HCV patients were diagnosed as having cirrhosis by laparoscopy. Among these cirrhotic patients, three HBV patients and one HCV patient were diagnosed as having F3 fibrosis with liver biopsy. Liver cirrhosis was likely to be underestimated in HBV patients with liver biopsy. The higher proportion of diffuse irregular liver surface among HBV patients with F3 stage fibrosis was probably due to sampling error with needle biopsy. But there were no underestimated HBV patient diagnosed as having cirrhosis by laparoscopy (Table 2). With regard to cirrhosis, the surface findings of HBV and HCV are different. HBV-related cirrhosis is associated with the presence of large regenerative nodules. Thus, the discrepancy between MRL and needle biopsy was the type of viral infection. But this gap in diagnosis of liver cirrhosis can be filled by evaluating liver surface irregularities by MRL.

The indication for laparoscopy is limited due to its invasiveness. But MRL is derived from the construction of images of hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI, patients with contraindication for laparoscopy (e.g. bleeding tendency, old age) can be evaluated safely by the non-invasive MRL, as long as there are no contraindications for Gd-EOB-DTPA-enhanced MRI. Moreover, this examination can be repeated easily unlike laparoscopic examination. Further studies are needed to determine the usefulness of repeat MRL in the follow up of liver fibrosis.

Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid is a liver-specific contrast material for MRI and its safety and usefulness for detection of HCC has been reported.[35,37] The treatment strategy for HCC is different from other solid tumors because HCC develops in the cirrhotic liver. The Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy has gained wide acceptance because of its stratification capacity and its treatment indication.[38] The BCLC staging system is based on factors related to tumor stage, liver functional reserve and performance status because the prediction of prognosis is related to not only tumor stage but also underlying liver function in patients with HCC. In the present study, MRL showed that the liver surface findings changed with the progression of liver fibrosis. Especially, advanced liver cirrhosis was associated with increased incidence of right lobe atrophy (Table 3). In other words, advanced liver cirrhosis indicates reduction of functional liver reserve. Because patients of Child–Pugh B and C have impaired liver function, radical treatment for HCC must be avoided. As mentioned above, Gd-EOB-DTPA-enhanced MRI is used for evaluation of HCC stage. MRL is technically Gd-EOB-DTPA-enhanced MRI; this "one-stop-shop" method provides both HCC staging and liver functional reverse. Although transient elastography and multiple resonance elastography are useful non-invasive techniques for evaluation of liver fibrosis, they cannot be used to determine the stage of HCC. Thus, MRL may have an advantage in this respect.

Our study has certain limitations. First, because the 3-D shape of the liver is derived in our technique from MRI using Gd-EOB- DTPA contrast material, the technique does not allow evaluation of the color of the liver surface. Fujioka *et al.* reported that liver color was useful for the diagnosis of primary biliary cirrhosis.[39] Therefore, MRL is not entirely a complete substitute for laparoscopy in this regard. However, our method allows evaluation of the shape of the entire liver non-invasively similar to laparoscopy even in patients contraindicated for laparoscopy. Second, this study is based on the results of patients with chronic HBV or HCV infection. Patients with chronic liver diseases of other causes were not evaluated. However, HBV and HCV infections are two of the major causes of chronic liver disease and hepatocarcinogenesis, and were able to demonstrate the usefulness of MRL in high-risk groups. Further studies are needed to evaluate the usefulness of MRL in patients with chronic hepatitis of other etiologies. Third, the number of patients evaluated by laparoscopy-guided liver biopsy was small. So, further studies with a large number of patients are needed to strengthen usefulness of MRL.

Chronic viral liver disease is regarded as a high-risk for HCC. Gd-EOB-DTPA-enhanced MRI is a useful screening technique for HCC. The data of MRL are derived from Gd-EOB-DTPA-enhanced MRI. MRL and Gd-EOB-DTPA-enhanced MRI can be conducted simultaneously. In conclusion, MRL is a potentially useful non-invasive method for the evaluation of chronic viral hepatitis.

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A genome-wide association study of HCV-induced liver cirrhosis in the Japanese population identifies novel susceptibility loci at the MHC region

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Background & Aims: We performed a genome-wide association study (GWAS) of hepatitis C virus (HCV)-induced liver cirrhosis (LC) to identify predictive biomarkers for the risk of LC in patients with chronic hepatitis C (CHC).

Methods: A total of 682 HCV-induced LC cases and 1045 CHC patients of Japanese origin were genotyped by Illumina Human Hap 610-Quad bead Chip.

Results: Eight SNPs which showed possible associations ($p < 1.0 \times 10^{-5}$) at the GWAS stage were further genotyped using 936 LC cases and 3809 CHC patients. We found that two SNPs within the major histocompatibility complex (MHC) region on chromosome 6p21, rs910049 and rs3135363, were significantly associated with the progression from CHC to LC ($p_{combined} = 9.15 \times 10^{-11}$ and 1.45×10^{-10}, odds ratio (OR) = 1.46 and 1.37, respectively). We also found that *HLA-DQA1*0601* and *HLA-DRB1*0405* were associated with the progression from CHC to LC ($p = 4.53 \times 10^{-4}$ and 1.54×10^{-4} with OR = 2.80 and 1.45, respectively). Multiple logistic regression analysis revealed that rs3135363, rs910049, and *HLA-DQA1*0601* were independently associated with the risk of HCV-induced LC. In addition, individuals with four or more risk alleles for these three loci have a 2.83-fold higher risk for LC than those with no risk allele, indicating the cumulative effects of these variations.

Conclusions: Our findings elucidated the crucial roles of multiple genetic variations within the MHC region as prognostic/predictive biomarkers for CHC patients.

Introduction

Two million people in Japan and 210 million people worldwide are estimated to be infected with the hepatitis C virus (HCV), which is known to be a major cause of chronic viral liver disease [1]. Patients with chronic hepatitis C (CHC) usually exhibit mild inflammatory symptoms, but are at a significantly high risk for developing liver cirrhosis (LC) and hepatocellular carcinoma [2]. More than 400,000 people at present suffer from LC, which is ranked as the 9th major cause of death in Japan. In addition, liver cancer causes approximately 32,000 deaths per year, making it the 4th most common cause of death from malignant diseases. Thus, HCV-related diseases are important public health problems [3].

Clinical outcomes after the exposure to HCV vary enormously among individuals. Approximately 70% of infected persons will develop chronic hepatitis [4], and about 20–30% of CHC patients will develop cirrhosis, but others can remain asymptomatic for decades [2]. The annual death rate of patients with decompensated cirrhosis is as high as 15–30% [5]. Moreover, more than 7% of LC patients develop hepatocellular cancer in Japan and Taiwan, while the frequencies are less than 1.6% among other ethnic groups [6,7]. These inter-individual and inter-ethnic differences have been attributed to various factors such as viral genotypes,

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Abbreviations: CHC, chronic hepatitis C; GWAS, genome-wide association study; HCV, hepatitis C virus; LC, liver cirrhosis; MHC, major histocompatibility complex; OR, odds ratio; PBC, primary biliary cirrhosis; SNPs, single nucleotide polymorphisms.

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Table 1. Characteristics of samples and methods used in this study.

Stage	Source	Platform	Number of samples	Female (%)	Age, yr (mean ± SD)
GWAS					
Liver cirrhosis	BioBank Japan	Illumina Human Hap 610	682	313 (46.3)	67.1 ± 9.7
Chronic hepatitis C[a]	Hiroshima University	Illumina Human Hap 610	1045	371 (35.5)	55.2 ± 11.0
Replication					
Liver cirrhosis	Tokyo University	Invader assay	716	334 (46.8)	64.4 ± 10.4
	Hiroshima University		220	98 (44.5)	64.7 ± 8.98
Chronic hepatitis C[a]	BioBank Japan	Invader assay	1670	780 (46.8)	59.7 ± 12.6
	Hiroshima University		2139	1061 (51.8)	58.8 ± 9.20

[a]Number of samples that qualified. CHC patients with severe liver fibrosis (F3 or F4) or lower platelet counts (<160,000) were excluded.

alcohol consumption, age at infection, co-infection of HIV or HBV [8–10], insulin resistance, steatosis, and metabolic syndrome [11]. Previous gene expression analyses also identified various genes associated with liver fibrosis among patients with CHC [12–14]. In addition, miRNAs such as mir-21 and mir-122 were shown to be correlated with live fibrosis [15,16].

Currently, the genome-wide association study is the most common method to identify genetic variations associated with disease risk [17–20]. In addition, the roles of genetic factors in HCV-related diseases have been elucidated. *IL28B* is associated with spontaneous clearance of HCV [21] as well as with the clinical response to the combination therapy of pegylated interferon and ribavirin [22,23]. Recently, our group has shown that SNP rs2596542 on *MICA* [24] and SNP rs1012068 on *DEPDC5* [25] are significantly associated with HCV-induced liver cancer. Although liver cirrhosis is the major risk factor of liver cancer, a fraction of CHC patients will develop HCC without accompanying LC. Therefore, the underlying genetic background would be different between HCV-induced LC and HCV-induced HCC. Previous studies identified the association of genetic variants in *HLA-DQ/DR/B* [26–28], *2–5AS* [29], *TLR3* [30], and *PNPLA3* [31] with the risk of liver fibrosis among patients with CHC. However, a comprehensive approach for HCV-induced LC has not been conducted so far. Here we performed GWAS of HCV-induced LC to identify predictive biomarkers for the risk of LC in patients with CHC.

Materials and methods

Ethics statement

All subjects provided written informed consent. This project was approved by the ethical committees at University of Tokyo, Hiroshima University, Sapporo Kosei General Hospital, Toranomon Hospital, and Center for Genomic Medicine, Institutes of Physical and Chemical Research (RIKEN).

Study population

The characteristics of each cohort are shown in Table 1. In this study, we conducted GWAS and replication analysis on a total of 1618 HCV-induced LC and 4854 CHC patients. All subjects had abnormal levels of serum alanine transaminase for more than 6 months and were positive for both HCV antibody and serum HCV RNA. Among 1618 LC and 4854 CHC samples, 342 LC patients (21.14%) and 2997 CHC patients (61.70%) underwent liver biopsy. The remaining 1276 LC and 1857 CHC patients were diagnosed by non-invasive methods including hepatic imaging (e.g., ultrasonography, computed tomography, arteriography or magnetic resonance imaging), biochemical data (serum bilirubin, serum albumin, platelet, or prothrombin time), and the presence/absence of clinical manifestations of portal hypertension (e.g., varices, encephalopathy or ascites). The patients with CHC or LC were recruited for this study regardless of their treatment history. We excluded from the analysis the followings CHC patients: (1) advanced liver fibrosis (F3 or F4 by New Inuyama classification) [32], (2) platelet count under 160,000 for patients without liver fibrosis staging, and (3) HBV co-infection. Characteristics of each study cohort are shown in Table 1. In brief, DNA of HCV-induced LC and CHC patients was obtained from Biobank Japan (http://biobankjp.org/) [33], the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/researchprofile/pdf/liverstudygroupe.pdf), Toranomon Hospital, and the University of Tokyo. All subjects were of Japanese origin.

SNP genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a standard method. In GWAS, we genotyped 682 LC and 1045 CHC samples using Illumina Human Hap 610-Quad bead Chip (Supplementary Fig. 1). Samples with low call rate (<0.98) were excluded from our analysis (six LC and two CHC samples). We then applied SNP quality control as follows: call rate $\geqslant$0.99 in LC and CHC samples, Hardy–Weinberg $p \geqslant 1 \times 10^{-6}$ in LC and CHC samples. Consequently, 461,992 SNPs on the autosomal chromosomes passed the quality control filters. SNPs with minor allele frequency of <0.01 in both LC and CHC samples were excluded from further analyses, considering statistical power in the replication analysis. Finally, we analyzed 431,618 SNPs in GWAS. Among the top ten SNPs showing $p < 1.0 \times 10^{-5}$, we selected nine SNPs for further analysis with LD threshold of $r^2 = 0.95$. In the replication stage, we genotyped 936 LC and 3809 CHC using multiplex PCR-based Invader assay (Third Wave Technologies).

Statistical analysis

The association of SNPs with the phenotype in the GWAS, replication stage, and combined analyses was tested by logistic regression analysis, upon adjusting for age at recruitment (continuous) and gender, by assuming additive model using PLINK [34]. In the GWAS, the genetic inflation factor λ was derived by applying logistic regressed p values for all the tested SNPs. The quantile–quantile plot was drawn using R program. The odds ratios were calculated using the non-susceptible allele as reference, unless stated otherwise. The combined analysis of GWAS and replication stage was verified by using the Mantel–Haenszel method. We set the significance threshold as follows; $p = 1 \times 10^{-5}$ in the GWAS stage (first stage) and $p = 6.25 \times 10^{-3}$ (=0.05/8) in the replication analysis. We considered $p < 5 \times 10^{-8}$ as threshold of GWAS significance in the combined analysis, which is the Bonferroni-corrected threshold for the number of independent SNPs genotyped in HapMap Phase II [35]. The heterogeneity across two stages was examined by using the Breslow–Day test [36]. We used Haploview software to analyze the association of haplotypes and LD values between SNPs. Quality control for SNPs was applied as follows: call rate $\geqslant$0.95 in LC and CHC samples, and Hardy–Weinberg $p \geqslant 1 \times 10^{-6}$ in CHC samples in replication stage. The statistical power was 19.51% in GWAS (the first stage) ($p = 1.00 \times 10^{-5}$), 97.98% in replication ($p = 0.05/8$), and 74.76% in the combined stage ($p = 5.00 \times 10^{-8}$) at minor allele frequency of 0.3 and OR of 1.3.

Imputation-based association analysis of HLA class I and class II alleles

We obtained an SNP or a combination of SNPs which could tag the HLA alleles in the Japanese population from a previous study [37]. Genotypes of tagging SNPs were imputed in the GWAS samples by using a Hidden Markov model programmed in MACH [38] and haplotype information from HapMap JPT samples

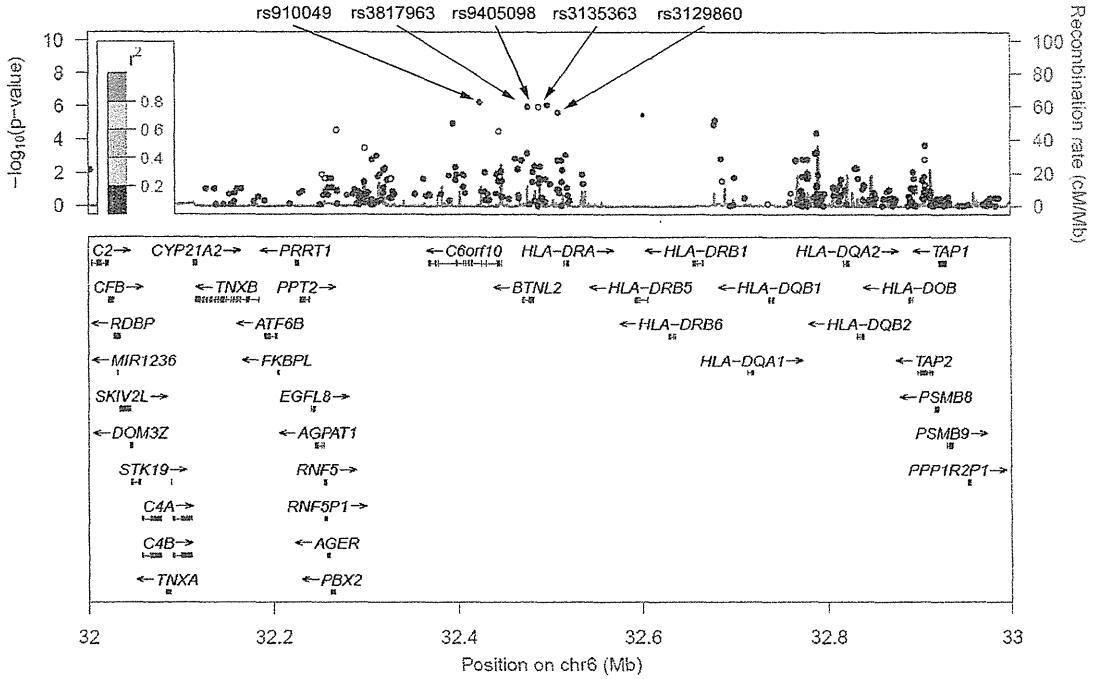

Fig. 1. Regional association plot at 6p21.3. (Upper panel) p Values of genotyped SNPs (circle) and imputed SNPs (cross) are plotted (as $-\log_{10}$ p value) against their physical position on chromosome 6 (NCBI Build 36). The p value for rs910049 at GWAS is represented by a purple diamond. Estimated recombination rates from HapMap JPT show the local LD structure. Inset; the color of the other SNPs indicates LD with rs3135363 according to a scale from $r^2 = 0$ to $r^2 = 1$ based on pair-wise r^2 values from HapMap JPT. (Lower panel) Gene annotations from the University of California Santa Cruz genome browser.

and 1000 genome imputation samples [39]. We applied the same SNP quality criteria as in GWAS, to select SNPs for the analysis. We employed the logistic regression analysis upon age and gender adjustment to assess the associations between HCV-induced LC and HLA alleles.

Software

For general statistical analysis, we employed R statistical environment version 2.9.1 (cran.r-project.org) or plink-1.06 (pngu.mgh.harvard.edu/~purcell/plink/). The Haploview software version 4.2 [40] was used to calculate LD and to draw Manhattan plot. Primer3 -web v0.3.0 (http://frodo.wi.mit.edu) web tool was used to design primers. We employed LocusZoom (http://csg.sph.umich.edu/locuszoom/) for regional plots. We used SNP Functional Prediction web tool for functional annotation of SNPs (http://snpinfo.niehs.nih.gov/snpfunc.htm) [41]. We used "Gene Expression Analysis Based on Imputed Genotypes" (http://www.sph.umich.edu/csg/liang/imputation) [42] for eQTL analysis. We used MACTH [43] web tool for searching potential binding sites for transcription factors (http://www.gene-regulation.com/index.htm).

Results

Genome-wide association study for HCV-induced liver cirrhosis

We performed a two-stage GWAS using a total of 1618 cases and 4854 controls (Supplementary Fig. 1). In the first stage, a whole genome scan was performed on 682 Japanese patients with HCV-induced LC and 1045 Japanese patients with CHC, using Illumina Human Hap 610-Quad bead Chip. The genotyping results of 431,618 single nucleotide polymorphisms (SNPs) obtained after our standard quality control were used for further analysis. CHC patients with severe liver fibrosis (F3 or F4 according to the New Inuyama classification [32]) or lower platelet counts (<160,000) were excluded from the control group. As progression from CHC to LC is strongly affected by age and gender, we performed logistic regression analyses including age and gender as covariates at all tested loci in our analyses. The genetic inflation factor lambda was 1.051, indicating that there is little or no evidence of population stratification (Supplementary Fig. 2A). Although no SNPs cleared the GWAS significance threshold ($p < 5 \times 10^{-8}$) at the first stage, we selected ten candidate SNPs showing suggestive association of $p < 1 \times 10^{-5}$ (Supplementary Fig. 2B and Supplementary Table 1). After excluding SNP rs6891116 due to almost absolute linkage with SNP rs10252674 ($r^2 = 0.99$), the remaining nine SNPs were further genotyped using an independent cohort, consisting of 936 LC and 3809 CHC cases, by multiplex PCR-based Invader assay as the second stage. We could successfully obtain genotype results for eight SNPs after the QC filter (call rate $\geqslant 0.95$ in LC and CHC samples, Hardy–Weinberg of $p \geqslant 1 \times 10^{-6}$ in CHC samples). The logistic regression analysis adjusted by age and gender revealed that five SNPs on chromosome 6q21.3 indicated a significant association with progression from CHC to LC after the Bonferroni correction ($p < 0.05/8 = 6.25 \times 10^{-3}$, Supplementary Table 2). A meta-analysis of the two stages with a fixed-effects model revealed that all of the five SNPs significantly associated with progression from CHC to LC (p values of 9.15×10^{-11}–1.28×10^{-8} with odds ratios (OR) of 1.30–1.46, Fig. 1 and Table 2). These five SNPs were located in the HLA class II region and were in strong linkage disequilibrium with each other (D' >0.75,

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Table 2. Summary of GWAS and replication analyses.

SNP	Stage	Allele (1/2)	Gene	Liver cirrhosis 11	12	22	RAF[a]	Chronic hepatitis C 11	12	22	RAF[a]	OR (95% CI)[b]	*p* value[c]	*p* value$_{het}$[d]
rs910049														
	GWAS	a/g	*C6orf10* (6p21.3)	24	217	435	0.196	25	224	794	0.131	1.73 (1.40-2.15)	5.39×10^{-7}	
	Replication			38	259	631	0.180	66	952	2790	0.142	1.37 (1.20-1.58)	7.59×10^{-6}	
	Combined[e]											1.46 (1.28-1.62)	9.15×10^{-11}	0.075
rs3817963														
	GWAS	a/g	*BTNL2* (6p21.3)	92	343	241	0.390	101	437	505	0.306	1.53 (1.29-1.81)	9.50×10^{-7}	
	Replication			130	395	395	0.356	409	1573	1816	0.315	1.22 (1.10-1.36)	2.66×10^{-4}	
	Combined[e]											1.30 (1.18-1.42)	1.28×10^{-8}	0.029
rs9405098														
	GWAS	a/g	No gene (6p21.3)	75	293	308	0.328	70	365	608	0.242	1.54 (1.30-1.84)	1.10×10^{-6}	
	Replication			100	361	462	0.304	249	1429	2129	0.253	1.30 (1.16-1.46)	5.64×10^{-6}	
	Combined[e]											1.37 (1.23-1.50)	1.04×10^{-10}	0.105
rs3135363														
	GWAS	c/t	No gene (6p21.3)	35	258	383	0.757	89	447	507	0.700	1.58 (1.32-1.90)	7.89×10^{-7}	
	Replication			73	322	540	0.750	389	1486	1929	0.702	1.30 (1.16-1.46)	7.94×10^{-6}	
	Combined[e]											1.37 (1.24-1.51)	1.45×10^{-10}	0.069
rs3129860														
	GWAS	a/g	No gene (6p21.3)	58	294	324	0.303	57	348	638	0.221	1.55 (1.29-1.82)	6.45×10^{-6}	
	Replication			88	339	507	0.276	208	1341	2246	0.231	1.28 (1.14-1.44)	2.53×10^{-5}	
	Combined[e]											1.36 (1.22-1.49)	1.07×10^{-9}	0.085

1618 (682 in GWAS and 936 in replication) liver cirrhosis and 4854 (1045 in GWAS and 3809 in replication) chronic hepatitis C samples were analyzed.
[a]RAF, risk allele frequency.
[b]OR, odds ratios; CI, confidence interval.
[c]*p* Values obtained by logistic regression analysis adjusted for age and gender under additive model.
[d]*p* Values of heterogeneities (Phet) across three stages were examined by using the Breslow–Day test.
[e]Combined odds ratio and *p* values for independence test were calculated by Mendel-hauzen and Laird method in the meta-analysis.

Supplementary Fig. 3). To further evaluate the effect of each variation on the progression from CHC to LC, we performed multiple logistic regression analyses. As a result, rs910049 (p of 1.91×10^{-3} with OR of 1.25) and rs3135363 (p of 1.49×10^{-4} with OR of 1.23) remained significantly associated with the progression risk from CHC to LC, while the remaining three SNPs failed to show significant associations ($p > 0.05$) (Supplementary Table 3). Thus, two SNPs, rs910049 and rs3135363, seem to be independent risk factors for HCV-induced LC.

Since reduced platelet level is associated with a poor prognosis among CHC patients [44] we excluded patients with platelet level of less than 160,000 from CHC groups to increase the risk of type 2 error in this study. We also conducted the analysis using only CHC patients diagnosed with liver biopsy. As a result, both SNPs reached genome-wide significance ($p < 5 \times 10^{-8}$), although the associations were reduced due to the smaller sample size (Supplementary Table 4).

Subgroup analyses, stratified by IFN treatment status, amount of alcohol consumption, and gender, were also performed, since these factors were shown to be associated with the prognosis of CHC patients [45–47]. A total of 334 LC patients (35.83%) and 2325 CHC (82.4%) were treated with IFN therapy. Although the frequency of IFN treatment was different between CHC and LC groups, these variations associated with the LC risk regardless of IFN treatment as well as gender and alcohol consumption (Supplementary Fig. 4A–C). When we included these factors as covariates, the association of these variations with HCV-induced LC was sustained, with OR of 1.48 and 1.56, and SNP rs3135363 still reached genome-wide significance ($p = 3.95 \times 10^{-9}$) (Supplementary Table 5).

The association of previously reported variations with HCV-induced LC

Non-synonymous SNP rs738409 (I148M) in the *PNPLA3* gene was shown to be associated with progression of LC in the previous prospective study in Caucasians [31]. SNP rs738409 was also associated with the severity of non-alcoholic fatty liver disease in Japanese [48]. Therefore, we analyzed SNP rs738409 in our case-control cohort, but rs738409 did not significantly associate with HCV-induced LC ($p = 0.24$ and OR = 1.10), although the risk G allele was more frequent among LC than CHC (Supplementary Table 6). Our result is similar to what observed among Caucasians in the previous study, in which rs738409 increased liver cancer risk among alcoholic cirrhosis but did not among hepatitis C cirrhosis [49]. Since biological studies demonstrated that its risk allele (G) abolishes the triglyceride hydrolysis activity of *PNPLA3* [50] *PNPLA3* variation would have a strong impact on non-viral cirrhosis.

Recently, GWAS in the Caucasian population identified the association of SNPs rs4374383, rs16851720 and rs9380516 with the progression of liver fibrosis after HCV infection [51]. However, SNPs rs4374383 and rs16851720 did not exhibit significant association ($p = 0.654$ and 0.231, respectively) in our sample set. Although SNP rs9380516 exhibited the association with p-value of 0.015, the risk allele showed an opposite result

Table 3. Results of three associated variations from candidate gene analyses.

Gene	Tagging SNP	Haplotype frequency		OR (95% CI)[a]		*p* value[b]
		Liver cirrhosis	Chronic hepatitis C			
*DQA1*0601*	rs2736182(T) + rs2071293(A)	0.038	0.019	2.80	1.38-3.32	4.53×10^{-4}
*DRB1*0405*	rs411326(C) + rs2395185(A) + rs4599680(A)	0.324	0.266	1.45	1.15-1.56	1.54×10^{-4}

Association was tested by comparing haplotype distribution between 682 liver cirrhosis and 1045 chronic hepatitis C samples in GWAS.
[a]OR, odds ratio; CI, confidence interval.
[b]*p* Values were obtained by case-control analysis of GWAS stage (*p* for haplotype were obtained by score test, implemented in R) (*DQA1*0601* and *DRB1*0405*). The *p* values obtained by logistic regression analysis adjusted for age and gender under additive model.

(Supplementary Table 6). Taken together, these SNPs would not be associated with liver fibrosis in the Japanese population.

Genes related to extracellular matrix turnover or immune response (*KRT 19*, *COL1A1*, *STMN2*, *CXCL6*, *CCR2*, *TIMP1*, *IL8*, *IL1A*, *ITGA2*, *CLDN 4*, and *IL2*) were shown to be implicated in liver fibrosis of chronic hepatitis C [14]. To further characterize these loci, we conducted imputation analyses in the GWAS sample set (682 cases and 1045 controls), using data from HAPMAP phase II (JPT), and found 163 SNPs in 9 loci. However, none of these SNPs indicated significant association with *p*-value of less than 0.01 (Supplementary Table 7). Thus, variations of these genes did not associate with progression from chronic hepatitis C to liver cirrhosis.

Imputation-based fine mapping of HLA region

The most significantly associated SNP rs3135363 is located within an intergenic region between *BTNL2* and *HLA-DRA*, and rs910049 is located in intron 7 of *C6orf10* gene (Supplementary Figs. 5 and 6). To further characterize these loci, we conducted imputation-based association analysis for the GWAS samples (682 LC and 1045 CHC samples) using data from HAPMAP Phase II (JPT), and could obtain the results of nearly 6000 SNPs in a 4-Mb genomic region. The regional association plots revealed that all modestly-associated SNPs are confined within a 700-kb region containing 21 genes, namely *TNXB*, *ATF6B*, *FKBPL*, *PRRT1*, *PPT2*, *EGFL8*, *AGPAT1*, *RNF5*, *RNF5P1*, *AGER*, *PBX2*, *C6orf10*, *BTNL2*, *HLA-DRA*, *HLA-DRB5*, *HLA-DRB6*, *HLA-DRB1*, *HLA-DQA1*, *HLA-DQA2*, *HLA-DQB1* and *HLA-DQB2* (Supplementary Fig. 5). Although 640 SNPs, including ten non-synonymous SNPs within the 4-Mb region, showed very modest associations ($p < 0.01$) with HCV-induced LC, none of these SNPs in this region revealed strong association with HCV-induced LC, after adjustment with the two SNPs, rs910049 and rs3135363 (Supplementary Fig. 7). Taken together, the associations observed in this region would reflect the association with rs910049 and rs3135363.

Previous reports indicated the association of *HLA-DRB1* and *HLA-DQ* alleles with HCV-induced chronic hepatitis in the Japanese population [26]. To investigate the association of HLA alleles with HCV-induced LC, we estimated the genotypes at the HLA region by applying the imputation results of HLA-tagging SNPs [37]. We could successfully determine 53 alleles of *HLA-A*, *B*, *C*, *DQA*, *DQB*, and *DRB* genes and find that *HLA-DQA1*0601* and *HLA-DRB1*0405* were strongly associated with HCV-induced LC (p values of 4.53×10^{-4} and 1.54×10^{-4} with ORs of 2.80 and 1.45) even after the Bonferroni correction ($p < 0.05/53 = 9.43 \times 10^{-4}$) (Table 3 and Supplementary Table 8A–E) [37].

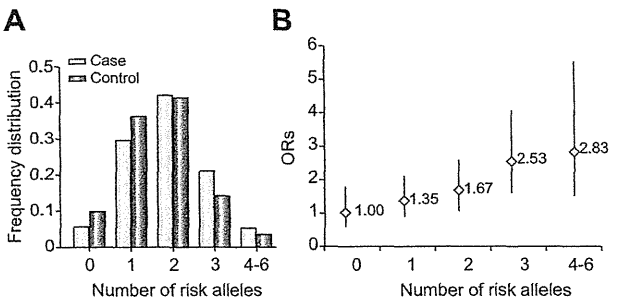

Fig. 2. Cumulative effects of liver cirrhosis risk alleles. (A) Frequency distribution divided by risk allele numbers (rs910049, rs3135353, and *HLA-DQA0601*) among liver cirrhosis (light blue bars) and chronic hepatitis C (dark blue bars) patients. (B) Plot of the increase odds ratio (OR) for liver cirrhosis according to the number of risk alleles. The ORs are relative to the subjects with no risk alleles (rs910049, rs3135353, and *HLA-DQA0601*). Vertical bars correspond to 95% confidence intervals. Horizontal line marks the null value (OR = 1).

Cumulative effect of multiple loci within the HLA region

SNPs rs3135363 and rs910049, *HLA-DQA1*0601*, and *HLA-DRB1*0405* are located within a 300-kb segment in the HLA class II region and show moderate linkage disequilibrium (Supplementary Fig. 8). To further evaluate these genetic factors, we performed multiple logistic regression analyses and found that rs910049 (p of 9.40×10^{-3} with OR of 1.38), rs3135363 (p of 3.94×10^{-4} with OR = 1.41), and *HLA-DQA1*0601* (p of 7.79×10^{-3} with OR of 1.54) were significantly associated with HCV-induced LC (Supplementary Table 9), indicating these three variations were independent risk factors for progression of CHC to LC.

To investigate the pathophysiological roles of rs910049 and rs3135363 in disease progression, we searched the eQTL database (http://www.sph.umich.edu/csg/liang/imputation) and found that risk alleles of rs910049 (A) and rs3135363 (T) were associated with lower expression of *HLA-DQA* (LOD of $\geqslant 6.86$ and 17.31, respectively) and *DRB1* (LOD of $\geqslant 12.01$ and 18.96, respectively), and with higher expression of *HLA-DQB1* (LOD of $\geqslant 6.76$ and 4.46, respectively) (Supplementary Table 10). Thus, rs910049 and rs3135363 are likely to affect the expression of HLA class II molecules and subsequently alter the risk of HCV-induced LC.

Finally, we examined the cumulative effects of rs910049, rs3135363, and *HLA-DQA1*0601*. Individuals with four or more risk alleles (8.8% of general population) have 2.83-fold higher risk of HCV-induced LC compared with those with no risk allele (15.0% of general population, Fig. 2).

Research Article

Discussion

We here demonstrated that multiple genetic variations in the MHC region were significantly associated with the risk of disease progression from CHC to LC, using a total of 1618 HCV-LC and 4854 CHC cases. Since a substantial proportion of patients with CHC show progression to LC in a certain time period, exclusion of CHC patients who have a high risk for LC from control subjects is essential to reduce the risk of false negative association. In this study, CHC patients with advanced fibrosis (F3 or F4 in stage) or with reduced platelet level (less than 160,000/μl) were excluded from the control samples, since these alterations are well-known risk factors for LC development [9,32]. Consequently, we were successfully able to identify the HCV-induced LC loci.

HLA genes are known to play critical roles in the regulation of our immune responses through controlling the antigen presentation to CD8 (class I) and CD4 (class II) T cells. Although previous studies indicated the association of HLA class I alleles such as *HLA-B57*, *HLA-A11*, and *HLA-C04* with persistent HCV infection [52,53], no SNPs in the HLA class I region exhibited strong association with HCV-induced LC. Here we identified three variations (rs910049, rs3135363, *HLA-DQA1*0601*) in the HLA class II region to be significantly associated with the progression risk from CHC to LC. Since two SNPs, rs910049 and rs3135363, had been indicated to affect expression levels of *HLA-DRB1* and *DQ*, our findings indicated the significant pathophysiological roles of HLA class II molecules in the development of HCV-induced liver fibrosis. Considering the function of *HLA-DQ* and *HLA-DR*, we suggest that the antigen presentation by HLA class II molecules is likely to play a critical role in the elimination of HCV-infected liver cells and subsequently prevent HCV-induced LC.

Direct acting antiviral drugs for HCV can cure up to 75% of patients infected with HCV genotype 1, and the lifetime risk of developing LC and HCC among HCV carriers was decreased during the two recent decades [54,13]. However, the amino acid sequence of the NS3 protease domain varies significantly between HCV genotypes and the antiviral efficacy differs in different HCV genotypes [55]. Moreover, protease inhibitors increased the incidence of adverse reactions such as anemia and skin rash [56]. Therefore, estimation of liver cirrhosis risk and prediction of treatment response would be essential to provide a personalized treatment and to achieve the optimal results. Due to the recent advances in pharmacogenetic studies, genetic factors associated with efficacy and adverse effects of anti-HCV treatment were identified. *IL-28B* is a powerful predictor of treatment outcome of pegylated interferon and ribavirin therapy [22], while a genetic variation in the *ITPA* gene was shown to be associated with ribavirin-induced anemia [57]. Since we conducted a retrospective study, and the majority of LC patients did not receive IFN treatment, we could not evaluate the treatment responses in our study design. However, SNPs identified in this study were associated with the LC risk independent of IFN treatment. Although the impact of each SNP was relatively weak compared with viral factors (HCV genotype, core and NS5A mutation [58]) and host factors (age, gender, obesity, and insulin resistance), we found that individuals with three or more risk alleles have a nearly three-fold higher risk of LC than those with no risk allele. Since lifetime risk of HCC development among HCV carriers is as high as about 27% for male and 8% for female [59], these three loci would have the strong effect on the clinical outcome of CHC patients. In general, the progression from chronic hepatitis C to liver cirrhosis usually takes more than 20–30 years. Therefore, a large scale prospective cohort study with more than 10-year follow-up is essential to evaluate the role of these variations as a prognostic biomarker. We would like to perform prospective analysis in future studies. We hope that our findings would contribute to clarify the underling molecular mechanism of HCV-induced liver cirrhosis.

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Conflict of interest

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

Authors' contributions

Y. U., K. K., K. C., and K.M. conceived and designed the study; Y. U., H. O., N. K., Y. K., R. M., N. H., and M. K. performed genotyping; A. T., P. H. Y. L., C. T., and N. K. performed quality control at genome-wide phase; M. O., R. T., M. O., K. K., D. M., H. A., J. T., H. K., Y. N., K. M. and M. K. managed DNA samples; Y. U. analyzed and summarized the whole results; Y. U., Y. N., and K. M. wrote the manuscript; Y. N. obtained funding for the study.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.12.024.

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Efficacy and Anticarcinogenic Activity of Ribavirin Combination Therapy for Hepatitis C Virus-Related Compensated Cirrhosis

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Key Words
Hepatitis C virus · Interferon · Ribavirin · Hepatocellular carcinoma · Cirrhosis · Biochemical response

Abstract
Objective: Anticarcinogenic activity of ribavirin combination therapy for hepatitis C virus (HCV)-related compensated cirrhosis is still unclear. ***Methods:*** In study 1, in 157 consecutive patients with HCV-related compensated cirrhosis, treatment efficacy with interferon plus ribavirin therapy was evaluated for 48 weeks of HCV genotype 1b (HCV-1b) or 24 weeks of HCV-2a/2b. In study 2, in 185 consecutive patients with HCV-related compensated cirrhosis, who showed no sustained virological response following the first course of interferon monotherapy, hepatocarcinogenesis rates were evaluated according to the additional treatment, and they were classified into three groups: no treatment, interferon monotherapy, and ribavirin combination therapy. ***Results:*** In study 1, in HCV-1b, rates of sustained virological response and sustained biochemical response were 21 and 56%, respectively. In HCV-2a/2b, rates of sustained virological response and sustained biochemical response were 70 and 78%, respectively. In HCV-1b, sustained biochemical response rates were significantly higher than those of sustained virological response. In study 2, the hepatocarcinogenesis rates in ribavirin combination therapy were significantly lower than those in interferon monotherapy and no treatment, respectively. ***Conclusion:*** Ribavirin combination therapy for HCV-related compensated cirrhosis reduces the risk of hepatocarcinogenesis in comparison with interferon monotherapy, and higher rates of sustained biochemical response might be associated with lower hepatocarcinogenesis rates.

Introduction

Hepatitis C virus (HCV) usually causes chronic infection, which can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1–5]. The life expectancy of patients with HCV-related cirrhosis is largely influenced by the development of hepatocellular carcinoma during the clinical course [3]. Because an effective and curative therapy for hepatocellular carcinoma remains

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Table 1. Profile and laboratory data at the start of ribavirin combination therapy in 157 patients with HCV-related compensated cirrhosis (study 1)

Demographic data	
Patients, n	157[1]
Sex (male/female), n	105/52
Age, years	58 (34–74)
Laboratory data	
Serum aspartate aminotransferase, IU/l	69 (7–235)
Serum alanine aminotransferase, IU/l	70 (14–585)
Leukocytes, /mm^3	4,100 (1,600–8,800)
Hemoglobin, g/dl	14.0 (9.4–17.6)
Platelet count, $\times 10^4$/mm^3	11.3 (6.1–32.2)
HCV genotype (1b/2a/2b), n	120/27/10
Levels of viremia, log IU/ml	6.1 (3.9–7.5)
Treatment	
Past history of interferon-based therapy, n	95 (60.5%)
PEG-IFNα-2b/IFNα-2b, n	110/47
Ribavirin dose, mg/kg	10.7 (2.7–15.1)
Duration of treatment, weeks	
Genotype 1b	48 (1–48)
Genotype 2a or 2b	24 (5–24)

Unless otherwise indicated, values represent median (range).

[1] 24 of the 157 patients with HCV-related compensated cirrhosis in study 1 were also included in study 2. They showed no sustained virological response following the first course of interferon monotherapy (≥24 weeks) and were treated additionally with ribavirin combination therapy (≥24 weeks).

limited at best, primary prevention of hepatocellular carcinoma in patients with chronic liver disease is of great importance at present.

Treatment of HCV-chronic hepatitis with interferon can induce viral clearance and marked biochemical and histological improvement [6, 7]. Furthermore, previous studies showed that interferon monotherapy reduced the risk of hepatocellular carcinoma [8–10]. However, an extended analysis of the Hepatitis C Antiviral Long-Term Treatment against Cirrhosis (HALT-C) cohort recently showed that long-term peginterferon (PEG-IFN) monotherapy could not reduce the incidence of hepatocellular carcinoma among patients with advanced hepatitis C who did not achieve sustained virological response, and patients with cirrhosis who received PEG-IFN monotherapy had a lower risk of hepatocellular carcinoma than controls [11]. Thus, it is controversial whether interferon monotherapy for patients with liver cirrhosis might reduce hepatocarcinogensis. Furthermore, it is still unclear whether ribavirin combination therapy for patients with liver cirrhosis might reduce the risk of hepatocellular carcinoma, and there are also no reports on whether ribavirin combination therapy could reduce the risk in comparison with interferon monotherapy.

The present study investigated the efficacy and anticarcinogenic activity of ribavirin combination therapy for HCV-related compensated cirrhosis, especially in comparison with interferon monotherapy.

Materials and Methods

Study Population

Two retrospective cohort studies were performed to investigate treatment efficacy and anticarcinogenic activity of ribavirin combination therapy for HCV-related compensated cirrhosis.

In the study 1 cohort, 157 consecutive patients of HCV-related compensated cirrhosis were recruited into the study protocol of interferon (PEG-IFNα-2b or IFNα-2b) plus ribavirin combination therapy for 48 weeks of HCV genotype 1b (HCV-1b) or 24 weeks of HCV-2a/2b, from 2001 to 2010 at Toranomon Hospital. In this retrospective study the rates of sustained virological response [HCV-RNA negativity at 24 weeks after the completion of therapy based on the COBAS TaqMan HCV test (Roche Diagnostics)] were evaluated as well as sustained biochemical response [normal level of serum alanine aminotransferase at 24 weeks after the completion of therapy (6–50 IU/l)]. Treatment efficacy was evaluated by intention-to-treat (ITT) analysis classified as treatment failure in patients who could not complete the treatment regimen and per protocol (PP) analysis. Table 1 summarizes the profiles and data of the 157 patients at the commencement of combination therapy with interferon plus ribavirin in study 1. They included 105 men and 52 women aged 34–74 years (median 58 years). 110 (70.1%) patients received PEG-IFNα-2b plus ribavirin, and the remaining 47 (29.9%) patients received IFNα-2b plus ribavirin. They received PEG-IFNα-2b at a median dose of 1.3 μg/kg (range 0.5–1.9 μg/kg) subcutaneously each week or IFNα-2b at a median dose of 6 million units (range 3–6 million units) intramuscularly each day (7 times per week for the initial 2 weeks followed by 3 times per week). They also received oral ribavirin at a median dose of 10.7 mg/kg (range 2.7–15.1 mg/kg) daily. In 56 of the 157 (35.7%) patients, the dose of ribavirin was reduced during treatment due to a fall in hemoglobin concentration. The median total duration of treatment in 120 patients of HCV-1b was 48 weeks (range 1–48 weeks), and that in 37 patients of genotype 2a or 2b was 24 weeks (range 5–24 weeks).

In the study 2 cohort (fig. 1), 185 consecutive patients of HCV-related compensated cirrhosis, who showed no sustained virological response following at the first course of interferon monotherapy (≥24 weeks) from 1987 to 2010 at Toranomon Hospital, were recruited. Hepatocarcinogenesis rates were evaluated according to the additional treatment (second course of treatment), and were classified into three groups: no treatment (106 patients), interferon monotherapy (≥24 weeks; 55 patients), and ribavirin combination therapy (≥24 weeks; 24 patients). 106 patients without treatment did not receive the additional treatment because of concerns about adverse effects, lack of time for treatment, physician recommendation based on the appearance of depression and car-

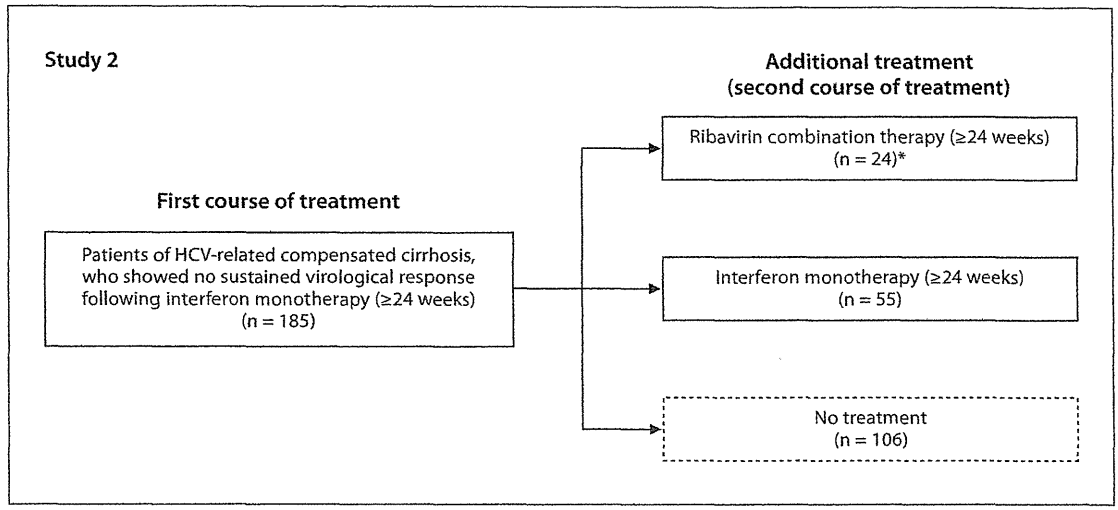

Fig. 1. For study 2, 185 patients with HCV-related compensated cirrhosis, who showed no sustained virological response following the first course of interferon monotherapy (≥24 weeks), were recruited. Hepatocarcinogenesis rates were evaluated according to the additional treatment (second course of treatment), and patients were classified into three groups: no treatment, interferon monotherapy (≥24 weeks), and ribavirin combination therapy (≥24 weeks). * 24 of 157 patients with HCV-related compensated cirrhosis in study 1 were also included in study 2.

diopulmonary disease during and after the first course of interferon monotherapy or the lower levels of serum alanine aminotransferase. The median follow-up time, from the end of the first course of interferon monotherapy until the last visit, was 6.4 years (range 0.0–21.0 years). 24 of the 157 patients in study 1 were also included in study 2; they showed no sustained virological response following the first course of interferon monotherapy (≥24 weeks) and were treated additionally with ribavirin combination therapy (≥24 weeks).

At the additional treatment of interferon monotherapy, 43 patients (78.2%) received IFNα alone, and the remaining 12 patients (21.8%) received IFNβ alone. They received interferon monotherapy including initial aggressive induction therapy (every day for 8 weeks followed by 3 times per week), with a median treatment duration of 44 weeks (range 24–382 weeks) at a median dose of 3 million units (range 3–10 million units) intramuscularly each day.

At the additional treatment of ribavirin combination therapy, 11 patients (45.8%) received PEG-IFNα-2b plus ribavirin, and the remaining 13 patients (54.2%) received IFNα-2b plus ribavirin. They received PEG-IFNα-2b at a median dose of 1.5 μg/kg (range 0.8–1.7 μg/kg) subcutaneously each week or IFNα-2b at a median dose of 6 million units (range 3–6 million units) intramuscularly each day (7 times per week for the initial 2 weeks followed by 3 times per week), with a median treatment duration of 26 weeks (range 24–48 weeks). They also received oral ribavirin at a median dose of 11.0 mg/kg (range 3.0–12.5 mg/kg) daily.

In the present studies, the patients were selected based on the following criteria. (1) Patients had compensated cirrhosis, but no decompensated cirrhosis or hepatocellular carcinoma. The diagnosis of compensated cirrhosis was based on clinical features (absence of signs for decompensation of ascites, encephalopathy, or gastrointestinal bleeding), laboratory tests, and peritoneoscopy or liver biopsy. (2) Patients were negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, Calif., USA), and positive for HCV-RNA by qualitative or quantitative analysis. (3) Patients were free of coinfection with human immunodeficiency virus. (4) Lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (5) Patients were free of other types of hepatitis, including hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (6) Each patient signed a consent form of the study protocol that had been approved by the human ethics review committee.

Laboratory Investigations

Blood samples were frozen at –80° within 4 h of collection and were not thawed until used for testing. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of the NS5 region [12]. HCV-RNA quantitative analysis was measured by branched DNA assay version 2.0 (Chiron Corp., Emeryville, Calif., USA), AMPLICOR GT HCV Monitor version 2.0 using the 10-fold dilution method (Roche Molecular Systems Inc., Pleasanton, Calif., USA), or COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). High viral load of viremia levels was defined as branched DNA assay ≥1.0 MEq/ml, AMPLICOR GT HCV Monitor ≥100 × 10^3 IU/ml, or COBAS TaqMan HCV test ≥5.0 log IU/ml. Low viral load was defined as branched DNA assay <1.0 MEq/ml, AMPLICOR GT HCV Monitor <100 × 10^3 IU/ml, or COBAS TaqMan HCV test <5.0 log IU/ml. The lower limit of HCV-RNA qualitative analysis (Amplicor, Roche Diagnostics, Mannheim, Germany) was 100 copies/ml, and that of

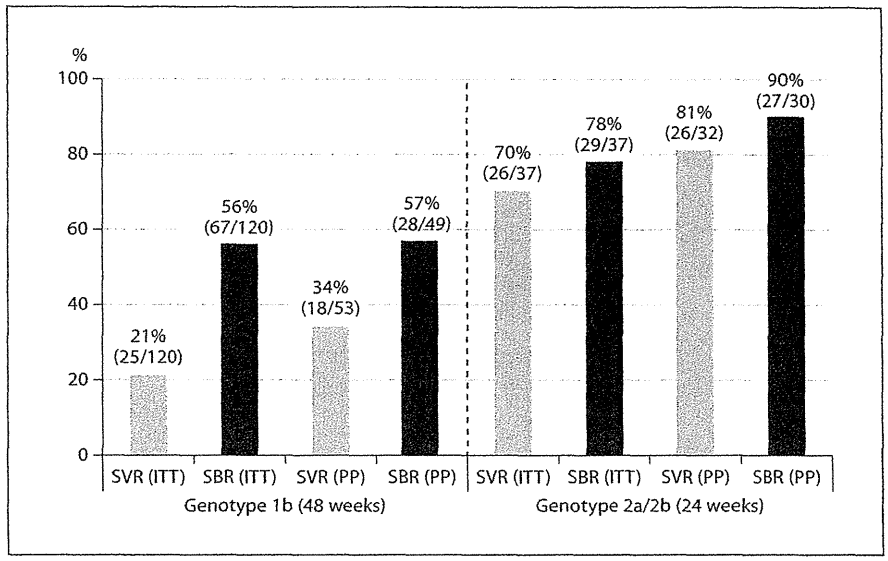

Fig. 2. In 157 patients with HCV-related compensated cirrhosis treatment efficacy with interferon plus ribavirin therapy was evaluated for 48 weeks of HCV genotype 1b or 24 weeks of genotype 2a/2b. In HCV genotype 1b, rates of sustained biochemical response (SBR) were significantly higher than those of sustained virological response (SVR; ITT analysis, $p < 0.001$, and PP analysis, $p = 0.028$).

COBAS TaqMan HCV test was 1.2 log IU/ml. The undetectable samples by HCV-RNA qualitative analysis or COBAS TaqMan HCV test were defined as negative HCV-RNA.

Follow-Up and Diagnosis of Hepatocellular Carcinoma

Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by careful interviews and medical examination at least once every month. Patient compliance with treatment was evaluated with a questionnaire. Blood samples were also obtained at least once every month before, during, and after treatment, and were also analyzed for levels of serum alanine aminotransferase and HCV-RNA at various time points.

Patients were examined for hepatocellular carcinoma by abdominal ultrasonography every 3–6 months. If hepatocellular carcinoma was suspected based on ultrasonographic results, additional procedures, such as computed tomography, magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy if necessary, were used to confirm the diagnosis.

Statistical Analysis

χ^2 test, Fisher's exact probability test, and Mann-Whitney's U test were used to compare the background characteristics between groups. Multiple comparisons were examined by the Bonferroni test. The cumulative hepatocarcinogenesis rates were calculated using the Kaplan-Meier technique, and differences between the curves were tested using the log-rank test. Statistical analysis of the hepatocarcinogenesis rates according to groups was calculated using the period from the end of the first course of interferon monotherapy until the appearance of hepatocellular carcinoma or until the last visit or until the start of the third course of interferon-based treatment. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with hepatocarcinogenesis. The hazard ratio (HR) and 95% confidence interval were also calculated. Potential predictive factors associated with hepatocarcinogenesis included the following 13 variables: age, sex, serum aspartate aminotransferase, serum alanine aminotransferase, platelet count, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, HCV genotype, levels of viremia, total duration of additional treatment, and group of additional treatment. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All p values <0.05 and <0.1 by the two-tailed test were considered significant ($p < 0.05$) and marginally significant ($p < 0.1$), respectively. Variables that achieved statistical significance ($p < 0.05$) on univariate analysis were tested by multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, Ill., USA).

Results

Efficacy of Ribavirin Combination Therapy (Study 1)

Treatment efficacy of a 48-week regimen of interferon plus ribavirin combination therapy in 120 patients infected with HCV-1b was evaluated. In ITT analysis, rates of sustained virological response and sustained biochemical response were 21% (25 of 120 patients) and 56% (67 of 120 patients), respectively. In the PP analysis, rates of sustained virological response and sustained biochemical response were 34% (18 of 53 patients) and 57% (28 of 49 patients), respectively (fig. 2). In both analyses, rates of sustained biochemical response were significantly higher than those of sustained virological response (ITT analysis, $p < 0.001$, and PP analysis, $p = 0.028$).

Table 2. Profile and laboratory data of 185 patients with HCV-related compensated cirrhosis according to additional treatment groups (study 2)

	No treatment	Interferon monotherapy (≥24 weeks)	Ribavirin combination therapy[1] (≥24 weeks)
Demographic data			
Patients, n	106	55	24
Sex (male/female), n	64/42	37/18	20/4
Age, years	56 (30–75)[a]	56 (35–76)[b]	51 (34–68)
Laboratory data			
Serum aspartate aminotransferase, IU/l	75 (26–285)	83 (35–213)	62 (30–160)
Serum alanine aminotransferase, IU/l	92 (17–400)	104 (30–316)	93 (36–250)
Platelet count, $\times 10^4/mm^3$	10.7 (2.5–18.2)[c]	10.8 (5.7–19.8)[d]	13.0 (5.2–23.5)
Total cholesterol, mg/dl	165 (103–273)[h]	152 (101–220)	160 (111–211)
High-density lipoprotein cholesterol, mg/dl	46 (25–93)	43 (21–65)	47 (28–56)
Low-density lipoprotein cholesterol, mg/dl	93 (38–168)	87 (45–139)	100 (34–135)
Triglycerides, mg/dl	96 (36–437)	80 (51–215)	108 (52–206)
HCV genotype (1b/2a or 2b), n	70/36	39/16	17/7
Levels of viremia (high viral load/low viral load), n	84/16	37/15[e]	24/0
Additional treatment			
Duration of additional treatment, weeks	–	44 (24–382)[f]	26 (24–48)
Sustained virological response (ITT), n	–	11 (20%)	7 (29%)
Sustained biochemical response (ITT), n	–	25 (45%)[g]	16 (67%)

Unless otherwise indicated, values represent median (range).

Demographic data and laboratory data, at the start of the first course of interferon monotherapy, are shown.

[a] $p = 0.013$, [b] $p = 0.030$, [c] $p = 0.002$, [d] $p = 0.015$, [e] $p = 0.006$, [f] $p = 0.044$, [g] $p = 0.083$ compared with ribavirin combination therapy by Bonferroni test, Mann-Whitney U test, or χ^2 test. [h] $p = 0.039$ compared with interferon monotherapy by Bonferroni test.

[1] 24 of 157 patients with HCV-related compensated cirrhosis in study 1 were also included in study 2. They showed no sustained virological response following the first course of interferon monotherapy (≥24 weeks), and were additionally treated with ribavirin combination therapy (≥24 weeks).

Treatment efficacy of a 24-week regimen of interferon plus ribavirin combination therapy in 37 patients infected with HCV-2a or 2b was evaluated. In the ITT analysis, rates of sustained virological response and sustained biochemical response were 70% (26 of 37 patients) and 78% (29 of 37 patients), respectively. In the PP analysis, rates of sustained virological response and sustained biochemical response were 81% (26 of 32 patients) and 90% (27 of 30 patients), respectively (fig. 2). In both analyses, rates of the sustained biochemical response were not significantly higher than those of the sustained virological response.

Profile, Laboratory Data, and Efficacy according to Additional Treatment Groups (Study 2)

Profile and laboratory data, at the start of the first course of interferon monotherapy of 185 patients with HCV-related compensated cirrhosis, are summarized in table 2. The age of patients with ribavirin combination therapy was significantly lower than that of patients with no treatment ($p = 0.013$; Bonferroni test) and interferon monotherapy ($p = 0.030$; Bonferroni test). The platelet count of patients of ribavirin combination therapy was significantly higher than that of patients without treatment ($p = 0.002$; Bonferroni test) and interferon monotherapy ($p = 0.015$; Bonferroni test). The total cholesterol level of patients with interferon monotherapy was significantly lower than that of patients without treatment ($p = 0.039$; Bonferroni test). Low viral load rates of patients with interferon monotherapy were significantly higher than those of patients with ribavirin combination therapy ($p = 0.006$; Bonferroni test). There were no other significant differences in clinical features at the start of the first course of interferon monotherapy among the three groups.

Additional treatment duration of only 1 patient, who was diagnosed with hepatocellular carcinoma during additional treatment, was evaluated using the period from the start of the second course of interferon monotherapy

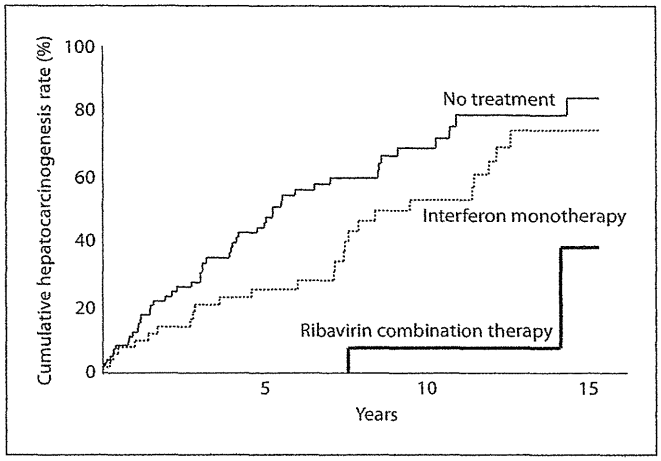

Fig. 3. Cumulative hepatocarcinogenesis rates in the three groups of additional treatment. The rates in no treatment were significantly higher than those in interferon monotherapy ($p = 0.047$; log-rank test) and ribavirin combination therapy ($p < 0.001$; log-rank test), and the rates in interferon monotherapy were significantly higher than those in ribavirin combination therapy ($p < 0.001$; log-rank test).

Table 3. Factors associated with hepatocarcinogenesis in 185 patients of HCV-related compensated cirrhosis identified by multivariate analysis (study 2): Cox proportional hazard model

Factors/category	Hazard ratio (95% confidence interval)	p
Additional treatment		
Ribavirin combination therapy	1	
Interferon monotherapy	4.47 (1.04–19.3)	0.045
No treatment	9.14 (2.19–38.2)	0.002
Age		
<55 years	1	
≥55 years	2.87 (1.76–4.67)	<0.001
Aspartate aminotransferase		
<58 IU/l	1	
≥58 IU/l	2.11 (1.20–3.74)	0.010

until the appearance of hepatocellular carcinoma. During additional treatment, the total duration of interferon monotherapy was significantly longer than that of ribavirin combination therapy ($p = 0.044$; Mann-Whitney U test). In ITT analysis, sustained virological response rates of ribavirin combination therapy (29%) were not different from those of interferon monotherapy (20%), but sustained biochemical response rates of ribavirin combination therapy (67%) tended to be higher than those of interferon monotherapy (45%; $p = 0.083$; χ^2 test) (table 2).

Predictive Factors Associated with Hepatocarcinogenesis by Multivariate Analysis

The data for the whole population sample were analyzed to determine those factors that could predict hepatocarcinogenesis. Hepatocarcinogenesis rates in older patients (≥55 years), in patients with higher levels of aspartate aminotransferase (≥58 IU/l), and lower levels of platelet count (<15.0 × 10^4/mm^3) were significantly higher than those in younger patients (<55 years), in patients with lower levels of aspartate aminotransferase (<58 IU/l), and higher levels of platelet count (≥15.0 × 10^4/mm^3), respectively ($p < 0.001$, $p = 0.006$, and $p = 0.017$; log-rank test). Furthermore, the rates in no treatment were significantly higher than those in interferon monotherapy ($p = 0.047$; log-rank test) and ribavirin combination therapy ($p < 0.001$; log-rank test), and the rates in interferon monotherapy were significantly higher than those in ribavirin combination therapy ($p < 0.001$; log-rank test) (fig. 3). Thus, univariate analysis identified four parameters that significantly correlated with hepatocarcinogenesis. These factors were entered into multivariate analysis, which then identified three parameters that significantly influenced hepatocarcinogenesis independently: additional treatment (no treatment; HR 9.14, $p = 0.002$), age (≥55 years; HR 2.87, $p < 0.001$), and levels of aspartate aminotransferase (≥58 IU/l; HR 2.11, $p = 0.010$) (table 3).

The data for 167 patients, except for 18 patients who showed a sustained virological response following additional treatment, were also analyzed to determine those factors that could predict hepatocarcinogenesis. Hepatocarcinogenesis rates in older age (≥55 years) and higher levels of aspartate aminotransferase (≥58 IU/l) were significantly higher than those in younger age (<55 years) and lower levels of aspartate aminotransferase (<58 IU/l), respectively ($p < 0.001$ and $p = 0.007$; log-rank test). Furthermore, the rates in ribavirin combination therapy were significantly lower than those in interferon monotherapy ($p < 0.001$; log-rank test) and no treatment ($p < 0.001$; log-rank test) (fig. 4). Thus, univariate analysis identified three parameters that significantly correlated with hepatocarcinogenesis. These factors were entered into multivariate analysis, which then identified three parameters that significantly influenced hepatocarcinogenesis independently: additional treatment (no treatment; HR 7.87, $p = 0.005$), age (≥55 years; HR 2.52, $p < 0.001$), and levels of aspartate aminotransferase (≥58 IU/l; HR 2.13, $p = 0.010$) (table 4).

Discussion

One of our previous studies indicated that the cancer-suppressive activity of interferon monotherapy in patients with HCV-RNA eradication was similar to that in patients with alanine aminotransferase normalization without HCV-RNA elimination [9]. Other studies also indicated a higher incidence and more rapid development of hepatocellular carcinoma in HCV patients with high levels of alanine aminotransferase [13, 14]. Collectively, these results suggest that the carcinogenic process in patients with chronic HCV infection is enhanced by high levels and fluctuations of alanine aminotransferase, and indicate a close relationship between suppression of inflammatory necrosis of hepatocytes and a lower incidence of hepatocellular carcinoma in patients with HCV-associated chronic liver disease. Recent studies based on interferon plus ribavirin combination therapy also showed that the attainment of sustained virological response or lower levels of alanine aminotransferase after ribavirin combination therapy could reduce the rates of hepatocellular carcinoma [15, 16], but the small numbers of patients with compensated cirrhosis (5% or less of all patients) were recruited. The present study 1 based on the patients with compensated cirrhosis showed that rates of sustained virological response and sustained biochemical response in HCV-2a/2b were high rates of 70 and 78%, and that rates of sustained biochemical response (57%) were significantly higher than those of sustained virological response (34%) in HCV-1b. Furthermore, the present study 2 based on the patients with compensated cirrhosis, who showed no sustained virological response following the first course of interferon monotherapy, also showed that sustained biochemical response rates of ribavirin combination therapy (67%) tended to be higher than those of interferon monotherapy (45%). Thus, in ribavirin combination therapy for compensated cirrhosis, higher rates of sustained biochemical response might be associated with lower rates of hepatocarcinogenesis. One limitation is that the present study was performed based on the small numbers of patients who showed no sustained virological response with interferon monotherapy. In further prospective studies a larger number of patients need to be investigated to confirm this finding.

Previous studies have shown that gender, age, fibrosis stage, alanine aminotransferase, and interferon regimen are important pretreatment predictors of hepatocarcinogenesis [9, 10, 17]. In the present study 2 based on the patients with compensated cirrhosis, higher age and aspartate aminotransferase were associated with higher hepatocarcinogenesis rates in the whole population sample and in the sample which excluded patients who showed sustained virological response following additional treatment. Furthermore, as treatment-related factors, the hepatocarcinogenesis rates in ribavirin combination therapy were significantly lower than those in interferon monotherapy. Thus, in patients with compensated cirrhosis representing a high-risk group of hepatocarcino-

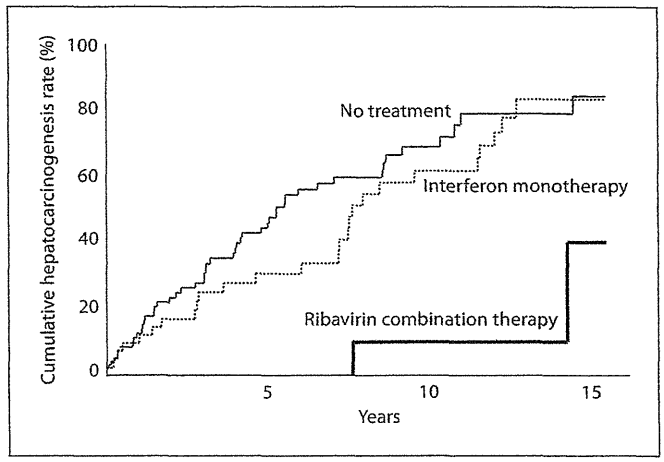

Fig. 4. Cumulative hepatocarcinogenesis rates in the three groups of additional treatment, except for patients who showed sustained virological response following additional treatment. The rates in ribavirin combination therapy were significantly lower than those in interferon monotherapy ($p < 0.001$; log-rank test) and no treatment ($p < 0.001$; log-rank test).

Table 4. Factors associated with hepatocarcinogenesis in 167 patients of HCV-related compensated cirrhosis, except for 18 patients who showed sustained virological response following additional treatment identified by multivariate analysis (study 2): Cox proportional hazard model

Factors/category	Hazard ratio (95% confidence interval)	p
Additional treatment		
Ribavirin combination therapy	1	
Interferon monotherapy	4.68 (1.08–20.3)	0.039
No treatment	7.87 (1.89–32.9)	0.005
Age		
<55 years	1	
≥55 years	2.52 (1.54–4.11)	<0.001
Aspartate aminotransferase		
<58 IU/l	1	
≥58 IU/l	2.13 (1.20–3.79)	0.010

genesis, ribavirin combination therapy might reduce the risk of hepatocellular carcinoma in comparison with interferon monotherapy. One reason for the higher anticarcinogenic activity by ribavirin combination therapy might be due to higher rates of sustained biochemical response. The other reason might be due to the difference in the background (lower age and higher levels of platelet count as an indicator of fibrosis stage) of patients with ribavirin combination therapy. Further studies of a larger number of patients matched for background, including age, sex, genotype, and platelet count, are required to investigate the rates of hepatocarcinogenesis and the mechanism of anticarcinogenic activity by ribavirin combination therapy for HCV-related compensated cirrhosis.

Two previous studies (PROVE1 and PROVE2) showed that the 12- and 24-week regimen of telaprevir/PEG-IFN/ribavirin could achieve sustained virological response rates of 35–60 and 61–69% in patients infected with HCV-1, respectively [18, 19]. However, a recent study (PROVE3) also showed that the sustained virological response rates were the lower rates of 39 and 38% with the 24- and 48-week regimen of triple therapy in previously nonresponding patients infected with HCV-1, who do not become HCV-RNA negative during or at the end of the initial PEG-IFN/ribavirin treatment, respectively [20]. Furthermore, the telaprevir-based regimen induces resistant variants [21–23] and has side effects including anemia and rash [18–20, 24]. Hence, patients, who do not achieve sustained virological response by triple therapy, need to be identified, in order to avoid unnecessary side effects and telaprevir-resistant variants. Recent studies identified amino acid substitutions at position 70 and/or 91 in the HCV-1b core region, advanced fibrosis stage, and higher levels of α-fetoprotein as pretreatment predictors of poor virological response to PEG-IFN/ribavirin combination therapy or triple therapy of telaprevir/PEG-IFN/ribavirin [23, 25–28], and these factors are also risk factors and surrogate markers of hepatocarcinogenesis [29–34]. Hence, ribavirin combination therapy for these patients might be an efficacious therapeutic regimen for sustained biochemical response and thus a reduction of the risk of hepatocarcinogenesis. Large-scale prospective studies should be conducted in the future to confirm this finding.

In conclusion, the present retrospective study indicated that ribavirin combination therapy for HCV-related compensated cirrhosis could reduce the risk of hepatocarcinogenesis in comparison with interferon monotherapy. Large-scale prospective studies need to be conducted in the future to confirm these findings.

Acknowledgments

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