IL-1B* gene polymorphism.

Takamatsu et al. reported that the presence of the *IL-1B* −31C/C genotype was found at a significantly higher frequency in patients with liver cirrhosis than in those without cirrhotic alcoholic liver disease [28]. The reason for this discrepancy between our data and those of this previous report is unclear, although one possible explanation is a difference of the pathogenesis of liver cirrhosis.

In summary, the findings of the present study suggest that polymorphism in the promoter region of the *IL-1B* gene (−31) is implicated in the regulation of liver fibrosis in patients with HBV infection. The interactions between HBV viral factors and host factors including cytokine polymorphisms may contribute to disease progression in HVB carriers. The interaction between *IL-1B* polymorphisms and liver fibrogenesis deserves further study.

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Clinical Studies

Sustained viral response prolonged survival of patients with C-viral hepatocellular carcinoma

Akamatsu M, Yoshida H, Shiina S, Teratani T, Obi S, Tateishi R, Mine N, Kondo Y, Kawabe T, Omata M. Sustained viral response prolonged survival of patients with C-viral hepatocellular carcinoma.
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Abstract: *Background:* We conducted this retrospective study to evaluate the position of interferon therapy in the curative treatment of hepatitis C virus-associated hepatocellular carcinoma (HCC). *Methods:* We compared overall and recurrence-free survival rates between 191 patients who received interferon therapy before HCC development (15 with sustained virologic response (SVR)), 53 who received interferon therapy after HCC ablation (17 with SVR), and 399 HCC patients with Child–Pugh class A liver function who did not receive interferon (controls). *Results:* The overall survival rate in the controls was 82.4%, 53.2%, and 28.3% at 3, 6, and 9 years, respectively, whereas that in patients who developed HCC after achieving SVR was 93.3%, 93.3%, and 93.3%; those with HCC after non-SVR, 87.8%, 56.5%, and 35.8%; SVR after HCC, 100%, 87.5%, and 59.7%; and non-SVR after HCC, 94.3%, 70.9%, and 53.2%. Cox proportional hazard regression analysis revealed that the risk of death was significantly reduced in patients with HCC after SVR and those with SVR after HCC, with a risk ratio of 0.124 (95% confidence interval (95% CI): 0.017–0.890, $P = 0.0378$) and 0.388 (95% CI: 0.169–0.887, $P = 0.0250$), respectively, compared with the controls. Improved survival was attributable mainly to sustained liver function among patients with SVR, and recurrence-free survival did not differ significantly. *Conclusion:* Interferon therapies before and after HCC development were both significantly associated with prolonged survival when SVR was achieved.

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Key words: hepatitis C virus – hepatocellular carcinoma – interferon – recurrence – survival

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Although the incidence of hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) is possibly declining owing to the introduction of neonatal vaccination, that of hepatitis C virus (HCV)-associated HCC is increasing in several industrialized countries where HCV infection spread recently, mainly due to blood transfusion or injecting drug abuse (1–4). The incidence of HCV-associated HCC is estimated to have been more than tripled in Japan since 1970s. Several groups demonstrated that interferon therapy against chronic hepatitis C reduces the risk of HCC development, down to one-fifth in sustained virologic responders (SVR) in particular (5–8). It is estimated that more than one-tenth of patients with chronic hepatitis C in Japan have already received interferon therapy. Nevertheless, HCV-associated HCC still claims about 30 000 victims each year (9).

Advances in medical and surgical treatments have substantially prolonged survival of HCC patients (10). Nevertheless, most patients with HCC eventually die from hepatic causes, such as recurrence of HCC and liver failure. This is hardly surprising since the remaining liver, often already complicated with cirrhosis, continues to suffer from viral hepatitis, remaining at high risk of development of HCC de novo and of further deterioration of liver function. However, HCC developed in interferon responders may take a different course as there is little inflammation in the liver. Similarly, interferon therapy after HCC treatment may also be beneficial. We previously conducted a randomized controlled trial

on interferon therapy after curative percutaneous ethanol injection therapy (PEIT) for HCV-associated HCC. That study showed significant improvement in survival in the treated group, although the study was limited by low efficacy of conventional interferon monotherapy only available then (11).

The combination with polyethylene glycol (PEG)-interferon and ribavirin, currently the treatment of choice, is more effective in treating chronic hepatitis C than conventional interferon monotherapy (12–14), and would be also more potent when used after treating HCC. However, it is not clear whether the antiviral therapy after HCC development is as good, in terms of survival and recurrence, as that before HCC development. Although incidence is reduced, HCC does develop among SVR to interferon therapy. However, their prognosis will not be affected by active hepatitis. If the antiviral therapy after HCC treatment does not provide comparable prognosis, the therapy should be oriented primarily to chronic hepatitis C patients before HCC development. In this retrospective study, we compared the prognosis of HCC patients who had a previous history of interferon therapy, those who received interferon therapy after HCC treatment, and the controls who were not treated with interferon to evaluate the clinical relevance of antiviral therapy after the treatment of HCV-related HCC.

Materials and methods

Patients

Between January 1993 and February 2004, a total of 1306 patients with HCC positive for HCV antibody were admitted to the authors' institution, excluding those with a history of heavy alcohol consumption (>80 g/day) or other liver diseases such as hepatitis B virus infection, autoimmune hepatitis, or primary biliary cirrhosis. Among them, 1020 patients were curatively treated, as confirmed by subsequent imaging, with percutaneous tumor ablation (PTA) or surgical resection (Table 1), and they constituted the subjects of this study. Among them, 191 had a previous history of receiving interferon therapy: 15 had attained SVR, as defined by undetectable HCV-RNA for more than 6 months after the cessation of interferon administration, and 176 had not. Anti-HCV antibody was determined by passive hemagglutination test (Abbott Japan, Tokyo, Japan) or enzyme linked immunosorbent assay (Ortho Diagnostic Systems, Tokyo, Japan). HCV-RNA was identified by reverse transcriptase -polymerase chain reaction (RT-PCR) according to the method of Kato and colleagues (15–17); HCV genotype, by the method of Okamoto et al. (18).

HCC was treated with surgical resection or PTA, that is, PEIT, percutaneous microwave coagulation therapy (PMCT), or radiofrequency ablation (RFA). The eligibility criteria for surgical resection were described elsewhere (19). The extent of resection was determined by the distribution of nodules, and the liver function reserve should match the extent of resection. PTA was contraindicated by any of intractable ascites, portal venous tumor invasion, and extrahepatic metastases. Platelet count should be greater than 50 000/μl, prothrombin time better than 50%, and the number of hepatic HCC nodules not greater than six. When a patient had indication for both surgery and PTA, the modality of treatment was determined by the patient's choice on fully informed consent. PEIT was performed

Table 1. Patients' characteristics

Group	A	B	C	D	E	
n	15	176	17	36	399	
IFN, Pre-HCC	SVR	Non-SVR	None	None	None	
IFN, Post-HCC	None	None	SVR	Non-SVR	None	*P* value
Age (years)	63 (53–71)	65 (37–83)	59 (57–68)	61 (37–75)	68 (41–86)	<0.0001
Gender (male:female)	13:2	118:58	13:4	25:11	271:128	0.5513
Tumor nodules (uni-/multinodular)	8:7	105:71	9:8	23:13	247:152	0.8730
Tumor size (mm)	22 (10–68)	21 (10–75)	21 (13–38)	21 (9–41)	24 (6–112)	0.1298
HCC treatment (PTA:SR)	(14:1)	(167:9)	(17:0)	(35:1)	(381:18)	0.8503
Child–Pugh class (A:B:C)	13:2:0	111:51:14	13:4:0	20:12:4	399:0:0	<0.0001
Total bilirubin (mg/dl)	0.8 (0.3–1.9)	0.9 (0.4–2.6)	0.9 (0.3–1.5)	0.8 (0.3–1.9)	0.7 (0.3–2.4)	0.0003
Albumin (g/dl)	3.6 (2.9–4.6)	3.7 (2.0–4.9)	3.7 (3.0–4.4)	3.6 (2.9–4.6)	3.8 (2.8–4.8)	<0.0001
ALT level (IU/l)	94 (37–280)	68 (4–220)	103 (33–264)	94 (37–280)	65 (13–358)	<0.0001
Platelet count (×10^3/μl)	9.2 (4.3–22.1)	10.1 (3.6–46.3)	8.3 (4.6–14.4)	9.2 (4.3–22.1)	11.2 (2.9–47.4)	0.0016
AFP positive (≥100 ng)	6 (40%)	46 (26.1%)	5 (29.4%)	6 (16.7%)	77 (19.3%)	0.1555

IFN, interferon therapy; SVR, sustained virologic response; SR, surgical resection; PTA, percutaneous tumor ablation; ALT, alanine aminotransferase; AFP, α-fetoprotein. Median (range) were shown.

under ultrasound (US) guidance using a 21-gauge needle (15 cm long; Silux, Tokyo, Japan) (20). PMCT was carried out under US guidance using a 15 cm long guide needle (14 gauge) according to the procedure described previously (21). RFA was executed under US guidance using a 15 -cm long guide needle (16 gauge) (22). PTA was repeated once or twice a week until complete necrosis of all HCC lesions with a safety margin of at least 5 mm was confirmed by dynamic computed tomography. If neither surgery nor PTA were indicated, transcatheter arterial chemoembolization therapy (TACE) was considered, although TACE was not regarded as curative treatment even when successfully performed.

After the completion of the initial treatment, interferon therapy was given to 53 patients with an interval of 2–3 months. These patients included 49 subjects of our previous controlled study. All patients who received post-HCC interferon therapy had had the primary tumor completely ablated, liver function in Child–Pugh class A, and a platelet count greater than 50×10^9 cells/l. Patients received 3–6 million units of interferon-α three times a week for 24–48 weeks. The virologic outcome was determined 6 months after the completion of interferon administration.

Observation

After the initial treatment for HCC was completed, each patient was regularly screened for HCC recurrence with abdominal ultrasound every 3 months, dynamic computed tomography every 4–6 months, and the evaluation of HCC tumor markers, α-fetoprotein (AFP), lectin fraction 3 of AFP (AFP-L3), and des-γ-carboxy prothrombin (DCP) every 1–2 months (23, 24). HCC recurrence was confirmed by magnetic resonance imaging and/or angiographic studies, and ultrasound-guided tumor biopsy was also performed when necessary (25). Patients with HCC recurrence received further treatment with PEIT, PMCT, or RFA when the indication criteria were still met: otherwise, TACE, systematic chemotherapy, or irradiation was performed as indicated.

Patients were divided into five groups. Patients in Group A ($n = 15$) and Group B ($n = 176$) had received interferon therapy before HCC development, with SVR (Group A) or without (Group B). Patients in Group C ($n = 17$) and Group D ($n = 36$) received interferon therapy after the completion of HCC treatment and achieved SVR (Group C) or did not (Group D). As the majority of patients in Groups A–D had liver function in Child–Pugh class A, the control group, Group E, were constituted of patients with Child–Pugh class A liver function who did not receive interferon therapy ($n = 399$) (Fig. 1). The end points in this study were HCC recurrence and survival.

Statistical procedures

Differences in frequency distribution were analyzed by χ^2 test, and continuous variables were compared with Kruskal–Wallis test. Cumulative incidence curves were plotted with the Kaplan–Meier method and the difference between the groups was assessed using the log-rank test. Survival time was defined as the interval between the end of the initial HCC treatment and the event, detection of recurrence or death. In the comparison of cumulative recurrence between Groups C and D (interferon therapy after HCC treatment), the recurrence-free survival time was counted from the commencement of interferon administration. We used the Cox's proportional hazard regression analysis in the survival time analysis to evaluate risk factors for HCC recurrence and overall survival. Survival time in Groups A–D were compared with that in Group E using dummy variables in Cox proportional hazard regression.

Results

Overall survival

Control patients in Group E, with Child–Pugh class A liver function and untreated with interferon, showed cumulative survival rates of 82.4%, 53.2%, and 28.3% at 3, 6, and 9 years, respectively. In contrast, patients in Group A, virologic responders to previous interferon therapy, showed a better survival, 93.3%, 93.3%, and 93.3% at the respective time point. The survival rates were 87.8%, 56.5%, and 35.8% in Group B; 100%, 87.5%, and 59.7% in Group C; and 94.3%, 70.9%, and 53.2% in Group D (Fig. 2).

Causes of deaths were summarized in Table 2. Tumor progression and liver failure were the main causes of death. In particular, liver failure was the cause of death in 12 cases (19.4%) in Group B, eight cases (40.0%) in Group D, and 28 cases (16.8%) in Group E, all of which were constituted of patients with continued HCV infection. In contrast, there were no deaths because of liver failure in Group A and Group C, the groups of patients who achieved SVR before or after HCC development.

As each group may have had different backgrounds, we confirmed the effect on survival in multivariate analysis adjusting for gender, age,

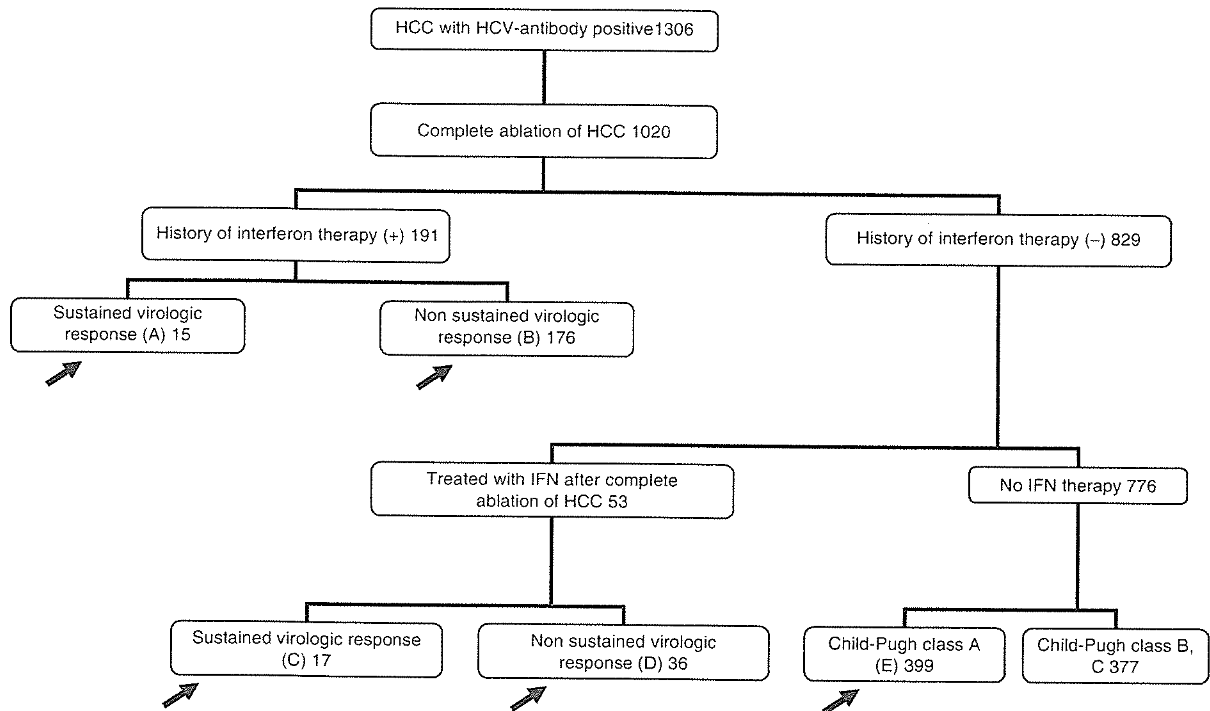

Fig. 1. Hepatitis C virus antibody-positive hepatocellular carcinoma patients admitted to our hospital. The five groups (arrowed) were included in this study.

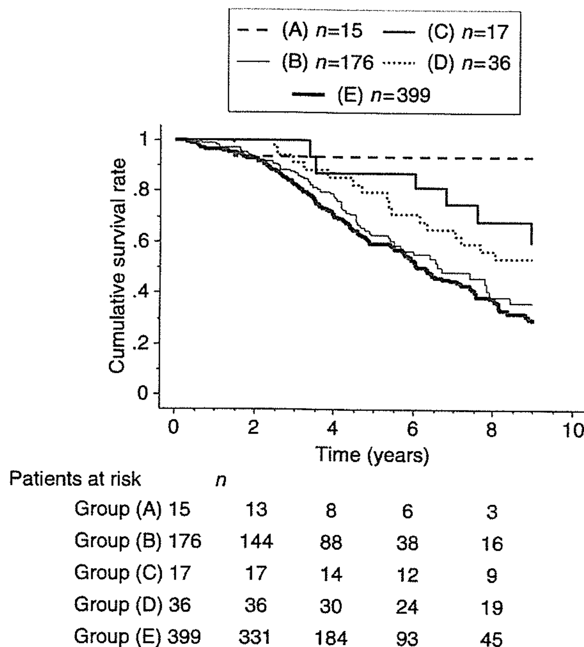

Fig. 2. Cumulative survival rates of hepatocellular carcinoma (HCC) patients. (A) Patients who had achieved sustained virologic response (SVR) before HCC development. (B) Patients who had received interferon therapy without achieving SVR before HCC development. (C) Patients who received interferon therapy after HCC treatment and achieved SVR. (D) Patients who received interferon therapy without achieving SVR after HCC treatment. (E) Patients with Child–Pugh class A liver function who did not receive interferon therapy (control group).

Table 2. Cause of death

Group (*n*)	Total death	Tumor progression (%)	Liver failure (%)	Variceal rupture (%)	Liver-unrelated death (%)
A (15)	1	1 (100)	0 (0)	0 (0)	0 (0)
B (176)	62	41 (66.1)	12 (19.4)	1 (1.6)	8 (12.9)
C (17)	8	7 (87.5)	0 (0)	0 (0)	1 (12.5)
D (36)	20	9 (45.0)	8 (40.0)	0 (0)	3 (15.0)
E (399)	167	100 (59.8)	28 (16.8)	9 (5.4)	30 (18.0)

total bilirubin concentration, tumor characteristics, and serum level of AFP (Table 3). Factors other than gender, age, and groups were chosen by step-wise selection. Groups A, C, and D showed significantly better survival with a relative risk of 0.124 (95% confidence interval (95% CI): 0.017–0.890, $P = 0.0378$), 0.388 (95% CI: 0.169–0.887, $P = 0.0250$) and 0.514 (95% CI: 0.293–0.900, $P = 0.0200$) compared with Group E.

HCC recurrence

Next, we examined the effect of interferon therapy before or after HCC treatment on recurrence. Crude analysis among these five groups did not show significant difference in recurrence. Then, we compared the recurrence rates between Group A and Group B, and between Group C and

Table 3. Predictors for overall survival

Variable	Risk ratio	95% confidence interval	P value
Gender (male vs. female)	1.314	1.314–1.748	0.0600
Age (by 1 year)	1.047	1.027–1.066	<0.0001
Total bilirubin	1.519	1.002–2.302	0.0489
Tumor criteria (advanced vs. early)	1.344	1.009–1.789	0.0431
AFP (≥100 ng/dl vs. below)	2.326	1.745–3.096	<0.0001
Groups			
E (baseline)	1		
A	0.124	0.017–0.890	0.0378
B	0.888	0.650–1.214	0.4576
C	0.388	0.169–0.887	0.0250
D	0.514	0.293–0.900	0.0200

AFP, α-fetoprotein; tumor criteria: early stage was defined as solitary; size ≤5 cm or number ≤3; size ≤3 cm. Risk ratios were calculated by multivariate Cox proportional hazard regression.

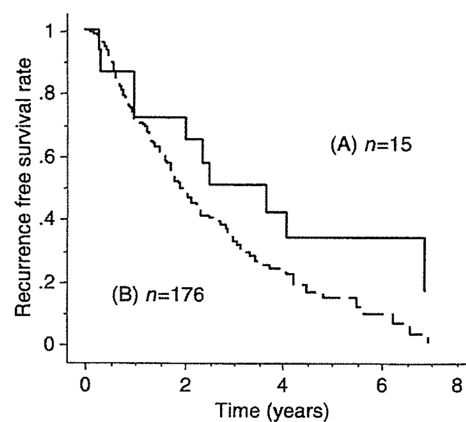

Fig. 3. Recurrence-free survival among patients with a previous history of interferon therapy. (A) Patients who had achieved sustained virologic response (SVR) before hepatocellular carcinoma (HCC) development and (B) Patients who had received interferon therapy without achieving SVR before HCC development. Recurrence-free survival was compared exclusively among those patients who attained complete ablation of the primary tumor. $P = 0.06$ by the log-rank test.

Group D separately. The recurrence-free survival appeared to be better in Group A than in Group B, but the difference did not attain statistical significance ($P = 0.06$) (Fig. 3). However, there was a trend of divergence between the two recurrence-free survival curves after the year-2.

The cumulative recurrence-free survival rates in Groups C and D were compared counting the time after the commencement of interferon therapy as patients in these groups were necessarily free of recurrence at that time point. As shown in Fig. 4, patients in Group C showed a better recurrence-free survival than those in Group D ($P = 0.04$). There was a trend of further divergence in later phase. The risk ratios for HCC recurrence as Group C (with SVR) to Group D (without SVR) were 0.595 in year-1 and 0.698 in year 2–3 but decreased to 0.451 in the later period, suggesting a decreasing trend of recurrence among the SVRs.

Discussion

The risk of HCC development among chronic hepatitis C patients differs widely according to the gender, age, fibrosis stages, and alcohol consumption. Considering the socioeconomic costs and the possibility of major untoward effects, interferon therapy may not be recommended to all patients with HCV infection but to selected ones who have a substantial risk of HCC development or liver failure (26). However, there is a small but not negligible possibility of HCC development among other patients. Interferon therapy after medical ablation (11) or surgical resection (27) of HCC was reported to improve survival. However, the prognosis of patients who achieved SVR after HCC treatment has not been compared with that of patients with HCC that developed after having achieved SVR. If there is substantial difference, antiviral therapy would be recommended preferentially before HCC development.

We previously reported that the risk of HCC was reduced to about one-fifth in chronic hepatitis C patients who achieved SVR by interferon therapy compared with those who did not (6). As the average SVR rate was about 30% with conventional interferon therapy of the 1990s Japan, the ratio of HCC patients who had achieved SVR to those who had failed to do so could be estimated to be 3:35, or about 1:10. In the current study, the observed ratio was 18:216 (patients with palliative treatment included), confirming that interferon therapy did reduce HCC development among patients with SVR. By the same reasoning, the proportion of patients who develop HCC after achieving SVR among patients with a past history of interferon therapy will relatively increase in future due to the improved SVR rate of recent treatment with pegylated interferon and ribavirin.

In addition to the prevention of HCC, interferon therapy can lead to the resolution of cirrhosis (28) and prevent death because of liver failure (29, 30) among chronic hepatitis C patients. These effects are to be expected also among patients who had developed and been treated for HCC, and, indeed, in the current

study we showed improved survival among patients who achieved SVR before (Group A) or after (Group C) HCC development, compared with the control group (Group E) (Fig. 2). Note that the control group was favorably biased in the survival analysis in that patients with Child–Pugh B/C liver function were excluded. Multivariate analysis confirmed that survival was significantly improved with a risk ratio of 0.124 in Group A and 0.388 in Group C (Table 3) compared with Group E. According to the risk ratios, the benefit of achieving SVR appears to be greater before HCC development than after it. Patients in Group D, who received interferon after HCC treatment but failed to achieve SVR, also showed improved survival compared with Group E, with a risk ratio of 0.514. Interferon therapy may have beneficial effects at least temporarily even if it eventually fails to achieve SVR.

In Groups A and C, those patients who achieved SVR before and after HCC development, respectively, tumor progression was the major cause of death and liver failure caused none. In contrast, liver failure caused 16.8% of total deaths in the control group although it was constituted exclusively of patients with Child–Pugh class A liver function at entry. Sustained liver function in patients with SVR may have contributed to better survival not only by suppressing death due to liver failure but also by keeping patients within the indication of treatment for recurrent HCC.

Interferon therapy prevents HCC development among chronic hepatitis C patients even with cirrhosis (6, 7). Thus, we expected that the rates of HCC recurrence would be reduced in patients with SVR. However, statistical analyses found no significant difference between Groups A and B, or between C and D. Nevertheless, the recurrence-free survival curves had a trend of divergence in the later period of observation (Figs 3 and 4). Recurrence of HCC has been theoretically divided into two categories, intrahepatic metastasis and multicentric carcinogenesis, and the former is thought to occur preferentially in the early period after treatment (31). The results of the current study may suggest that SVR achieved before or after HCC development may suppress multicentric carcinogenesis but does not affect already existing intrahepatic metastasis.

The current study also indicated that interferon therapy that failed to achieve SVR had little effect on prognosis, and that the primary purpose of interferon therapy should be SVR whenever possible. In this regard, the recently introduced combination therapy with PEG interferon and ribavirin is highly encouraging. We previously

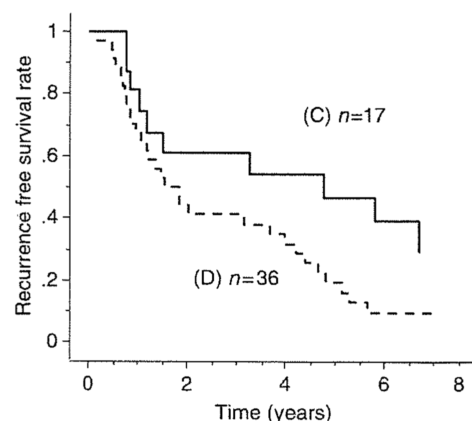

Fig. 4. Recurrence-free survival among patients who received interferon therapy after hepatocellular carcinoma (HCC) treatment. (C) Patients who received post-HCC interferon therapy and attained sustained virologic response (SVR) and (D) those who failed to attained SVR. Since all patients had no recurrence at the start of post-HCC interferon therapy, the survival time was measured from that time point. $P = 0.04$ by the log-rank test.

showed that HCV genotypes and virus load did not affect the incidence of recurrence after HCC treatment (32). However, they do affect the efficacy of interferon therapy, and the expectable benefit of interferon therapy is greater when SVR is more likely to be achieved, i.e., when the genotype is non-Ib and serum virus load is lower.

In conclusion, we showed that prognosis was significantly improved in patients who were treated with interferon before or after HCC development with SVR. Interferon therapy should be considered in chronic hepatitis C patients accompanied by HCC risk, and also in HCC patients after complete tumor removal.

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MDM2 Promoter SNP309 Is Associated with the Risk of Hepatocellular Carcinoma in Patients with Chronic Hepatitis C

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Abstract **Purpose:** A single nucleotide polymorphism (SNP) in the promoter region of *MDM2* gene, SNP309, has recently been shown to be associated with accelerated tumor formation in both hereditary and sporadic cancers in humans. However, the association of SNP309 with hepatocellular carcinoma is unknown. We evaluated the association of SNP309 with the risk of hepatocellular carcinoma development among Japanese patients with chronic hepatitis C virus infection.

Experimental Design: We genotyped the SNP309 at the *MDM2* promoter in 435 Japanese patients with chronic hepatitis C virus infection, including 187 patients with hepatocellular carcinoma and 48 healthy subjects, using a fluorogenic PCR. Presence of SNP was also confirmed by direct sequencing of the MDM2 promoter region.

Results: The proportion of G/G genotype of the SNP309 in patients with hepatocellular carcinoma (33%) was significantly higher than that in patients without hepatocellular carcinoma (23%), with an odds ratio (95% confidence interval) of 2.28 (1.30-3.98). A multivariate analysis revealed that *MDM2* SNP309 (G/G versus T/T), age >60 years, male gender, presence of cirrhosis, serum α-fetoprotein >20 μg/L, and serum albumin <3.2 g/dL were independently associated with the hepatocellular carcinoma development at odds ratio of 2.27, 2.46, 3.08, 4.15, 4.87, and 6.33, respectively.

Conclusions: The *MDM2* promoter SNP309 is associated with the presence of hepatocellular carcinoma in Japanese patients with chronic hepatitis C.

It has been estimated that hepatitis C virus (HCV) infects at least 170 million people worldwide,[1] which often leads to the dreadful sequels of cirrhosis, end-stage liver disease, and hepatocellular carcinoma (1-4). The risk of hepatocellular carcinoma development increases with the severity of inflammation and liver fibrosis (5-7). Several factors, such as alcohol intake, older age at time of infection, male gender, and coinfection with the hepatitis B virus or HIV, are known to accelerate disease progression in HCV infection (5, 7, 8). In addition, host genetic factors have been reported to affect the outcome of HCV infection (9-15).

Recently, we reported that genetic polymorphisms in *interleukin-1β* and *UDP glucuronosyltransferase 1A7* genes were associated with the development of hepatocellular carcinoma in Japanese patients with chronic HCV infection (13, 14), which was followed by similar reports by other groups as well (16). We further did a large-scale search of gene polymorphisms associated with the hepatocellular carcinoma development in a much larger population of HCV patients and found that three single nucleotide polymorphisms (SNP) in three genes (*SCYB14*, *GFRA1*, and *CRHR2*) were significantly associated with hepatocellular carcinoma development in Japanese patients with chronic HCV infection (15).

The association of both the germ-line and somatic inactivating mutations of the *p53* gene with increased tumor development is well known (17-20). Gene polymorphisms at critical nodes of the p53 pathways have also been reported to be associated with development of cancers (21-23). Recently, a SNP (SNP309, rs2279744) in the promoter region of *MDM2*, a negative regulator of p53, has been shown to be associated with accelerated tumor formation in both hereditary and sporadic cancers in humans (24). However, it is not known whether the SNP309 is associated with the development of hepatocellular carcinoma as well.

In the present study, we investigated the association of the *MDM2* promoter SNP309 with development of hepatocellular carcinoma among Japanese patients with chronic HCV infection.

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[1] http://www.who.int/inf-fs/en/fact164.html.

Patients and Methods

Patients. We studied 435 consecutive Japanese patients with chronic HCV infection who consulted the outpatient clinic of the University of Tokyo Hospital between August 2001 and June 2003 (239 men and 196 women; ages 22-84 years; median, age 62 years; 187 with hepatocellular carcinoma and 248 without hepatocellular carcinoma). To obtain an estimate of the genetic distribution of the SNP309 in the general Japanese population, we also obtained DNA samples from 48 healthy individuals who visited our hospital (41 men and 7 women; ages 23-53 years; median age, 34 years) with no history of liver diseases. The genomic DNA of these patients was made available after obtaining written informed consent for genotyping. Approval was obtained from the institutional ethics committee, and all the procedures followed institutional guidelines (25). Patients selected for the study were those who tested positive for HCV antibody by the second-generation enzyme immunoassay (Ortho Diagnostics, Tokyo, Japan), and HCV RNA was measured using the Amplicor HCV assay version 1 (Roche, Tokyo, Japan). All patients were negative for the hepatitis B surface antigen (Abbott Laboratories, North Chicago, IL). HCV genotypes were determined using a genotyping assay (SRL Laboratory Co., Tokyo, Japan). Any patients with an ethanol intake of ≥80 g/d for >10 years were considered to have a positive history of alcohol abuse. The following clinical variables were obtained for each patient at the time of whole-blood collection: age, gender, serum albumin level, serum total bilirubin level, serum alanine aminotransferase level, serum α-fetoprotein (AFP) level, prothrombin time, platelet count, and serum viral load measured using the Amplicor HCV monitor assay. The diagnosis of hepatocellular carcinoma was made by several imaging methods (ultrasonography, computed tomography, arteriography, or magnetic resonance imaging) and confirmed histologically by sonography-guided fine-needle biopsy in all 187 patients. All patients were shown not to have other cancers by an initial screening examination.

SNP genotyping. Genomic DNA was extracted from 100 μL whole blood using the SepaGene kit (Sanko Junyaku, Tokyo, Japan) according to the manufacturer's instructions. Extracted DNA was dissolved in 20 μL Tris-HCl buffer (10 mmol/L, pH 8.0) containing 1 mmol/L EDTA and was stored at −30°C until use.

We did the SNP genotyping using the Taqman SNP genotyping assays and ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA). The PCR contained genomic DNA (10 ng), 1× Taqman universal PCR master mix, forward and reverse primers (900 μmol/L each), 200 nmol/L VIC-labeled probe, and 200 nmol/L FAM-labeled probe. The primers and probes were designed according to the Custom Taqman SNP genotyping Assay protocol (Applied Biosystems) and were as follows: forward primer 5′-CGGGAGTTCA-GGGTAAAGGT-3′, reverse primer 5′-ACAGGCACCTGCGATCATC-3′, VIC-labeled probe 5′-CTCCCGCGCCGAAG-3′, and FAM-labeled probe 5′-TCCCGCGCCGCAG-3′. The probes were MGB probes designed specifically for Taqman Allelic Discrimination (Applied Biosystems). The PCR was carried out in 96-well plate with a reaction volume of 25 μL. Each 96-well plate contained 88 samples of unknown genotype, two samples of each of the T/T, G/G, or T/G genotypes as positive control and two no-template controls. Thermal cycle conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 92°C for 15 seconds and 60°C for 1 minute. Completed PCR plates were read on an ABI PRISM 7000 sequence detector and analyzed using the Allelic Discrimination Sequence Detection Software (Applied Biosystems).

As confirmatory check of the SNP genotyping, a short segment of intron 1 of the *MDM2* gene of randomly selected samples was amplified by PCR using the following primers: 5′-CGGGAGTTCAGGG-TAAAGGT-3′ and 5′-AGCAAGTCGGTGCTTACCTG-3′ (24) and was checked by direct sequencing. Because the results of allelic discrimination were 100% concordant with the direct sequencing, the rest of the genotyping was done using the Taqman systems only.

Statistical analysis. The association between the clinical variables (age, gender, cirrhosis, HCV serotype, serum viral load, serum albumin, total bilirubin level, serum alanine aminotransferase level, serum AFP, prothrombin time, platelet count, and alcohol intake) and the presence of hepatocellular carcinoma were evaluated using the two-tailed t test, the Wilcoxon test, and the χ^2 test where appropriate. The association between different SNP genotypes and the presence of hepatocellular carcinoma was evaluated using the χ^2 test. Possible confounding effects among the variables were adjusted using a multivariate logistic regression model (26), and odds ratios (OR) and 95% confidence intervals (95% CI) were calculated. All data analyses were done using JMP software (version 5.1.2, SAS Institute, Inc., Cary, NC). The Hardy-Weinberg equilibrium of alleles at individual loci was evaluated using HWE.[2] For all tests, $P < 0.05$ was considered significant.

Results

Patient characteristics. The characteristics of the total 435 patients with chronic HCV infection involved in the study are shown in Table 1. There were no significant differences in the HCV serotype, viral load, serum alanine aminotransferase levels, and alcohol use between the group of patients with and without hepatocellular carcinoma. In patients with hepatocellular carcinoma, however, age, proportion of male gender, presence of cirrhosis, serum total bilirubin, and serum AFP levels were higher and serum albumin levels, prothrombin time, and platelet counts were lower than in patients without hepatocellular carcinoma.

MDM2 promoter SNP309 polymorphisms in HCV-infected patients and healthy subjects. We examined the SNP309, located in the promoter region (intron 1) of the *MDM2* gene, in all 483 subjects (435 patients and 48 healthy individuals) using the Taqman SNP genotyping method. The presence of SNP was also confirmed by direct sequencing of randomly selected samples. The distributions of the SNP309 genotypes, alleles, and at-risk alleles with regard to the presence or absence of hepatocellular carcinoma are shown in Table 2. Data from the 48 healthy subjects are also shown for comparison. There was no difference in the distribution pattern of the SNP309 (T/T, T/G, and G/G genotypes) among healthy individuals and patients with chronic HCV infection. However, when the HCV patients were segregated into two groups based on presence or absence of hepatocellular carcinoma, the genotype frequencies and allelic frequencies of SNP309 were significantly different between the groups of patients with and without hepatocellular carcinoma. The at-risk alleles were also associated with the presence of hepatocellular carcinoma. For instance, the frequency of distribution of T/T versus G/G genotype among those who had developed hepatocellular carcinoma (16% versus 33%) was significantly different from those who had not developed hepatocellular carcinoma (27% versus 23%; $P = 0.004$). In other words, a higher number of patients with hepatocellular carcinoma were found to have the G/G genotype compared with the patients without

[2] ftp://linkage.rockfeller.edu/software.

Table 1. Patient demographics

Variable*	Total (n = 435)	Without hepatocellular carcinoma (n = 248)	With hepatocellular carcinoma (n = 187)	P
Sex (male)	247 (57)	128 (52)	119 (64)	0.001 †
Age (y)	62 (22-84)	59 (22-80)	65 (38-84)	<0.0001 ‡
Cirrhosis	189 (43)	56 (23)	133 (71)	<0.0001 †
HCV serotype 1	285 (77)	158 (77)	127 (77)	0.993 †
HCV load (IU/mL)	434 (0.5-1910)	435 (0.5-1910)	430 (0.5-1340)	0.514 ‡
Albumin (g/dL)	3.9 (2.3-5.0)	4.1 (2.3-5.0)	3.6 (2.3-4.7)	<0.0001 ‡
Total bilirubin (mg/dL)	0.8 (0.2-6.7)	0.7 (0.2-6.7)	0.9 (0.3-3.5)	<0.0001 ‡
Alanine aminotransferase (units/L)	60 (14-465)	55 (14-433)	65 (15-465)	0.142 ‡
AFP (μg/L)	11.5 (1.0-6107)	6 (1.0-425)	35 (3-6107)	<0.0001 ‡
Prothrombin time (%)	77 (7.3-100)	84 (7.3-100)	72 (13-100)	<0.0001 ‡
Platelet count (×10^4/μL)	12.7 (1.7-39)	15 (2.6-34)	9.6 (1.7-39)	<0.0001 ‡
Alcohol (>80 g/d)	15 (3)	7 (3)	8 (4)	0.189 ‡

*Age, albumin, total bilirubin, alanine aminotransferase, AFP, prothrombin time, platelet count, and HCV load are shown as median (range). Male, cirrhosis, alcohol, and HCV serotype 1 are shown as frequency (%).
†Calculated using the χ^2 test.
‡Calculated using the Wilcoxon test.

hepatocellular carcinoma, whereas the T/T genotype was more common among the nonhepatocellular carcinoma group than the hepatocellular carcinoma group.

The proportion of G/G genotypes of the *MDM2* SNP309 among patients with hepatocellular carcinoma (33%) was higher than in patients without hepatocellular carcinoma (23%) and the healthy subjects (27%). Of the T/T, T/G, and G/G genotypes, 32%, 43%, and 52% had hepatocellular carcinoma, respectively. Having a G allele gradually increased the proportion of patients with hepatocellular carcinoma (P = 0.0135). The *MDM2* SNP309 genotype T/G and G/G as opposed to genotype T/T increased the risk of hepatocellular carcinoma with the OR (95% CI) of 1.62 (0.98-2.68; P = 0.057) and 2.28 (1.30-3.98; P = 0.004), respectively. The attributable risks of *MDM2* SNP genotype T/G to T/T and G/G to T/T were 11% and 20%, respectively.

We also checked the effect of SNP309 on the rate of fibrosis progression and presence of cirrhosis; however, there were no significant difference among the three genotypes of SNP309 with regard to these variables.

Factors associated with presence of hepatocellular carcinoma in HCV-infected patients. The following factors were significantly associated with the presence of hepatocellular carcinoma according to univariate analyses: *MDM2* SNP309 (P = 0.0137), age >60 years (P < 0.0001), male gender (P = 0.001), presence of cirrhosis (P < 0.0001), serum albumin <3.2 g/dL (P < 0.0001), total bilirubin >0.7 mg/dL (P < 0.0001), AFP >20 μg/L (P < 0.0001), prothrombin time <70% (P < 0.0001), and platelet count <12.5 × 10^4/μL (P < 0.0001). To evaluate the effect of *MDM2* SNP309 polymorphisms on the presence of hepatocellular carcinoma, a multivariate logistic regression analysis was done with these nine variables. Six variables (SNP309 genotypes, presence of cirrhosis, age >60 years, male gender, and serum AFP >20 μg/L and serum albumin <3.2 g/dL) were included in the final model with OR (95% CI) of 2.27 (1.11-4.70; T/T versus G/G), 4.15 (2.46-7.08), 2.46 (1.42-4.31),

Table 2. SNP309 genotype frequencies in patients with HCV and healthy subjects

SNP309	Patients with HCV					Healthy subjects (n = 48)*
	Total (n = 435)*	Nonhepatocellular carcinoma (n = 248)*	Hepatocellular carcinoma (n = 187)*	OR (95% CI) hepatocellular versus nonhepatocellular carcinoma	P	
Genotype						
T/T	97 (22)	66 (27)	31 (16)	1.00		9 (19)
T/G	220 (51)	125 (50)	95 (51)	1.62 (0.98-2.68)	0.057	26 (54)
G/G	118 (27)	57 (23)	61 (33)	2.28 (1.30-3.98)	0.004	13 (27)
Allele						
T	414 (48)	257 (52)	157 (42)	1.00		44 (46)
G	456 (52)	239 (48)	217 (58)	1.49 (1.13-1.9)	0.004	52 (54)
At-risk allele						
T/G + T/T	317 (73)	191 (77)	126 (67)	1.00		35 (73)
G/G	118 (27)	57 (23)	61 (33)	1.62 (1.06-2.48)	0.025	13 (27)
T/T	97 (22)	66 (27)	31 (16)	1.00		9 (19)
G/G + T/G	338 (78)	182 (77)	156 (84)	1.83 (1.13-2.94)	0.013	39 (81)

*Values expressed as *n*(%).

Table 3. Factors associated with presence of hepatocellular carcinoma in HCV-infected patients in multivariate analysis

Factor	Category	OR (95% CI)	*P*
SNP309	T/T	1.00	
	T/G	1.53 (0.81-2.93)	0.192
	G/G	2.27 (1.11-4.70)	0.025
Cirrhosis	Presence	4.15 (2.46-7.08)	<0.0001
Age	>60 y	2.46 (1.42-4.31)	0.0015
Sex	Male	3.08 (1.80-5.41)	<0.0001
AFP	>20 μg/L	4.87 (2.87-8.27)	<0.0001
Albumin	<3.2 g/dL	6.33 (1.91-29.4)	0.0065

3.08 (1.80-5.41), 4.87 (2.87-8.27), and 6.33 (1.91-29.4), respectively (Table 3).

Discussion

In this study, we identified a potential genetic marker for susceptibility to hepatocarcinogenesis in patients with chronic HCV infection. We evaluated the relationships between the *MDM2* SNP309 genotypes and the outcome of chronic HCV infection. Our results showed an effect of the *MDM2* SNP309 polymorphisms on the presence of hepatocellular carcinoma after controlling for other confounding clinical variables. The proportion of patients with hepatocellular carcinoma gradually increased from the SNP309 genotype of T/T to T/G to G/G. The SNP309 allele G was found to be associated with the presence of hepatocellular carcinoma. Our multivariate model confirmed the association between the SNP309 G/G genotype and the presence of hepatocellular carcinoma. Further, our results showed that the genotype frequencies in patients with cirrhosis but without hepatocellular carcinoma were similar to those in patients without cirrhosis and hepatocellular carcinoma, suggesting that this polymorphism is not as strongly associated with the presence of cirrhosis.

The *MDM2* SNP309 was recently identified by Bond et al., who, using samples of breast cancers and soft-tissue sarcomas, showed that the G/G genotype of the SNP309 has a strong effect on tumorigenesis on humans (24). We studied the effect of the SNP309 on hepatocellular carcinoma development in patients with chronic hepatitis C and found that G/G genotype is significantly associated with hepatocellular carcinoma. The frequency of SNP309 in our study population of Japanese patients (27%) was higher than in the Caucasians (12%) as was originally reported by Bond et al. (24). Reports from Chinese populations also show a higher frequency of the G/G genotypes, much closer to our findings (27). This may be due to a racial difference of the study population. Using 88 patients of hereditary cancer (Li-Fraumeni syndrome) and 105 sporadic soft-tissue sarcomas, Bond et al. also showed that SNP309 associates with at least 9 years earlier onset of tumors in both hereditary and sporadic cancers (24). Similar findings have been recently reported by at least one other group (28). We also studied the effect of SNP309 on the age of onset of hepatocellular carcinoma; however, we did not find a significant age difference between T/T and G/G genotypes. Owing to the limitations of a cross-sectional design, our present study is unable to comment on whether SNP309 actually leads to an accelerated hepatocellular carcinoma development in HCV-infected patients. A prospective controlled study can be done to evaluate this phenomenon in future.

The SNP309 lies in the Sp1-binding site in the promoter region of *MDM2* gene. A T-to-G substitution in this region increases the binding affinity of the transcriptional activator Sp1, which results in high levels of MDM2 RNA and protein (24, 29). The heightened MDM2 levels are poor prognostic factors in many human cancers, including lung cancers and breast cancers (30, 31). MDM2 is a key negative regulator of p53, the most important tumor suppressor (32, 33), which targets p53 for proteosomal degradation (33–35). A high MDM2 level (as is seen in the SNP309 G/G genotype) leads to the attenuation of the p53 DNA damage response that allows increased cell proliferation and inhibition of apoptosis, providing advantageous signals for tumor cell survival (29, 34, 36). Studies using *MDM2* transgenic mice have shown that 100% of the MDM2-overexpressing mice developed spontaneous tumors in a lifetime (37). These studies, together with numerous accounts of MDM2 overexpression or amplification in a variety of human cancers, support the idea that heightened levels of MDM2, as seen in SNP309 G/G genotype, could positively affect tumor formation.

In Japan, >80% of hepatocellular carcinoma are caused by the HCV infection alone (3, 38, 39). Persistence of the viral infection in hepatic cells is strongly associated with the hepatocellular carcinoma development (2, 5, 6). In a recent report, Arva et al. showed that the SNP309 G allele results into MDM2-p53 complex that is transcriptionally inactive (36). In a related but different study, we have found that although a strong p53 expression suppresses replication of the HCV *in vitro*, the viral replication is significantly enhanced when *p53* gene expression is suppressed.[3] Inactivating gene mutations of p53 are common in patients with hepatocellular carcinoma (40, 41) but are not as frequent as in other malignancies, such as ovarian, esophageal, or colon cancers (42). A high prevalence of G alleles of the SNP309 in the hepatocellular carcinoma patients, on the other hand, implies that the p53 functions in the HCV patients could have been indirectly suppressed by the heightened MDM2 levels, making them more vulnerable to cancer development. Together, these data support a model whereby SNP309 enhances the affinity of the transcriptional activator Sp1 to the promoter of the *MDM2* gene, resulting in heightened transcription. Heightened levels of MDM2 lead to the direct inhibition of p53 transcriptional activity, which, in patients with chronic hepatitis C, could lead to an unrestricted replication and proliferation of the HCV inside hepatic cells, ultimately leading to tumor development. It has been suggested that the G allele can serve as a rate-limiting event in the formation of a tumors in humans based on the Knudson model (29, 43). Based on our results, we can assume a codominant model (i.e., one G allele increases the risk of having hepatocellular carcinoma and two G alleles further increase the risk).

Despite the limitations of a cross-sectional study, our analysis showed a prominent effect of the SNP309 G/G genotype on the risk of developing hepatocellular carcinoma. Most HCV-related hepatocellular carcinomas arise from a background of cirrhosis,

[3] Unpublished data.

but it is noteworthy that the association of the SNP309 and hepatocellular carcinoma was completely independent of the presence of cirrhosis. Given that many patients are referred to our hospital for the treatment of hepatocellular carcinoma, our study population may be biased toward patients with hepatocellular carcinoma. Our multivariate model, however, included most of the previously reported risk factors for hepatocellular carcinoma (i.e., age, male gender, presence of cirrhosis, AFP level, and serum albumin; refs. 13-15, 44, 45) as well as the polymorphisms of the SNP309. This implies that our results can be generalized to the Japanese population. The uncertainty of the ORs, owing to the study design, should be resolved in subsequent prospective controlled studies. In conclusion, *MDM2* promoter SNP309 is associated with the risk of hepatocellular carcinoma among Japanese patients with chronic HCV infection. The G allele of *MDM2* SNP309 could serve as an important marker to identify the subgroup of chronic HCV patients at a higher risk of hepatocellular carcinoma.

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Association of Transforming Growth Factor–β1 Functional Polymorphisms with Natural Clearance of Hepatitis C Virus

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Transforming growth factor (TGF)–β1 suppresses the proliferation and cytotoxicity of natural killer (NK) cells, which play critical roles in resolving hepatitis C virus (HCV) infection, especially during the acute phase. We examined 230 anti–HCV antibody–positive subjects for HCV RNA and the −509T/C genotype in the TGF-β1 gene promoter. The −509CC genotype and the −509C allele were significantly associated with higher HCV clearance rates ($P = .01$) and with lower transcriptional activity. The genetic effect remained significant even after adjustment for a history of transfusion. Low TGF-β1 producers might have less suppression of NK cells and be more likely to resolve HCV infection.

Viral infection triggers a series of host immune responses. Among hepatitis C virus (HCV)–infected patients, ~15%–20% experience natural clearance, most likely during the acute infectious stage, during which increased an level of interferon (IFN)–γ is a positive marker of the resolving infection [1]. NK cells produce IFN-γ, and their proliferation and cytotoxicity are critical for viral clearance [2]. Because NK cells are suppressed by default, an activation signal should overcome inhibitory regulation. Recently, a weaker inhibitory combination of the NK cell receptor killer cell immunoglobulin-like receptor (KIR)–2DL3 and HLA-C1 was reported to indicate a higher rate of resolution of HCV infection [3]. Moreover, the interleukin (IL)–10 −1082GG genotype, which prompts the production of a higher amount of IL-10, was associated with a lower clearance rate [4]. Considering that IL-10 inhibits the development and activation of NK cells with an IFN-γ–secreting phenotype [5], oversuppression of NK cell cytotoxicity may result in persistent HCV infection.

Transforming growth factor (TGF)–β is another well-known suppressor of NK cells that inhibits IFN-γ and IL-12 production and blocks the proliferation and cytotoxicity of NK cells [6]. The −509T allele is associated with a higher plasma concentration of TGF-β1 [7], and TGF-β1 dysregulation has been shown to be involved in the progression of liver cirrhosis and hepatocellular carcinoma [8]. However, TGF-β1 gene variants have not been studied in association with the natural clearance of HCV. Here, we demonstrate that the −509C/T mutation in the TGF-β1 gene is associated with the natural clearance of HCV and with promoter activity.

Subjects and methods. Study subjects were from northern Japan, where there is a very high prevalence (~40% of inhabitants) of anti-HCV antibodies among the population [9]. The study was approved by the ethical review committee of Yamagata University, and written informed consent was obtained from all subjects recruited. Subjects with a history of antiviral IFN therapy or who were positive for hepatitis B surface antigen were excluded; 230 anti-HCV antibody–positive subjects were enrolled in the study. There were no users of illicit drugs in the study population, and the routes of HCV transmission are still obscure in this community except in those with a history of receiving blood transfusion. Reuse of syringes and needles and the use of folk remedies, such as acupuncture, may be factors in community-acquired HCV infection, but the routes of HCV transmission remain unknown in most cases.

All subjects were tested for their serum HCV RNA status using an Amplicor HCV RNA detection kit (Amplicor HCV version 2.0; Roche Diagnostics). The persistence of resolution was confirmed over a minimum of 12 months by independent sampling.

The −509T/C (rs1800469) single-nucleotide polymorphism (SNP) was determined using the TaqMan assay (Hubit Genomix), and genotypes for the 2904T/G (rs2241715) and 5738G/T (rs2241717) SNPs were obtained from an earlier study [10]. The 869C/T (rs1982073) genotype was determined by direct sequencing, and the IL-10 genotypes, −592A/C (rs1800872) and −1082G/A (rs1800896), were determined using melting-

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curve analysis. The detailed protocol is available from the authors on request.

A TGF-β1 reporter construct, pHTG-luc(-845), which contains an 845-bp genomic fragment of the human TGF-β1 gene, and an expression vector for HCV core protein, PCXN2/HCV-core [11], were provided by Dr. Hiroyoshi Taniguchi (Tokyo University, Japan). Because pHTG-luc(-845) contains C at position -509, it was designated pTGFB(-509C) in the study. Site-directed mutagenesis was used to obtain pTGFB(-509T), using a QuikChange multisite-directed mutagenesis kit (Stratagene) with the following primer pair: forward, 5′-CAACAGG-ACACCTGAAGGATGGAAGGGTCAG-3′, and reverse, 5′-CT-GACCCTTCCATCCTTCAGGTGTCCTGTTG-3′.

Human hepatoma cell line HepG2 cells were seeded at a density of 2.5×10^5 cells/well on 6-well cell culture plates on the day before transfection. We transfected 600 ng of pTGFB(-509C), pTGFB(-509T), or pGL3-basic luciferase reporter vector and 10 ng of pRL-TK (Promega), with or without 100 ng of pCXN2 or pCXN2/HCV-core, using Lipofectamine 2000 and Opti-MEM (Invitrogen); then we replaced the medium 4 h after transfection. The cells were harvested 48 h after transfection. Luciferase activity was measured and normalized with *Renilla* luciferase activity, to compensate for transfection efficiency. The 20-μg lysate of the transfected cells was subjected to SDS-PAGE, and Western blotting was performed using an HCV core–specific mouse monoclonal antibody with horseradish peroxidase–conjugated goat anti–mouse IgG (Santa Cruz Biotechnology).

Allele and genotype frequencies of SNPs between the subjects with persistent infection and those whose infection resolved were tested with Fisher's exact test. Odds ratios (ORs) were computed using logistic-regression models. Promoter activities were standardized to a negative control (pGL3-basic vector) and compared using Student's t test. The D' and r^2 were calculated for all pairwise combinations of the 4 SNPs, using the LDSUPPORT program (available for academic use from N. Kamatani, Tokyo Women's Medical University, Tokyo, Japan) based on the EM algorithm. All other analyses were performed with SAS software (version 9.1.3; SAS Institute). Differences of $P < .05$ were considered to be significant.

Results. HCV antibody–positive subjects (84 men and 144 women) were a mean of 66.8 years old (range, 32–88 years old) and were tested for serum HCV RNA levels. In 46 subjects, HCV RNA was not detected, whereas the other 184 tested positive. One half of subjects with persistent HCV infection (92/184 [50%]) had genotype 2b infection, 77 (41.9%) had genotype 1b infection, and 12 (6.5%) had genotype 2a infection. The frequency distribution was not significantly different between subjects with and without a history of blood transfusion (data not shown). The mean age and sex ratios were not significantly different between the groups with resolved versus persistent infection. Twenty-two percent of subjects with persistent infection (33/151) had a history of blood transfusion, compared with only 9.5% of the subjects whose infection resolved (4/42).

Every pair of -509T/C, 869C/T, 904T/G, and 5738G/T SNPs were in tight linkage disequilibrium ($D' > 0.9$; $r^2 > 0.8$). The results for these genotypes and their haplotypes showed essentially the same as those of -509T/C; thus, we set this genotype as a representative. The genotype frequency of the -509T/C variant did not deviate from Hardy-Weinberg equilibrium. The

Table 1. Association between the transforming growth factor–β1 509T/C single-nucleotide polymorphism and hepatitis C virus clearance.

		Type of infection, no. (%)			
Variable	Subjects, no.	Persistent	Self-limiting	OR (95% CI)	P
Genotype	230	184 (91)	46 (23)		
TT	64	56 (30.4)	8 (17.4)	1.0 (Referent)	
TC	109	89 (48.4)	20 (43.5)		
CC	57	39 (21.2)	18 (39.1)	2.4 (1.2–4.8)	.0133[a]
Allele					
Total	460	368	92		
T	237	201 (54.6)	36 (39.1)	1.0 (Referent)	
C	223	167 (45.4)	56 (60.9)	1.9 (1.2–3.0)	.0084
No transfusion	386	302	84		
T	200	165 (54.6)	35 (41.7)	1.0 (Referent)	
C	186	137 (45.4)	49 (58.3)	1.7 (1.0–2.8)	.0364
Transfusion	74	66	8		
T	37	36 (54.5)	1 (12.5)	1.0 (Referent)	
C	37	30 (45.5)	7 (87.5)	8.4 (0.98–72.1)	.0524

NOTE. Odds ratios (ORs) were computed using logistic regression analyses. A positive OR indicates a protective association with resolution of infection. CI, confidence interval.

[a] TT and TC vs. CC.

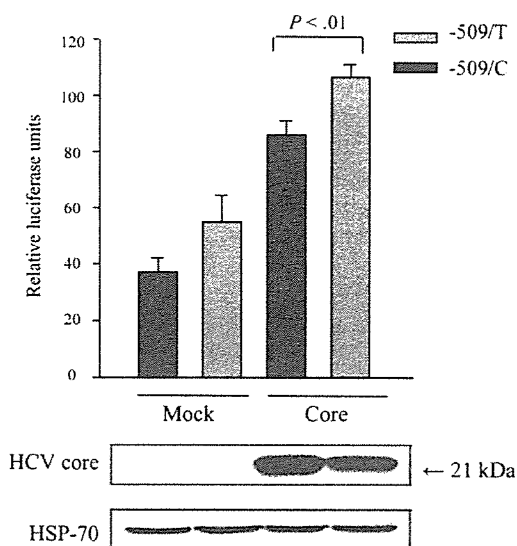

Figure 1. Promoter activity and the −509T/C single-nucleotide polymorphism in hepatocytes with and without hepatitis C virus (HCV) core protein coexpression: transforming growth factor–β1 gene promoter activity. Data are shown as means and SDs of 3 measurements of luciferase activity standardized to corresponding *Renilla* luciferase activity. Results are representative of 3 experiments, each using at least 3 wells for each condition. Promoter activity under the condition of HCV core protein coexpression was significantly higher in subjects with the −509T allele than in those with the −509C allele (P = .0067, Student's t test). HCV core protein expression was confirmed using Western blotting. Heat-shock protein (HSP)–70 is shown as a control for the total amount of protein.

frequencies of the −509CC genotype and −509C allele were significantly higher in subjects whose infection resolved (table 1). Multivariate logistic-regression analysis that included a history of blood transfusion, which was a significant negative predictor (OR, 0.44; P = .0351), showed that the −509C allele was an independent predictor of HCV clearance (OR, 1.89; P = .0076).

The IL-10 genotypes, −592A/C (rs1800872) and −1082G/A (rs1800896), were not associated with HCV clearance (data not shown). The allele frequencies did not deviate from those of a previously reported healthy Japanese population [4], but no subjects carried the −1082GG genotype, which corresponds to a higher IL-10 level and a lower clearance rate, and only a few subjects possessed the −1082AG genotype (n = 14). In subjects with the −592AA genotype, the lower production of IL-10 and higher rate of resolution of infection [4] were not associated with clearance in our study population (OR, 0.96; P = .89). A multivariate logistic-regression analysis that included the IL-10 genotypes did not show any effect of the association of −509T/C with HCV resolution. Therefore, we did not include the IL-10 genotypes in the final model.

When we stratified the subjects into 2 groups according to their transfusion history, the frequency of the −509C allele was significantly higher in subjects with self-limiting infection, compared with subjects who had persistent infection, in those with no history of blood transfusion (table 1). Among subjects who had received blood transfusions, this tendency was more apparent, although the significance did not reach statistical significance. Seven (87.5%) of 8 alleles in subjects whose infection resolved were the C allele, compared with 30 (45.5%) of 66 alleles in subjects with persistent infection. Age and sex were not associated with the clearance of HCV and were not included in the final model.

To investigate whether the −509T/C polymorphism is involved in the promoter activity of the TGF-β1 gene, we performed an in vitro reporter assay. The promoter reporter construct possessing −509C had ~30% lower promoter activity, compared with that of the −509T reporter construct. Given that the frequency of the −509C allele was significantly higher in subjects whose infection resolved (P = .01), there was an inverse relationship between the TGF-β1 gene promoter activity and natural clearance of HCV.

Because hepatocytes infected with HCV can also secrete TGF-β1 and the HCV core protein can enhance its production [11], we examined TGF-β1 gene-promoter activity of the −509T or −509C alleles under the coexpression of HCV core protein. The in vitro promoter assay showed that the −509C allele had significantly lower promoter activity than the −509T allele (~20%; $P < .01$) under the coexpression of HCV core protein (figure 1). The magnitude of the activation induced by HCV core protein coexpression did not differ between the −509T and −509C alleles (P = .29).

Discussion. In the present study, we investigated the association of TGF-β1 gene polymorphisms with the natural clearance of HCV. Our results clearly showed that the −509C allele is associated with a higher clearance rate of HCV as well as with lower promoter activity.

Recently, several candidate loci for the natural clearance of HCV have been reported, including HLA, KIR [3], and IL-10 [4]. In whites in the United Kingdom, subjects with the IL-10 −592AA genotype were more likely to clear their infection (13.3% vs. 7.0% of subjects with self-limiting and persistent infection, respectively), whereas subjects with the −1082GG genotype had a higher risk of persistent infection (13.8% vs. 25.0%) [4]. However, we did not find any association with IL-10 promoter genotypes. The discrepancy may arise as a result of differences in genotype frequency between the populations. In the Japanese population, the frequency of the −1082G allele is much lower than that in whites (7% vs. 54%) (see http://www.hapmap.org/cgi-perl/snp_details?name=rs1800896), and there were no −1082GG homozygous and few AG heterozygous subjects in our study population.

TGF-β is a potent immunosuppressive cytokine that inhibits

the function of NK cells. Recently, it has been reported that TGF-β down-regulates NKG2D receptor expression at the transcriptional level and that RNA interference that targets the TGF-β gene enhances NK cell activity [12, 13]. Thus, it is tempting to speculate that high TGF-β1 producers might oversuppress their immune system, particularly during the early stages of infection. In the present study, we have demonstrated that promoter activity was significantly lower in subjects with the −509C allele than in subjects with the −509T allele ($P<.01$) in both the presence and absence of the HCV core protein (figure 1). Although TGF-β1 might suppress the in vitro replication of the HCV replicon [14], our results reflect, to some extent, the in vivo condition of HCV-infected hepatocytes. However, whether the different levels of TGF-β1 gene expression and/or secretion between alleles actually contribute to differing NK activity or IFN-γ secretion in vivo remain unclear. Because the autocrine effect of the TGF-β1 produced by NK cells might not be high enough to impede the activation of NK cells by various cytokines [15], the in vivo interaction of the immune cells, including NK cells and hepatocytes, is an interesting issue.

Transfusion seems to be one of the inhibitory factors for natural clearance [3]. In our study population, a protective tendency of the −509C allele was found even in subjects with a history of blood transfusion, although the difference was not statistically significant (table 1), and the OR remained significant even after adjustment for a history of transfusion. Further study with a larger population is necessary to elucidate this interaction more clearly. The HCV genotype may also contribute to the natural clearance rate. Although the HCV genotype of subjects whose infection resolved was not available for the present study, the frequency of genotype 2b infection was relatively higher in subjects with persistent infection (50%), compared with the average in Japan (~10%–15%). We do not have any data to support the correlation between HCV genotype and the natural clearance rate. A prospective study of newly infected patients may help to clarify the association.

Our results suggest that a lower production of TGF-β1 is correlated with a higher incidence of HCV clearance. TGF-β1 might play an important role in the clearance of HCV viremia during the acute phase of infection. The role of TGF-β1 in HCV clearance warrants further investigation.

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ELSEVIER

Serum Levels of Interleukin-6 and Its Soluble Receptors in Patients with Hepatitis C Virus Infection

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ABSTRACT: Interleukin-6 (IL-6) is an important cytokine in liver regeneration, and elevated levels of IL-6 have been demonstrated in patients with chronic liver diseases (CLD). Many biological effects of IL-6 depend on naturally occurring soluble IL-6 receptors. In the present study we measured the concentrations of IL-6 and its soluble receptors in the sera of patients with CLD related to hepatitis C virus (HCV) infection. We studied 77 patients with varying degrees of HCV-related CLD. Serum levels of IL-6 and its soluble receptors (sIL-6R, sgp130) were measured by enzyme-linked immunosorbent assay. Serum IL-6 and sIL-6R were elevated in patients with CLD compared with healthy subjects. Serum levels of sgp130 did not differ between patients with chronic hepatitis and healthy subjects. However, in patients with liver cirrhosis, sgp130 was significantly elevated and was positively correlated with total bilirubin and negatively correlated with cholinesterase and prothrombin time. Our study demonstrated that in patients with HCV-related CLD, serum IL-6 and its soluble receptor levels are correlated with both liver function impairment and the degree of liver fibrosis. These observations suggest that the balance of IL-6 and its soluble receptors may correspond to the state of liver damage in patients with CLD. *Human Immunology 67, 27–32 (2006).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: Hepatitis C virus; Interleukin-6; Liver cirrhosis; Soluble interleukin-6 receptor

ABBREVIATIONS

CLD	chronic liver disease
CH	chronic hepatitis
IL-6	interleukin-6
sIL-6R	soluble interleukin-6 receptor
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HGF	hepatocyte growth factor
LC	liver cirrhosis
TNF-α	tumor necrosis factor-α

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine stimulating a variety of cell types, including hepatocytes [1–3]. IL-6 also modulates the hepatic expression of acute-phase proteins during inflammation [4,5]. Apart from its role in inflammation, IL-6 has been found to be essential for liver regeneration [6,7]. Results from IL-6 knockout mice have also indicated that IL-6 might be involved in triggering hepatocyte proliferation after hepatectomy [8]. The pathophysiological role of IL-6 in acute or chronic liver disease has been studied intensively [9]. Although IL-6 was consistently found to be elevated in liver diseases, such as chronic hepatitis and cirrhosis [10,11], the clinical relevance and molecular function of IL-6 in the pathogenesis of liver disease are only incompletely understood.

IL-6 mediates its diverse biological effects by interacting with a receptor complex consisting of a specific

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