respectively. Because two mice in group A and two mice in group C died before the second period, two remaining mice in group C and one back-up mouse were assigned to group A (n=2) and group B (n=1). During the second period, mice that received high or low doses were crossed over to the alternative treatment. Serum samples were collected before the first dose was administered and 5 h after every two doses were administered. Plasma samples were also collected at the same time on days 1, 3 and 5 in the first period and days 1, 3 and 4 in the second period. The mice were sacrificed 8 h after administration of the final dose, and serum, plasma and liver samples were collected.

Experiment 5: viral kinetics with BID dosing After infection of 45 mice, 12 HCV-infected mice maintained steady-state and high viral loads (1.2×10^6–8.5×10^7 copies ml^{-1}) for more than 6 months. The median survival time of this cohort was 131 days after infection. These mice were treated with 200 mg telaprevir kg^{-1} BID at 19:00 and 9:00 h for 3 days. The mice were divided into two groups and serum samples were collected just before the second dose was administered and 4 (n=6) or 8 (n=6) h after every two doses were administered.

Serum RNA extraction and HCV RNA quantification. HCV RNA was isolated from 10 µl serum under denaturing conditions using a SepaGene RV-R kit (Sanko Junyaku). The dried precipitates were dissolved in 10 µl diethylpyrocarbonate-treated water. Extracts were duplicated and assayed by quantitative real-time RT-PCR using TaqMan EZ RT-PCR core reagents (Applied Biosystems). Nucleotide positions of the probe and primer sets refer to HCV H77 strain (GenBank accession no. AF009606). The TaqMan probe 5′-6-FAM-CTGCGGAACCGGTGAGTACAC-BHQ-1-3′ (nt 148–168) was purchased from Biosearch Technologies, and the forward (5′-CGGGAGAGCCATAGTGG-3′; nt 130–146) and reverse (5′-AGTACCACAAGGCCTTTCG-3′; nt 272–290) primers were purchased from Sigma-Aldrich. The 25 µl RT-PCR mixture contained 0.2 nmol forward and reverse primers ml^{-1}, 0.3 nmol TaqMan probe ml^{-1} and 5 µl extracted RNA, and was monitored using a PRISM 7900HT sequence detection system (Applied Biosystems). The thermal profile was 2 min at 50 °C, 30 min at 60 °C for reverse transcription and 5 min at 95 °C, followed by 45 cycles of 20 s at 95 °C and 1 min at 62 °C. The HCV replicon I_{389}neo/NS3-3′/5.1 (Lohmann *et al.*, 1999) RNA was transcribed *in vitro* using a T7 RiboMax Express Large Scale RNA Production System (Promega) and purified twice using gel filtration. The concentration of this transcribed RNA was determined by absorbance at 260 nm and serially diluted 10-fold to prepare a standard curve for each assay.

Liver RNA extraction and HCV RNA quantification. A Wizard SV total RNA Isolation System (Promega) was used to obtain a DNase I-treated total RNA sample. The total RNA concentration was determined by absorbance at 260 nm. Total RNA samples were assayed by duplex real-time RT-PCR for relative quantification of HCV RNA using endogenous control gene expression of human β_2-microglobulin (hβ_2m; GenBank accession no. NM_004048), the TaqMan probe 5′-CAL Fluor Orange 560-AGTGGGATCG-AGACATGTAAGCAGCATCAT-BHQ-1-3′ (nt 401–430), and the forward and reverse primer set of 5′-TTGTCACAGCCCAA-GATAGTT-3′ (nt 379–399) and 5′-TGCGGCATCTTCAAACC-3′ (nt 434–450). To adjust the efficacy of PCR amplification of both target genes, the reaction condition was modified from the HCV single-probe assay. The temperature for extension was 60 °C, the concentration of the HCV probe was 0.24 nmol ml^{-1} and the reaction mixture contained the TaqMan probe/primer set for hβ_2m: 0.2 nmol primers ml^{-1} and 0.12 nmol TaqMan probe ml^{-1}. Because both target genes double after one cycle of PCR, a difference in Ct between HCV and hβ2m ($\Delta Ct = Ct_{HCV} - Ct_{h\beta2m}$) theoretically indicates a relative quantity of HCV RNA per control gene expression of $2^{-\Delta\Delta Q}$.

Determination of drug concentration. Plasma and liver samples were analysed using chiral liquid chromatography followed by tandem mass spectrometry. After reconstitution, sample extracts were separated by normal-phase chromatography on a 2×250 mm Hypersil CPS-1 column (Thermo Hypersil-Keystone) with a mobile phase of heptane : acetone : methanol (82 : 17 : 1). Analyte concentrations were determined by turbo ion spray liquid chromatography/tandem mass spectrometry in the positive-ion mode. Analysis was performed at SRL or Mitsubishi Chemical Medience.

Statistical analysis. The HCV RNA level in serum was normalized by logarithmic conversion. Statistical analysis was performed with a mixed linear model using SAS (SAS Institute). Mean differences between two groups were evaluated with Student's t-test. The difference compared with vehicle control at each time point was evaluated by Dunnett's multiple comparisons test. Linear and non-linear regression analyses were performed using GraphPad Prism 5 (GraphPad Software).

Viral dynamics model analysis. The basic mathematical model for the analysis of HCV infection *in vivo*, which is a system of three ordinary differential equations for uninfected cells (T), productively infected cells (I) and free virus (V), has been reviewed elsewhere (Perelson & Ribeiro, 2008). Briefly, one of the three equations ($dV/dt = pI - cV$), where viral particles are produced at rate p per infected cell and cleared at rate c per virion, was solved. During treatment for 2–3 days, if one assumes that the number of I is approximately constant and equal to its pre-treatment value and that the viral level was at its set-point value (V_0), then $pI = cV_0$. Using this relationship in the equation $dV/dt = (1-\varepsilon)pI - cV$, where ε is the effectiveness in blocking virion production, yields $dV/dt = (1-\varepsilon)\,cV_0 - cV$, $V(0) = V_0$ with the solution $V(t) = V_0(1-\varepsilon+\varepsilon e^{-ct})$. Because the log change of viral load at time [$\log \Delta V(t)$] equals $\log V(t)/V_0$, the solved equation [$\log \Delta V(t) = \log(1-\varepsilon+\varepsilon e^{-ct})$] was fitted to the values obtained in this study via non-linear least-squares regression in order to estimate ε and c.

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Research Article

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Predictive value of the *IL28B* polymorphism on the effect of interferon therapy in chronic hepatitis C patients with genotypes 2a and 2b

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Background & Aims: Common *IL28B* locus polymorphisms (SNPs rs8099917 and rs12979860) have been reported to affect peg-interferon plus ribavirin combination therapy (PEG-RBV) for hepatitis C virus (HCV) genotype 1b, but few reports have examined their effect on other two common genotypes, 2a and 2b.

Methods: We analyzed predictive factors for sustained virological response (SVR) in a retrospective study of 719 patients with either genotype 2a (530) or 2b (189). Of these patients, 160 were treated with PEG-RBV and 559 were treated with interferon monotherapy. We evaluated predictive factors including HCV RNA, histological findings, *IL28B* SNP genotypes (rs8099917, rs12979860, and rs12980275), and the effect of treatment regimen and prior treatment history.

Results: HCV RNA viral load, treatment regimen, and rs8099917 genotypes independently contributed to the effect of the therapy. For patients treated with PEG-RBV, rs8099917 and viral load were independent predictive factors for SVR in genotype 2b but not in genotype 2a. Conversely, in patients treated with interferon monotherapy, viral load and rs8099917 were independent predictive factors for SVR in genotype 2a but not in genotype 2b. The favorable rs8099917 genotype is also associated with a steep decline in viral load by the second week of treatment.

Conclusions: Initial viral load and rs8099917 genotype are significant independent predictors of SVR in genotype 2 patients.

Keywords: Interferon therapy; Single nucleotide polymorphism; Ribavirin; Hepatitis C.

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; PEG-IFN, pegylated interferon; RBV, ribavirin; PEG-RBV, pegylated interferon plus ribavirin combination therapy; SNP, single nucleotide polymorphism; SVR, sustained viral responder; NR, non-responder.

Introduction

Hepatitis C virus (HCV) infection is a major worldwide cause of chronic liver diseases, affecting an estimated 170 million people [1]. Chronic HCV infection may progress to hepatocellular carcinoma (HCC) or liver cirrhosis (LC) [2–6], and in Japan, 60–70% of patients with HCC or LC are HCV carriers [7]. There are two major genotypes (1 and 2) and three sub-genotypes (1b, 2a, and 2b) in Japan as well as in many other countries [8]. Although pathological features of these genotypes are similar [9,10], interferon therapy is more effective against genotype 2 than genotype 1 [11,12]. Compared to the less than 50% of genotype 1 patients who respond to therapy [13–19], more than 80% of genotype 2 patients who received 24-week peg-interferon and ribavirin (PEG-RBV) combination therapy achieved sustained virological response (SVR), defined as absence of HCV RNA six months after the cessation of therapy. Because of this otherwise high success rate, the small subset of genotype 2 patients who fail to respond to therapy should be examined more closely. Although treatment-resistant genotype 2 sub-populations have been reported [20–22], the mechanism underlying variable response to treatment is unclear. Multiple viral (e.g., HCV genotype, amino acid substitutions in the NS5A and core region [22–26]) and host factors (e.g., age [14], body mass index [27], and insulin resistance

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Table 1. Baseline characteristics of patients with HCV genotypes 2a and 2b.

	All (n = 719)	2a (n = 530)	2b (n = 189)
Sex (M/F)	403/316	301/229	102/87
Age	57 (49-64)	56 (48-64)	59 (50-66)
Body weight (kg)	59.8 (51-71.4)	60.15 (53.75-71.65)	57.4 (48.5-70)
BMI (kg/m^2)	23.2 (20.3-25.7)	24.48 (21.43-26.4)	21.78 (19.89-24.79)
Fibrosis (F0-2/F3-4)	484/101	359/68	125/33
Treatment (IFN/PEG-RBV)	559/160	477/53	82/107
Treatment naïve (Y/N)	689/30	523/7	166/23
HCV RNA (log IU/ml)	5.3 (4.7-5.9)	5 (4.6-5.7)	5.9 (5.5-6.5)
rs8099917 (TT/GT/GG)	572/135/11	425/97/7	147/38/4
rs12979860 (CC/TC/TT)	565/137/11	422/98/7	143/39/4
rs12980275 (AA/GA/GG)	543/158/16	402/116/10	141/42/6
SVR/non-SVR	455/264	340/190	115/74

IFN, interferon monotherapy; PEG-RBV, peg-interferon plus ribavirin combination therapy; SVR, sustained viral responder.

[28]) have been reported to affect the outcome of interferon therapy in genotype 1-infected patients but such factors have not been closely examined in genotype 2 patients.

Single nucleotide polymorphisms (SNPs) and other genetic factors have been reported to be useful in predicting the outcome of interferon therapy. Polymorphisms in MxA [29,30], interferon alpha-receptor 1 [31], and osteopontin [32] have also been reported to be associated with interferon response. We also identified a MAPKAPK3 SNP [33] that is a predictive factor for interferon mono-therapy. Recently, several groups have reported an association between several SNPs in the *IL28* locus and the effect of PEG-RBV combination therapy for genotype 1b [34–38] but only a few studies have examined the role of these SNPs in the treatment of other genotypes. In this study, we analyzed predictive factors for SVR in genotype 2a and 2b patients treated with PEG-RBV. Because PEG-RBV was only approved for use in Japan in 2005, we also examined predictive factors in patients who were treated with interferon monotherapy, which is still used in the event of an adverse reaction to ribavirin.

Patients and methods

Patients and study design

We studied 719 Japanese patients with chronic hepatitis C (positive for HCV RNA for more than 6 months) who received interferon therapy with or without ribavirin between 2002 and 2008. Patients were treated at Toranomon Hospital in Tokyo, Hiroshima University Hospital, and hospitals belonging to the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html). All patients were negative for hepatitis B surface antigen, had no evidence of other liver diseases, such as auto-immune hepatitis or alcoholic liver disease, and had not received immunosuppressive therapy before enrollment in the study. All patients gave written informed consent to participate in the study in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and according to the process approved by the ethical committees of Hiroshima University and the SNP Research Center at the Institute of Physical and Chemical Research (RIKEN) in Yokohama.

PEG-RBV patients received weekly injections of peg-interferon-alpha-2b at 1.5 g/kg body weight for 24 weeks. Ribavirin was administered orally, and the dosage was determined based on the patient's body weight (600 mg for <60 kg, 800 mg for 60–80 kg, 1000 mg for >80 kg). Patients receiving interferon monotherapy were treated daily with 6 million units of IFN intramuscularly for 8 weeks, followed by the same dose three times a week for 16 weeks, for a total of 528 million units. Successful treatment was ascertained based on sustained virological response (SVR), defined as HCV RNA-negative six months after cessation of therapy. Fibrosis stage and activity were diagnosed by pathologists at each hospital according to the criteria of Desmet et al. [39]. Patients were classified as interferon treatment naïve or experienced based on prior interferon treatment but only parameters related to the most recent therapy were used in the analysis.

SNP Genotyping and quality control

We genotyped each patient for three *IL28B* SNPs previously reported to be associated with therapy outcome: rs8099917, rs12979860, and rs12980275. Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip or the Invader assay, as described previously [40,41]. We were unable to determine genotypes for one of the 796 patients for rs8099917, six of the patients for rs12979860, and two for rs12980275.

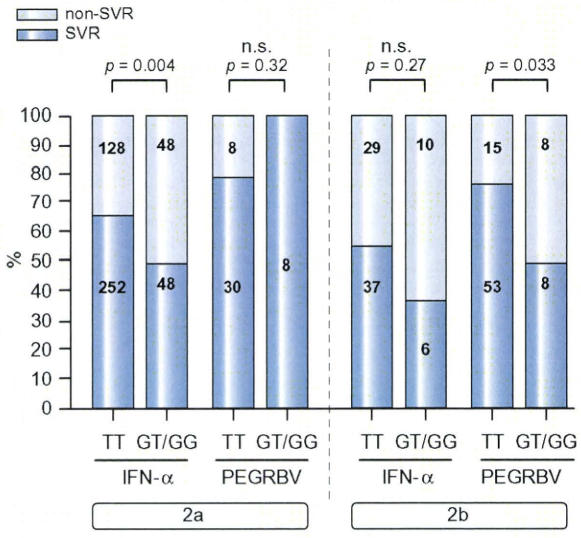

Fig. 1. Effect of interferon therapy on patients with genotype 2a and 2b infection. Sustained viral responders (SVR) and non-responders (non-SVR) were analyzed by IL28B SNP rs8099917 genotype, viral genotype, and treatment type. All patients were interferon-naïve.

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Table 2. Predictors for SVR in treatment-naïve patients treated with peg-interferon plus ribavirin combination therapy.

		Simple		Multiple			
Genotype	Variable	n	*p*	n	OR	(95% CI)	*p*
2a + 2b	Age	130	0.42				
	Sex	130	0.62				
	Genotype	130	0.21				
	Viral load	127	0.002 **	127	0.19	(0.06-0.55)	0.002 **
	Fibrosis	110	0.25				
	rs8099917	130	0.23				
	rs12980275	129	0.79				
2a	Age	46	0.77				
	Sex	46	0.62				
	Viral load	44	0.16				
	Fibrosis	39	0.75				
	rs8099917	46	0.8				
	rs12980275	45	0.77				
2b	Age	84	0.14				
	Sex	84	0.58				
	Viral load	83	0.01 *	83	0.13	(0.03-0.62)	0.01 *
	Fibrosis	71	0.08				
	rs8099917	84	0.03 *	83	0.23	(0.06-0.80)	0.02 *
	rs12980275	84	0.21				

*p <0.05; **p <0.01; ***p <0.001.

HCV RNA levels

HCV RNA levels, corresponding to initial viral load, were measured using one of several RT-PCR-based methods (the original Amplicor method, the high range method, or the TaqMan RT-PCR test). The measurement ranges of these assays were 0.5–850 KIU/ml, 5–5000 KIU/ml, and 1.2–7.8 log IU, respectively. Saturated samples were diluted with PBS and reanalyzed. All values were reported as log IU/ml.

Statistical analysis

Genotype-based associations were tested using the Cochran–Armitage trend test. Combined analysis was performed using the Mantel–Haenszel method. Simple and multiple regression analyses were used to examine the association between viral and clinical factors using p <0.05 as the criterion for inclusion in the multivariate model. HCV RNA was converted into a binary variable based on the median. Multivariate logistic regression analysis was performed using the Design package in *R* (http://www.r-project.org) with fast backward elimination and validation based on AIC score for model construction.

Results

Clinical characteristics are summarized by genotype in Table 1. The SVR rate was slightly but not significantly higher among patients with genotype 2a (340 out of 530; 64%) compared to genotype 2b patients (115 out of 189; 61%) (p = 0.43). Patients who were treated with PEG-RBV had a slightly but not significantly higher rate of SVR (111 out of 160; 69%) than patients treated with interferon monotherapy (344 out of 559, 61%) (p = 0.08). Because the number of patients treated with interferon monotherapy (559) greatly exceeds the number of patients treated with PEG-RBV (160), patients were analyzed separately by treatment type. Because 30 out of the 719 patients (4%) had received prior interferon treatment, only treatment-naïve patients were included in the analyses mentioned below, followed by a separate analysis of the effect of prior interferon treatment on SVR rate.

IL28B polymorphisms

Minor allele frequencies for rs8099917, rs12979860, and rs12980275 were 0.109, 0.112, and 0.132, respectively. The frequency of the rs8099917 risk allele was lower in SVR patients than non-SVR patients (0.089 vs. 0.14; p = 1.03e-05). The risk allele frequency among all patients was slightly higher than in the HapMap-JPT population (0.109 vs. 0.093; p = 0.01) but lower than in the HapMap-CEU population (0.109 vs. 0.183; p = 1.6e-05). We compared rs8099917 allele and genotype frequencies with 900 healthy Japanese subjects but found no significant differences. 67% of patients (372 out of 552) with the favorable rs8099917 TT genotype achieved SVR, compared to 51% (70 out of 136) of patients with GT or GG genotypes. Fig. 1 shows the joint effects of treatment type, viral genotype, and rs8099917 genotype. In every case results for rs8099917 and rs12979860 are the same, but both factors cannot be included in a multivariate model simultaneously due to multicollinearity, so results for rs8099917 are presented due to the higher genotyping success rate.

Predictive factors for SVR in patients treated with PEG-RBV

Among treatment-naïve patients treated with PEG-RBV, 78% (83 out of 106) of patients with rs8099917 TT achieved SVR compared

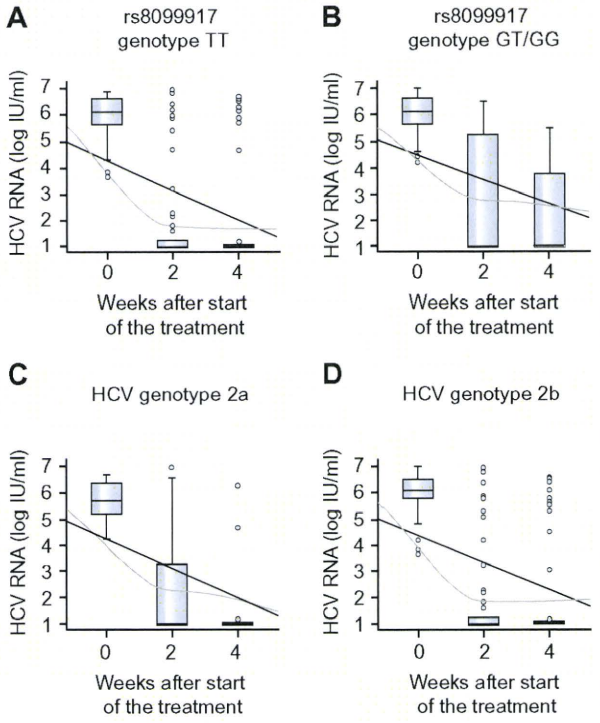

Fig. 2. Effect of rs8099917 genotype and HCV genotype on change in HCV RNA levels. HCV RNA levels at 0, 2, and 4 weeks after the start of peg-interferon plus ribavirin combination therapy in treatment-naïve patients. (A and B) Change in viral load for patients with the protective TT genotype for rs8099917 (A) compared to patients with the GT or GG genotypes (B). (C and D) Change in viral load for patients with HCV genotype 2a (A) versus genotype 2b (B).

to 67% (16 out of 24) of patients with non-TT genotypes ($p = 0.29$). In univariate and multivariate analyses, only viral load was an independent predictive factor for SVR ($p = 0.002$; Table 2), but when we examined genotypes 2a and 2b separately, rs8099917 genotype ($p = 0.02$) and viral load ($p = 0.01$) were both significant independent predictors of SVR for patients with genotype 2b, whereas no significant univariate or multivariate predictors were found for patients with genotype 2a. Notably, however, all 8 patients with genotype 2a with rs8099917 GT/GG achieved SVR (Fig. 1). The same pattern held for patients with rs12979860 TC/TT (9 SVR, 0 non-SVR) and rs12980275 GA/GG (11 SVR, 0 non-SVR) genotypes. Moreover, none of these patients was homozygous for the risk allele at each SNP.

Change in HCV RNA levels for patients treated with PEG-RBV

HCV RNA levels at the start of PEG-RBV therapy and after 2 and 4 weeks of treatment are plotted by rs8099917 genotype and viral genotype in Fig. 2. Under multivariate analysis, rs8099917 genotype was an independent predictive factor for change in HCV RNA level by week 2 ($p = 0.036$) but viral genotype was not significant ($p = 0.15$). For changes in HCV RNA levels by week 4, neither the rs8099917 genotype nor the viral genotype was significant ($p = 0.17$ and $p = 0.22$, respectively).

Predictive factors for SVR in patients treated with interferon monotherapy

Among patients treated with interferon monotherapy, 65% of patients with rs8099917 TT achieved SVR, compared to only 48% of patients with GT or GG genotypes ($p = 0.002$). Viral load and the rs8099917 and rs12980275 genotypes were significant univariate predictors of SVR, and under multivariate analysis viral load and rs8099917 remained as independent predictors (Table 3). When genotypes 2a and 2b were analyzed separately, viral load ($p = 0.001$) and rs8099917 genotype ($p = 0.014$) were independent predictive factors for SVR in patients with genotype 2a but no significant univariate of multivariate terms were found for genotype 2b.

Effect of prior interferon treatment

Thirty out of the 719 patients (4%) had previously received treatment with interferon. Among these patients, only 40% achieved SVR, compared to the 64% SVR rate among treatment-naïve patients. Initial viral load was the only independent predictor of SVR in these patients, whereas in treatment-naïve patients, viral load, rs8099917 genotype, and treatment type (PEG-RBV vs interferon monotherapy) were independent predictors of SVR (Table 4).

Development of resistance to interferon therapy

Over the course of therapy five patients developed resistance to PEG-RBV treatment. In each case the patient showed an initial drop in viremia followed by viral breakthrough. Three out of the five patients were heterozygous (T/G) for the rs8099917 genotype and two out of the five were homozygous for the favorable allele (T/T).

Discussion

As the effect of *IL28B* polymorphism has not been reported separately for genotype 2 and its subtypes so far, we investigated whether the polymorphism influences treatment outcome in patients with HCV genotype 2a and 2b infections. In addition to previously reported effects for genotypes 1 and 4, our results demonstrate that polymorphisms in the *IL28B* locus are also predictive for SVR in genotype 2 (Table 2). We also showed that the favorable *IL28B* SNP genotype is associated with a rapid decrease in HCV RNA levels, which is itself a predictive factor for SVR [42]. Several studies have reported that polymorphisms at the *IL28B* locus affect the outcome of peg-interferon and ribavirin combination therapy in patients with HCV genotype 1b [34–36,38]. In particular, associations with therapy outcome have been reported for two SNPs in strong linkage disequilibrium, rs8099917 (T/G), and rs12979860 (C/T). Only a few studies have examined the effect of the SNP on the treatment outcome for other genotypes. Rallón et al. reported that the rs12979860 genotype is associated with treatment outcome for genotypes 1 and 4 but not genotype 3 in patients with HIV/HCV co-infection [43]. Similarly Rauch et al. reported an association between rs8099917 polymorphism and NVR for genotypes 1 and 4 (difficult-to-treat) but not for genotypes 2 and 3 (easier-to-treat) but the effect due to genotype 2 alone is unclear [38]. In a recent study, Mangia et al. also exam-

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Table 3. Predictors for SVR in treatment-naïve patients treated with IFN monotherapy.

		Simple			Multiple			
Genotype	Variable	n	*p*		n	OR	(95% CI)	*p*	
2a + 2b	Age	559	0.35						
	Sex	559	0.17						
	Genotype	559	0.068						
	Viral load	507	0.0002	***	506	0.59	(0.45-0.77)	0.0001	***
	Fibrosis	450	0.61						
	rs8099917	558	0.001	**	506	0.52	(0.33-0.82)	0.005	**
	rs12980275	558	0.009	**					
2a	Age	477	0.19						
	Sex	477	0.2						
	Viral load	425	0.001	**	424	0.6	(0.44-0.81)	0.001	***
	Fibrosis	382	0.37						
	rs8099917	476	0.003	**	424	0.53	(0.32-0.88)	0.014	*
	rs12980275	476	0.01	**					
2b	Age	82	0.67						
	Sex	82	0.56						
	Viral load	82	0.47						
	Fibrosis	68	0.53						
	rs8099917	82	0.19						
	rs12980275	82	0.44						

*p <0.05; **p <0.01; ***p <0.001.

ined genotypes 2 and 3 and found a significant association between rs12979860 genotype and rapid virological response (RVR) at week 4 for genotype 2 [44]. While rs12979860 was not directly associated with SVR in their study, rs12979860 genotype was significantly associated with SVR among those patients who failed to achieve RVR. In this study, we found a significant association between rs8099917 genotype and RVR in multivariate analysis for genotype 2b ($p = 0.028$, data not shown) but not for genotype 2a. When RVR was included as a factor in multivariate logistic regression analysis for genotype 2b, RVR and rs8099917 genotype were both retained in the final model but only RVR was significant (RVR: $p = 4.9e\text{-}05$; rs8099917: $p = 0.0850$; data not shown). When only non-RVR patients were included, no factors were significant; however, there were only six patients who achieved SVR without RVR and only one patient who achieved RVR but then failed to achieve SVR.

Although SVR rate was generally higher for genotype 2a, as reported previously [20,21], we found few differences between genotypes 2a and 2b. However, when analyzed separately, the results suggest an interesting interaction between the *IL28B* genotype, the viral genotype, and treatment type. In particular, we found that rs8099917 was a predictive factor for genotype 2a treated with IFN but not PEG-RBV, and conversely for genotype 2b treated with PEG-RBV but not IFN. This result is likely due to the relatively small sample sizes, but nonetheless all 8 (100%) of the genotype 2a PEG-RBV patients lacking the favorable rs8099917 genotype achieved SVR, compared to less than 50% for IFN therapy or either type of treatment with genotype 2b. In fact, each patient was heterozygous for each of the three *IL28B* SNPs examined. A further complication is that each of the five patients who developed resistance to interferon therapy was infected with genotype 2a, and two of these patients had the favorable rs8099917 TT genotype while the others were heterozygous (GT). More detailed analysis will be required to interpret these results.

Because PEG-RBV therapy was not covered by insurance in Japan until 2005, we also present data comparing the effects of *IL28B* polymorphisms on treatment with the older IFN monotherapy versus the more recent PEG-RBV combination therapy. Although the small sample sizes within each patient group likely underestimate the effect of SNP genotype, we found that rs8099917 influences response to IFN monotherapy in patients with genotype 2a and also influences the response to PEG-RBV therapy in patients with genotype 2b. Although PEG-RBV is currently the standard treatment for chronic hepatitis C infection, interferon monotherapy may still be used in the case of intolerance to ribavirin; therefore, it is important to understand the direct effects of interferon with and without ribavirin. Moreover, even with the advent of protease inhibitors and other antiviral drugs undergoing clinical trials, they are likely to be co-administered with interferon to prevent the otherwise rapid emergence of resistant quasispecies [45].

In summary, we showed that the *IL28B* SNP genotype is an important predictive factor for SVR and early viral dynamics in patients with HCV genotypes 2a and 2b.

Conflict of interest

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

Table 4. Comparison of predictive factors for SVR based on prior treatment with interferon.

		Simple			Multiple			
	Variable	n	*p*		n	OR	(95% CI)	*p*	
All	Age	719	0.70						
	Sex	719	0.28						
	Genotype	719	0.42						
	Viral load	663	6.00E-02	***	662	0.63	(0.51-0.79)	4.30E-05	***
	Fibrosis	585	0.83						
	rs8099917	718	0.002	**	662	0.57	(0.38-0.85)	0.0055	**
	rs12980275	717	0.03	*					
	Treatment	719	0.054						
Naïve	Age	689	0.58						
	Sex	689	0.18						
	Genotype	689	0.62						
	Viral load	634	0.0011	**	633	0.53	(0.41-0.69)	2.00E-06	***
	Fibrosis	560	0.95						
	rs8099917	688	0.00059	***	633	0.5	(0.33-0.77)	0.0015	**
	rs12980275	687	0.013	*					
	Treatment	689	0.0013	**	633	3.01	(1.82-4.99)	1.80E-05	***
Experienced	Age	30	0.91						
	Sex	30	0.75						
	Genotype	30	0.14						
	Viral load	29	0.032	*	29	0.21	(0.05-0.87)	0.032	*
	Fibrosis	25	0.53						
	rs8099917	30	0.12						
	rs12980275	30	0.1						
	Treatment	30	N/A						

*p <0.05; **p <0.01; ***p <0.001.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Regular surveillance by imaging for early detection and better prognosis of hepatocellular carcinoma in patients infected with hepatitis C virus

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Abstract
Purpose This study evaluated the usefulness of regular check-ups by ultrasonography and contrast-enhanced imaging for early detection of hepatocellular carcinoma (HCC) in a retrospective analysis.
Patients and methods From April 2001 to March 2007, 240 consecutive patients with HCC who were infected with hepatitis C virus (HCV) were divided into three groups. Patients diagnosed with HCC by repeated imaging constituted Group A (surveillance group). Group B comprised patients in whom HCC was detected during scheduled doctor visits for liver disease or other diseases such as diabetes. Group C comprised non-screened patients.
Results The prevalence of solitary tumors decreased from Group A through Group B to Group C (66, 48 and 24%, respectively, $P < 0.001$). The proportion of patients in stages I and II decreased from 83% (103/124) in Group A to 53% (42/79) in Group B and 24% (9/37) in Group C ($P < 0.001$). The proportion of patients who were treated with curative procedures, such as resection or ablation, was highest at 80% (99/124) in Group A, and lower at 53% (42/79) in Group B and 27% (10/37) in Group C ($P < 0.001$). The cumulative survival rate was better in Group A than B ($P < 0.05$), and in Group B than C ($P < 0.001$). Periodical medical check-ups without imaging did not necessarily detect early-stage disease, even when HCC-related markers including des-γ-carboxy prothrombin were tested.
Conclusions Regular surveillance with ultrasonography and contrast-enhanced imaging is useful for detecting early-stage HCC and increase chances for curative treatments in patients with HCV-related chronic liver disease.

Keywords Hepatocellular carcinoma · Early detection · Curative procedures · Survival rates · Surveillance

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide [1], and patients with HCC continue to suffer an unsatisfactory prognosis. Surveillance for HCC should aim at decreasing mortality, and early detection is vital for therapeutic success. Serum levels of alpha-fetoprotein (AFP) and ultrasonography are widely accepted screening tests for early diagnosis of HCC [2–11].

However, serological markers including des-γ-carboxy prothrombin (DCP) and glycosylated AFP have shown limited success in detecting HCC in early stages [12–14]. Recent advances in imaging technologies allow the detection of early HCC, as reported in the guideline of the American Association for the Study of Liver Diseases [14]. Patients need to be surveyed for HCC, taking into consideration the incidence of HCC and cost-effectiveness. The discovery of hepatitis B virus (HBV) and hepatitis C virus (HCV), responsible for the majority of HCC cases [15], has enabled providers to identify the population at risk for HCC.

In Japan, HBV and HCV infections are associated with HCC in 15 and 80% of the cases, respectively [16, 17]. This retrospective study focused on HCV-associated HCC in Japan, and compared the efficacy of three methods for diagnosing HCC diagnosis. As the results show, regular repeated imaging was useful for early detection of HCC in patients infected with HCV.

Patients and methods

Patients

From April 2001 to March 2007, 338 consecutive patients were diagnosed with HCC in our institution. Among them, 240 patients infected with HCV were enrolled in this study. We retrospectively examined the procedure of diagnosis from clinical records and classified patients into one of three groups according to the method for diagnosing HCC. A total of 124 patients were diagnosed with HCC by regular imaging procedures such as ultrasonography, and they were categorized into the surveillance group (Group A). Hepatic damages such as rough surface pattern of the liver and dullness on the liver edge, as well as the detection of obvious varices on the first ultrasonography, led them to receive repeated imaging procedures. In 82% (102/124) of Group A patients, the interval between the latest imaging and diagnosis of HCC was within 6 months. The average interval between the latest imaging and diagnosis of HCC was 4.3 months [median, 3.6 months (range 2–11 months)]. They also received tests for HCC-related markers at least every 3 months. Group B comprised 79 patients who had been diagnosed with HCC during scheduled doctor visits for HCV-related liver disease or other diseases such as diabetes. These patients were not enrolled in a surveillance program at the time, and had not undergone any imaging procedures for at least 1 year before the diagnosis of HCC, while they received tests for HCC-related markers at least every 3 months. Among them, 26 patients received imaging due to elevated levels of HCC-related markers, such as AFP and DCP. In the remaining 53 patients in Group B, imaging was performed incidentally; they had not received imaging over the previous 1 year. The 37 patients who had not been screened for HCC were classified into Group C. They were diagnosed with HCC when symptoms developed (32 patients) or incidentally during a diagnostic workout for unrelated medical conditions such as traffic accident (5 patients). The study conformed to the ethical guidelines of the declaration of Helsinki, and was approved by the Institutional Review Board.

Surveillance strategy

Figure 1 outlines the surveillance program. Briefly, detection of any mass on ultrasonography instigated repeated imagings if the nodule diameter was up to 1 cm, or a dynamic study if the diameter exceeded 1 cm. HCC nodules are characterized by an intense contrast uptake during the arterial phase of dynamic computed tomography (CT) or magnetic resonance imaging (MRI), with the contrast washed away during the delayed or venous phase [12–14]. In the present study, the specific pattern of arterial uptake followed by venous washout was considered to represent HCC, since the value of "washout" in the venous phase has been recognized recently. If the vascular pattern on CT or MRI was not specific for HCC in a nodule with a diameter >1.5 cm, angiographically assisted CT or biopsy was undertaken to establish the diagnosis. Patients with nodules <1.5 cm in diameter who did not reveal HCC by angiographically assisted CT or biopsy underwent repeated surveillance procedures, and subsequent enlargement of the nodule during follow-ups indicated shifting to a dynamic study.

Diagnosis of cirrhosis

The diagnosis of chronic liver disease was made at the time of HCC detection by the following procedures.

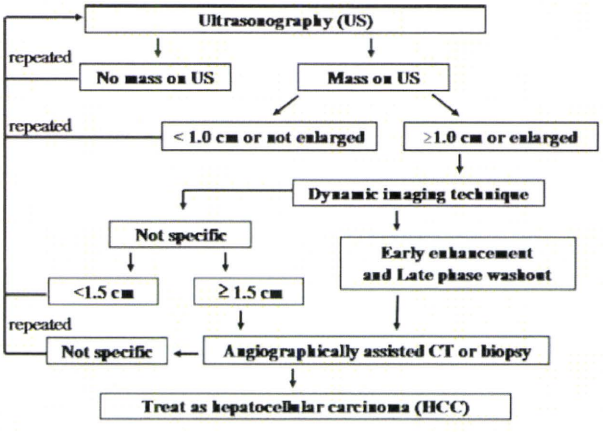

Fig. 1 Flow chart for the surveillance program including repeated imaging procedures

Histological findings were obtained in surgical specimens from 85 patients, and cirrhosis was diagnosed in 61 and chronic hepatitis or liver fibrosis in the remaining 24. Gastrointestinal varices in an additional 24 patients were considered diagnostic of cirrhosis. The remaining 131 patients were diagnosed to have cirrhosis according to the histologic scoring system [18].

Staging

Cancer stage was assessed by ultrasonography and dynamic CT or MRI. A total of 193 patients (80%) underwent angiography and/or angiographically assisted CT to obtain further details prior to resection, ablation or transarterial chemoembolization. In those patients, staging was also assessed by imaging on angiography and/or angiographically assisted CT. All patients underwent a chest X-ray, while additional investigations to detect metastases were performed only when extrahepatic involvement was suspected. Staging was not assessed by histologic findings on surgically resected specimens, even when they were available. Staging was determined according to the Liver Cancer Study Group of Japan classifications [19]. Staging was made also by the Milan criteria [20].

Treatment selection

Hepatic resection was indicated particularly to the patients with localized HCC who had maintained hepatic reserve capacity. When resection was contraindicated or refused by patients, the most appropriate treatment was selected according to the tumor status and liver function preserved [21]. Percutaneous ablation by ethanol injection [22] or radiofrequency ablation (RFA) [23] was considered in patients who had 1–3 nodules <3 cm in diameter, and were without vascular invasion or extrahepatic metastases. Transarterial chemoembolization [24] was offered to patients with either a paucifocal nodule not treatable by percutaneous ablation or multiple tumors not accompanied by thrombosis in main portal veins or extrahepatic metastasis. For the patients in Child-Pugh class C, transarterial chemoembolization was not recommended. In this study, resection and ablation were considered curative procedures based on their high efficacy.

Statistical analysis

The following 11 parameters were analyzed: age, sex, AFP, DCP, prothrombin activity, serum albumin level, total bilirubin level, liver state, tumor stage, HCC treatment and survival. Efficacy of the imaging program was evaluated by comparing clinical manifestation and prognosis among patients in the three groups. Differences in the distributions of tumor stage, tumor markers, and HCC treatment were evaluated by chi-squared test or Student's t test. Survival was calculated from the time of treatment start in patients who received it, and from the time of cancer diagnosis in patients without treatment. Data were censored at the time of death or the last follow-up visit. Survival was calculated according to the Kaplan–Meier method, and survival curves were compared by log-rank test. P values less than 0.05 were considered statistically significant.

Results

Background characteristics

There is no difference between Groups A and B in background of the patients except the programs with or without imaging. Table 1 details the background characteristics of all patients. Although the prevalence of cirrhosis was similar among the three groups, patients in Group C had poorer hepatic reserve with respect to albumin and total bilirubin levels ($P < 0.001$). The prevalence of non-cirrhotic liver in patients under 74 years was 26% (42/161), and 42% (33/79) in patients over 75 years. These differences were statistically significant ($P < 0.01$).

Features of HCC

The majority of HCC nodules were diagnosed by dynamic study including angiographically assisted CT, while HCC nodules in only 4 (1.7%) were confirmed by fine needle biopsy. Table 2 compares characteristics of HCC among the three groups. The frequency of solitary tumors was 66% (82/124) in Group A, 48% (38/79) in Group B, and 24% (9/37) in Group C, with a significant difference among three groups ($P < 0.001$). Nodules measuring less than 2 cm were detected in 64% (80/124) of patients in Group A, 25% (20/79) of those in Group B, and only 5% (2/37) of those in Group C ($P < 0.001$). The frequency of non-advanced tumor state decreased from 88% (109/124) in Group A, to 52% (41/79) in Group B, and to 27% (10/37) in Group C ($P < 0.001$). Cut-off values were set at 200 ng/ml and 40 mAU/ml, respectively, on AFP and DCP. In Group A, 47% (58/124) of the cases were negative for both, 46% (57/124) were positive for either, and 7% (9/124) were positive for both. In Group B, 11% (9/79) of the patients were negative for both, while 65% (51/79) were positive for ether, and 24% (19/79) were positive for both. In Group C, 11% (4/37) of the patients were negative for both, 57% (21/37) were positive for either, and 32% (12/37) were positive for both. These differences were statistically significant ($P < 0.001$). Thus, most patients in Groups B and C were positive for

Table 1 Background characteristics of patients

	Group A (surveillance) (n = 124)	Group B (scheduled doctor visits) (n = 79)	Group C (non-screened) (n = 37)	P value
Age at diagnosis of HCC (years)				
Median (range)	69.7 (49–89)	72.8 (49–87)	69.6 (50–87)	<0.05[b]
Gender				
Men	79 (64%)	52 (66%)	28 (76%)	NS
Women	45 (36%)	27 (34%)	9 (24%)	
History of blood transfusion	28 (22%)	19 (24%)	6 (16%)	NS
Excessive alcohol intake[a]	25 (20%)	20 (25%)	15 (49%)	NS
Liver state				
Not cirrhotic	34 (27%)	31 (39%)	10 (27%)	NS
Cirrhosis	90 (73%)	48 (61%)	27 (73%)	
Prothrombin activity (%)				
Median (range)	86 (48–125)	88 (57–135)	83 (39–124)	NS
Albumin (g/dl)				
Median (range)	3.6 (2.1–4.6)	3.8 (2.8–5.1)	3.4 (2.5–4.5)	<0.001[c]
Total bilirubin (mg/dl)				
Median (range)	0.9 (0.3–2.7)	0.8 (0.2–6.8)	1.4 (0.3–6.8)	<0.001[c]

NS not significant

[a] Excessive alcohol intake was defined as consumption of more 86 g alcohol/day

[b] Significant difference between Group B and Group A or Group C

[c] Significant difference between Group C and Group A or Group B

Table 2 Characteristics of the HCC nodule

	Group A (surveillance) (n = 124)	Group B (scheduled doctor visits) (n = 79)	Group C (non-screened) (n = 37)	P value
Solitary	82 (66%)	38 (48%)	9 (24%)	<0.001[b]
Size of main nodule				
–2 cm	80 (64%)	20 (25%)	2 (5%)	<0.001[b]
2.1–3 cm	31 (25%)	14 (18%)	6 (16%)	
3.1–5 cm	12 (10%)	33 (42%)	8 (22%)	
5.1 cm–	1 (1%)	12 (15%)	21 (57%)	
Vascular thrombus	4 (3%)	9 (11%)	10 (27%)	<0.001[b]
Distant metastases	1 (1%)	1 (1%)	5 (14%)	<0.001[c]
Tumor marker[a]				
Both negative	58 (47%)	9 (11%)	4 (11%)	<0.001[d]
Either positive	57 (46%)	51 (65%)	21 (57%)	
Both positive	9 (7%)	19 (24%)	12 (32%)	
Within the Milan criteria	109 (88%)	41 (52%)	10 (27%)	<0.001[b]

[a] HCC related tumor marker: AFP, DCP. Arbitrary cutoff values of 200 ng/ml and 40 mAU/ml were used for AFP and DCP, respectively

[b] Significant difference among all three groups

[c] Significant difference between Group C and Group A or Group B

[d] Significant difference between Group A and Group B or Group C

either or both AFP and DCP. Most patients in Group C who were in early tumor stages were diagnosed with HCC incidentally.

Figure 2a shows the distribution of tumor stages according to the Liver Cancer Study Group of Japan [19]. Proportions of patients in stages I and II were highest in the

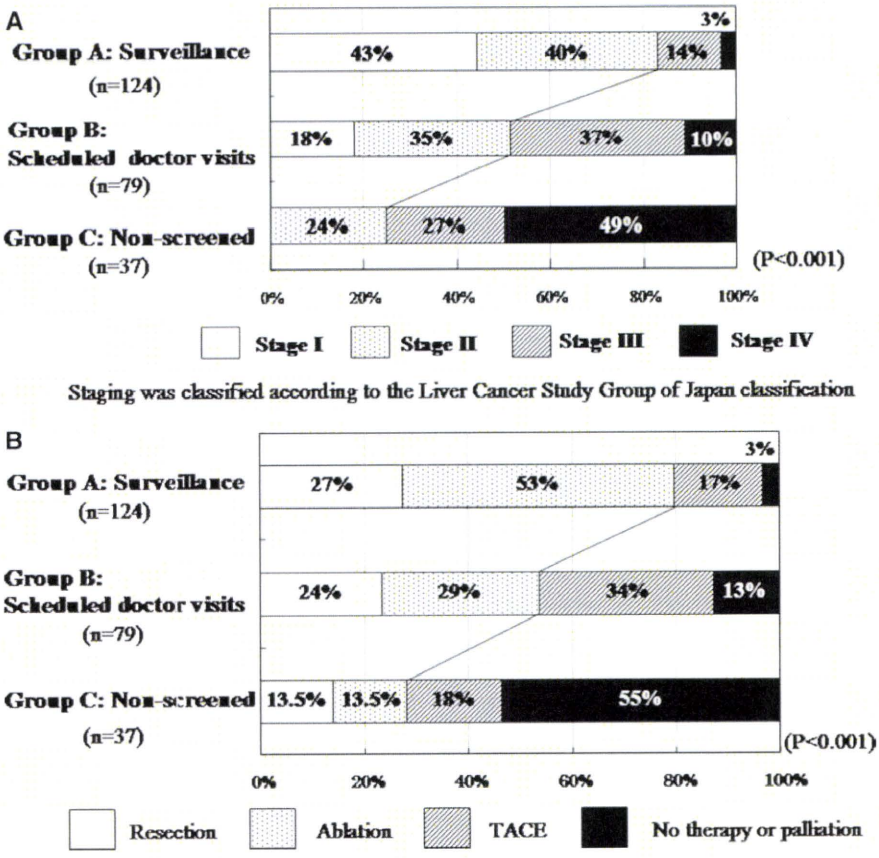

Fig. 2 **a** distribution of tumor stage according to the Liver Cancer Study Group of Japan [19]. **b** Distribution of treatment selected based on tumor stage and hepatic reserve

surveillance group (Group A); they decreased progressively through Group B to Group C ($P < 0.001$). The incidence of vascular thrombosis increased from 3% (4/124) in Group A to 11% (9/124) in Group B, and to 27% (10/37) in Group C ($P < 0.001$). Distant metastases were more frequent in Group C [14% (5/37)] than in Groups A and B [1% (1/124) and 1% (1/79), respectively] ($P < 0.001$). In Group A, the proportions of stages I and II was comparable between the patients with an interval between the latest imaging and diagnosis of HCC within 6 months and those with that of longer than 6 months [84% (86/102) vs. 77% (17/22)].

Treatment selection

Figure 2b shows the distribution of treatments selected based on the tumor stage and hepatic reserve. The proportion of patients treated with curative procedures, such as resection and ablation, was highest in Group A, and was lower in Groups B than C ($P < 0.001$). In Group C, the majority of patients received systemic chemotherapy or conservative care in hospice (palliation); most patients treated with curative procedures were diagnosed incidentally.

Survival

The median follow-up period was 35 months (range 3–94 months). During follow-ups, 148 patients died. Causes of death were cancer-related in 110 cases, liver failure in 6 (unrelated to treatment), gastrointestinal bleeding in 8, and others in the remaining 24. The distribution was similar between Groups A and B, while cancer-related causes were most prevalent (96%) in Group C. Figure 3a compares overall survival rates among the three groups. The cumulative survival rate was higher in group A than B ($P < 0.05$), and higher in group B than C ($P < 0.001$). Although survival rates of patients treated by curative procedures, such as resection and ablation, tended to be higher than the overall survival rate, there were no significant differences in the survival rates among patients in the three groups (Fig. 3b).

Discussion

For achieving better outcomes in patients with HCC, it is necessary to increase their eligibility for curative treatment. In the present study, 83% of patients under regular

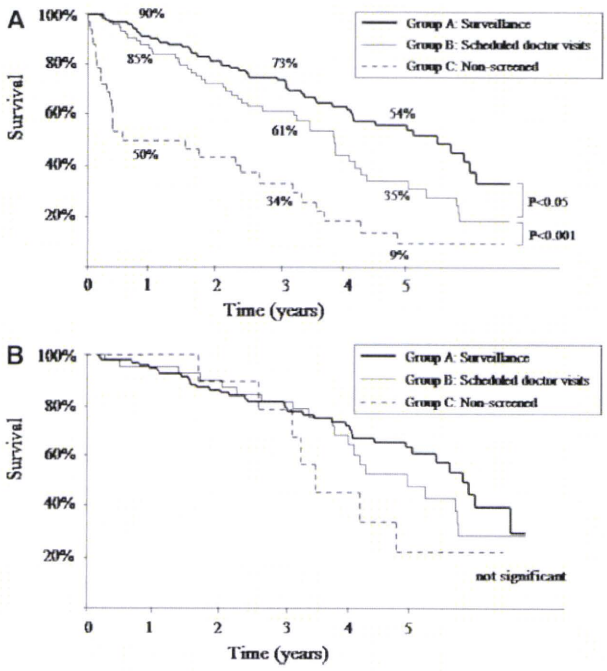

Fig. 3 **a** Survival rates in the three groups with different surveillance procedures. **b** Survival rates of the patients in three groups who had received curative treatments, such as resection and ablation

surveillance (Group A) were diagnosed with HCC at stage I or II, and the majority of them were indicated to curative treatments including surgical resection and RFA. As the results, patients in the surveillance group had a significantly better prognosis than those in the other groups without regular imaging screening (Group B) or none at all (Group C). Other reasons for the difference in prognosis among the three groups may include the following. Since the severity of underlying liver disease is a critical factor influencing the efficacy of surveillance programs, surveillance is reported to have few effects on improving the prognosis of patients with advanced cirrhosis [4, 10]. Although prevalence of cirrhosis was no different among the three groups, hepatic reserve was poorer in Group C than Group A or Group B. The dismal prognosis of patients in Group C, therefore, was attributed to either or both advanced tumor stage and poorer hepatic function. Indeed, analysis of only the patients who had received curative treatments, such as resection or ablation, revealed no significant differences in the survival among the three groups. However, the proportion of patients who had received curative treatment differed among the three groups with distinct diagnostic procedures.

Performance of surveillance would depend on the treatment selected and its efficacy. The 5-year survival of patients with a solitary HCC < 5 cm or up to 3 nodules <3 cm (Milan criteria [20]) exceeds 70% after transplantation, and that after resection surpasses 50% [12–14]. In general, transplantation offers the best long-term survival, and should be considered. In Japan, however, it is quite difficult for HCC patients to receive liver transplantation due to the shortage of donors [16], and liver resection is regarded as safe with less than 1% mortality [25]. Due to these background considerations, transplantation was not performed in the present study.

Should patients within the Milan criteria have undergone transplantation, differences in the outcome between Group A and Group B would have been reduced. In actuality, differences in the proportion of patients within the Milan criteria were lower that those in the distribution of stage I or II between them. The 5-year survival after resection was accomplished by 61% of patients with stage-II HCC and 73% of those with stage-I HCC; the staging was in accord with the definition of the Liver Cancer Study Group of Japan [16]. Thus, survival after resection in patients in Group A was comparable to that reported in transplanted patients within the Milan criteria. Indeed, the 5-year survival of patients in Group A who received curative treatments reached 63%. At present, the lack of sufficient liver donation is a worldwide problem in performing liver transplantation. Our results may indicate that surveillance by regular imaging can gain an excellent outcome where and when transplantation is hardly feasible, especially in patients with small HCC that can be treated by RFA or surgical resection.

With respect to HCC-related serological markers, most patients in Group A were negative for either AFP or DCP when they were diagnosed with HCC, in remarkable contrast to the majority of patients in Group B or C who were positive for either or both markers. In Group B, one-third of patients were tested for tumor markers during their scheduled doctor visits. However, the distribution of tumor stages was comparable between the patients with and without tumor-marker testing. Although yearly office visits would be helpful in early detection of HCC, periodical medical check-ups without screening by imaging may not necessarily detect early-stage disease, even if HCC-related markers such as AFP and DCP are tested for. This is the first report of poor performance of tumor markers including DCP in detecting early-stage HCC, and it suggests that various imaging procedures help detect HCC at a stage before levels of tumor markers elevate. Our results support the AASLD guideline that AFP alone should not be used for HCC screening when ultrasonography is not available [14]. On the other hand, it should be noted that 17% of patients in Group A in this study were diagnosed with HCC in stage III or IV, and 86% (18/21) of them were positive for either AFP or DCP. We therefore propose that HCC surveillance by regular imaging should be complemented with intermittent tests for tumor-markers, insofar as their elevated levels may reflect invisible nodules. As an

extension to this, repeated imaging with intermittent measurements of two different HCC-related tumor markers are included in the algorithm of the HCC surveillance program; it is described in Evidence-Based Clinical Practice Guidelines for HCC supported by the Japanese Ministry of Health, Labor and Welfare [26].

In a cirrhotic liver, small lesions detected by ultrasonography are likely to represent HCC. Even lesions not typical of cancer might transform into bona fide HCC during subsequent follow-ups. Generally, the incidence of HCC increases with the nodule size. In the present study, lesions >1 cm in diameter were examined by dynamic study, together with follow-ups by imaging at 3–6 month intervals, even when the appearance was atypical of HCC. Lesions >1.5 cm should be evaluated by dynamic study, preferably in combination with angiographically assisted CT or biopsy. Since the incidence of hypervascularity and moderately or poorly differentiated histology increases in HCC >1.5 cm [27–30], a 1.5-cm threshold in diameter may improve early diagnosis of HCC.

The AASLD guidelines recommend at-risk patients be screened by ultrasonography at 6–12-month intervals [14]. In our study, patients in Group B who had not undergone imaging for at least one year before the diagnosis often presented with advanced disease. A surveillance interval <12 months is therefore desirable. Although most patients in Group A were diagnosed with HCC within 6 months after the latest imaging, the proportion of stage I or II was similar between patients with the interval between the latest imaging and diagnosis of HCC below and above 6 months. However, optimal frequency of imaging was not determined in the present study. Further studies are required to determine the optimal screening interval.

Surveillance with imaging is feasible only in populations at risk for HCC, because radiological procedures are highly labor-intensive in comparison with serological testing. Major causes of cirrhosis in patients with HCC include HBV, HCV, alcoholic liver disease, exposure to aflatoxin, and possibly nonalcoholic steatohepatitis (NASH). Persistent infection with HBV or HCV is the most common cause of chronic liver disease including HCC, and increases the risk of HCC by approximately 20-fold. Heavy alcohol use and aflatoxin ingestion are environmental carcinogenic factors, and act synergistically with other risk factors [12–15]. In evaluating risks for HCC, geographic variations in incidence has to be taken into account. A recent study suggested an increased risk of HCC among patients with metabolic diseases such as diabetes or NASH [31–35]. However, the rate of HCC development in patients with NASH-related cirrhosis was significantly lower than that in those with HCV-related cirrhosis [33]. Thus, it remains uncertain how to assign surveillance programs to patients with metabolic disease.

In conclusion, surveillance programs including regular ultrasonography are useful for identifying HCC in early stages. HCC detected early is frequently indicated to curative treatments, such as resection and RFA, and is associated with better survival. Recently, several studies demonstrated that elderly patients infected with HCV developed HCC despite low-grade fibrosis stages [36, 37]. Elderly patients with HCV would be at high risk for the development of HCC, even though they do not show progression to cirrhosis. In the present study, most patients over 75 years were non-cirrhotic. Management of HCC should include early detection programs in all patients with HCV-related chronic liver disease including elderly patients in Japan.

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ITPA Polymorphism Affects Ribavirin-Induced Anemia and Outcomes of Therapy—A Genome-Wide Study of Japanese HCV Virus Patients

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CLINICAL ADVANCES IN LIVER, PANCREAS, AND BILIARY TRACT

BACKGROUND & AIMS: Ribavirin-induced anemia is one of the major causes of discontinuation and dose reduction during anti-hepatitis C virus therapy. Factors influencing this anemia, especially host genetic factors, are poorly understood. In this study we investigated predictive factors in hepatitis C virus patients treated with combination therapy. **METHODS:** We performed a 2-step genome-wide screening followed by replication analysis and fine-mapping using a total of 923 Japanese hepatitis C virus 1b–infected patients treated with pegylated-interferon plus ribavirin. We also applied logistic regression analysis to search for possible independent associations of clinical parameters and genetic variants with treatment-induced hemoglobin (Hb) decline as well as treatment outcomes. **RESULTS:** We identified a variant, located upstream of the inosine triphosphate pyrophosphatase gene on chromosome 20p13 that is significantly associated with treatment-induced anemia (combined $P = 6.0 \times 10^{-14}$). Resequencing and fine-mapping revealed several single nucleotide polymorphisms (SNPs) strongly associated with Hb decline, including the nonsynonymous SNP rs1127354 ($P = 3.5 \times 10^{-44}$), which was recently reported for other ethnic groups. Another reported SNP, the splicing variant-related SNP rs7270101, was not polymorphic in the Japanese population. Stratified analysis based on rs1127354 genotype revealed that inosine triphosphate pyrophosphatase expression is not correlated with Hb decline, suggesting that rs1127354 is a direct causal variant in the Japanese population. Multivariate analysis demonstrated that age, baseline Hb, baseline platelet count, and rs1127354 were independently associated with severe anemia (Hb <10 g/dL). **CONCLUSIONS: A missense substitution in inosine triphosphate pyrophosphatase gene affects ribavirin-induced anemia in hepatitis C virus–infected Japanese patients.**

Keywords: Hemolysis; Side Effect; SNP.

Hepatitis C virus (HCV) is one of the major causes of liver cirrhosis and hepatocellular carcinoma.[1] Sustained viral response, defined as negative for HCV RNA for 24 weeks after cessation of therapy, can be achieved by the current treatment regimen of pegylated-interferon (PEG-IFN) combined with ribavirin, but this outcome can be attained in <50% of patients infected with genotype 1 HCV.[2,3] Hemolysis is a common side effect of ribavirin and is the major reason for dose reduction. Age,[4,5] female sex,[4] baseline platelet level,[6] baseline hemoglobin (Hb) level,[5] dose,[4] plasma concentration[7] of ribavirin, and haptoglobin phenotype[6] have been reported to contribute to ribavirin-induced anemia and dose reduction. The extent of anemia caused by ribavirin varies greatly among individuals, suggesting a genetic influence. Recently, using a genome-wide association technique, Fellay et al reported that functional variants in inosine triphosphate pyrophosphatase (ITPA), including one coding and one intronic variant, were associated with treatment-induced anemia in HCV-infected patients.[8]

In this report, we describe the results of a genome-wide scan and fine-mapping of anemia in Japanese HCV patients treated with PEG-IFN and ribavirin combination therapy. We replicated the finding of Fellay et al that a nonsynonymous SNP in the ITPA gene is correlated with incidence of anemia. Moreover, we provide evidence suggesting that this variant is also associated with the outcomes of treatment with PEG-IFN and ribavirin combined therapy.

Abbreviations used in this paper: **GWAS, genome-wide association study; Hb, hemoglobin; HCV, hepatitis C virus; ITPA, inosine triphosphate pyrophosphatase; LD, linkage disequilibrium; PCR, polymerase chain reaction; PEG-IFN, pegylated-interferon; SNP, single nucleotide polymorphism.**

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Table 1. Clinical Characteristics of the Patients

	GWAS-1	GWAS-2	Replication-1
n	453	212	258
Age, *y*, mean (SD)	57.4 (11.2)	58.7 (10.8)	55.3 (12.7)
Sex, M/F, n	269/184	108/104	134/124
Weight, *kg*, mean (SD)	60.2 (10.3)	59.4 (10.4)	60.8 (11.8)
Body mass index,[a] mean (SD)	23 (2.9)	23.1 (2.9)	23.2 (3.3)
Baseline platelet count, $\times 1000/mm^3$, mean (SD)	158 (52)	154 (57)	165 (60)
Baseline hemoglobin level, *g/dL*, mean (SD)	14 (1.4)	13.9 (1.5)	13.9 (1.6)
Initial ribavirin dose, *mg/d*, n (%)			
200	30 (6.6)	3 (1.4)	19 (7.4)
400	42 (9.3)	28 (13.2)	38 (14.7)
600	200 (44.2)	89 (42)	118 (45.7)
800	170 (37.5)	84 (39.6)	76 (29.5)
1000	11 (2.4)	8 (3.8)	7 (2.7)
Hemoglobin level at week 4, mean (SD)	12 (1.7)	11.9 (1.6)	12.0 (1.7)
Hemoglobin decline at week 4, mean (SD)	2 (1.5)	2.0 (1.6)	1.9 (1.6)
Severe anemia at week 4, n (%)	42 (9.3)	17 (8.0)	32 (12.4)
Fibrosis, mild (F0-F2)/severe (F3-F4), n (%)	175/219 (44.0)	78/92 (45.9)	94/123 (43.3)
HCV genotype	1b	1b	1b

GWAS, genome-wide association study; HCV, hepatitis C virus; M/F, male/female; SD, standard deviation.
[a]Calculated as kg/m^2.

Patients and Methods

Study Population

In this study, we adopted a 2-step genome-wide association study (GWAS) consisting of a screening phase (GWAS-1 and -2) and a subsequent replication analysis (Replication-1) using a total of 923 subjects. Samples of the GWAS-1 were genotyped in the context of another genome-wide study concerning the genetics of treatment response to HCV therapy. The demographic features of the subjects are shown in Table 1. All patients were infected with HCV genotype 1b and treated with PEG-IFN plus ribavirin between 2003 and 2008 at either Toranomon Hospital Department of Hepatology or at Hiroshima University-affiliated hospitals. All patients had abnormal levels of serum alanine transaminase for more than 6 months and were positive for both anti-HCV antibody and serum HCV RNA. All patients were negative for hepatitis B surface antigen, had no evidence of other liver diseases, and had not received immunosuppressive therapy before enrollment in the study. Patients received weekly injections of PEG-IFN-α-2b at 1.5 μg/kg body weight and oral administration of ribavirin for 48 weeks. The amount of ribavirin was adjusted based on the subject's body weight (600 mg for <60 kg, 800 mg for 60−80 kg, 1000 mg for >80 kg). Patients with hemoglobin concentrations of <12 g/dL were given reduced dose of ribavirin (200 mg lower than standard dose determined by body weight) to prevent early discontinuation of the therapy. All subjects in the present study gave written informed consent to participate in the study according to the process approved by the Ethical Committee at the SNP Research Center, the Institute of Physical and Chemical Research (RIKEN), Yokohama, and conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

Genotyping and Quality Control

To identify causal variants for ribavirin-induced anemia, we applied a 2-stage approach for GWAS screening. In GWAS-1 we analyzed 453 samples using the Illumina HumanHap 610-Quad Genotyping BeadChip (San Diego, CA). Genotyping data were subjected to quality control before analysis. Genotyping for GWAS-2 and Replication-1 was performed using the Invader assay (Third Wave Technologies, Madison, WI), the TaqMan assay (Applied Biosystems, Foster City, CA), or by direct sequencing as described previously.[9,10]

Fine-Mapping Analysis

After the association analysis, fine-mapping was performed on the region surrounding the top marker. Using Phase II HapMap JPT genotype data, linkage disequilibrium (LD) blocks were defined by the Haploview program (http://www.broadinstitute.org/mpg/haploview/).[11] Resequencing around the top marker was also performed by direct sequencing of DNA from 48 unrelated Japanese HCV patients from the enrolled subjects. Using the Haploview program, tag SNPs were selected from among the identified SNPs based on a selection criteria of r^2 >0.8, and the tag SNPs were genotyped for all enrolled patients.

HCV RNA Level

The HCV RNA level was analyzed before interferon therapy, using reverse transcription polymerase chain reaction (PCR)−based methods (the high range method or the TaqMan reverse transcription-PCR test).