Research Article

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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.

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

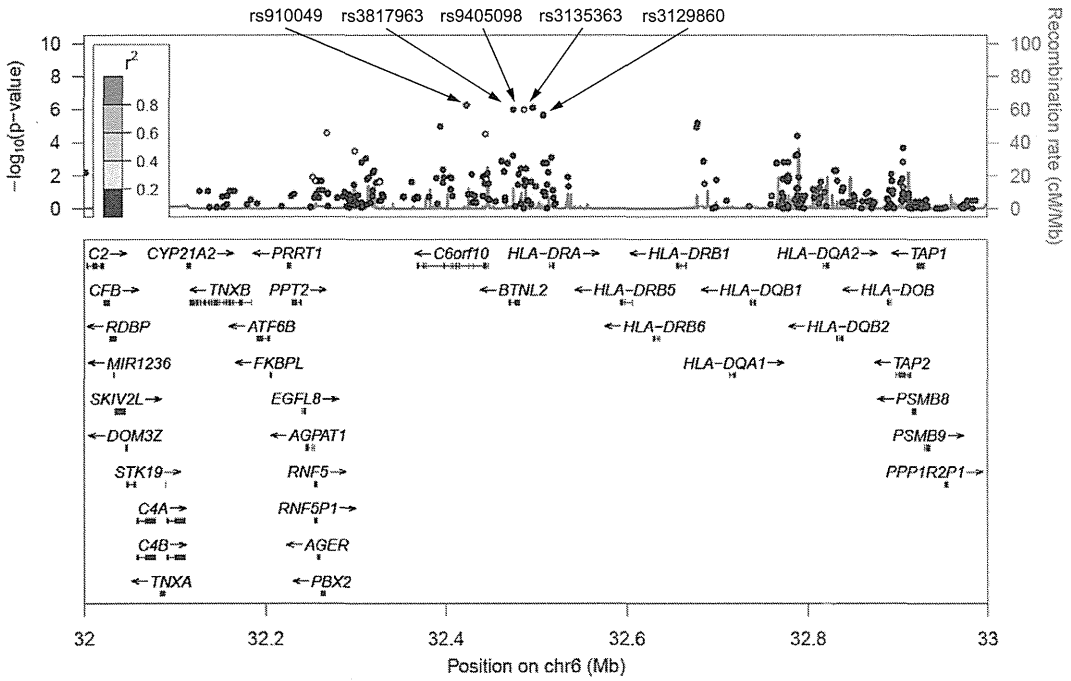

Fig. 1. Regional association plot at 6p21.3. (Upper panel) *p* Values of genotyped SNPs (circle) and imputed SNPs (cross) are plotted (as $-\log_{10} p$ value) against their physical position on chromosome 6 (NCBI Build 36). The *p* value for rs910049 at GWAS is represented by a purple diamond. Estimated 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,

Table 2. Summary of GWAS and replication analyses.

SNP	Stage	Allele (1/2)	Gene	Liver cirrhosis				Chronic hepatitis C				OR (95% CI)[b]	*p* value[c]	*p* value$_{het}$[d]
				11	12	22	RAF[a]	11	12	22	RAF[a]			
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].

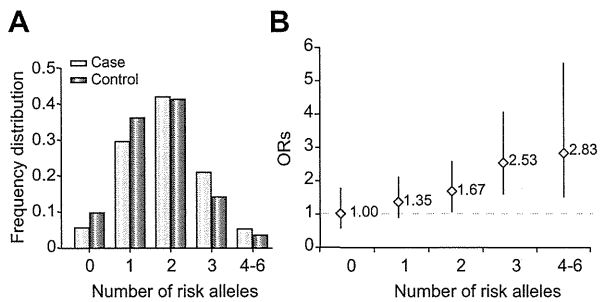

Fig. 2. Cumulative effects of liver cirrhosis risk alleles. (A) Frequency distribution divided by risk allele numbers (rs910049, rs3135353, and *HLA-DQA0601*) among liver cirrhosis (light blue bars) and chronic hepatitis C (dark blue bars) patients. (B) Plot of the increase odds ratio (OR) for liver 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).

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.

Acknowledgments

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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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Research Article

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The AMPK-related kinase SNARK regulates hepatitis C virus replication and pathogenesis through enhancement of TGF-β signaling

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Background & Aims: Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. The biological and therapeutic importance of host cellular cofactors for viral replication has been recently appreciated. Here we examined the roles of SNF1/AMP kinase-related kinase (SNARK) in HCV replication and pathogenesis.
Methods: The JFH1 infection system and the full-length HCV replicon OR6 cell line were used. Gene expression was knocked down by siRNAs. SNARK mutants were created by site-directed mutagenesis. Intracellular mRNA levels were measured by qRT-PCR. Endogenous and overexpressed proteins were detected by Western blot analysis and immunofluorescence. Transforming growth factor (TGF)-β signaling was monitored by a luciferase reporter construct. Liver biopsy samples from HCV-infected patients were analyzed for SNARK expression.
Results: Knockdown of SNARK impaired viral replication, which was rescued by wild type SNARK but not by unphosphorylated or kinase-deficient mutants. Knockdown and overexpression studies demonstrated that SNARK promoted TGF-β signaling in a manner dependent on both its phosphorylation and kinase activity. In turn, chronic HCV replication upregulated the expression of SNARK in patients. Further, the SNARK kinase inhibitor metformin suppressed both HCV replication and SNARK-mediated enhancement of TGF-β signaling.
Conclusions: Thus reciprocal regulation between HCV and SNARK promotes TGF-β signaling, a major driver of hepatic fibrogenesis. These findings suggest that SNARK will be an attractive target for the design of novel host-directed antiviral and antifibrotic drugs.

Keywords: SNARK; NUAK2; HCV; Metformin; Fibrosis; TGF-beta; SMAD; Kinase.
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Abbreviations: SNARK, sucrose-non-fermenting protein kinase 1/AMP-activated protein kinase-related protein kinase; TGF-β, transforming growth factor beta; JFH1, Japanese fulminant hepatitis 1; HTAs, host-targeting antivirals; HCC, hepatocellular carcinoma; SVR, sustained virological response; CsA, cyclosporin A.

Introduction

Chronic infection with hepatitis C virus (HCV) is a major cause of chronic liver disease and hepatocellular carcinoma (HCC) and the leading reason for liver transplantation worldwide. HCV infects approximately 170 million individuals worldwide [1]. Current therapy with pegylated interferon (IFN)-α in combination with ribavirin produces sustained virological response (SVR) in fewer than half of the patients infected with genotype 1 HCV, and does so with high rates of often unacceptable side effects [2]. In recent years, it has become increasingly evident that HCV propagation is highly dependent on host cellular cofactors which in turn represent promising antiviral targets [3]. It is hoped that these strategies will lead to rational host-targeting antivirals (HTAs) [4]. Moreover, HCV interferes with host cellular signaling pathways causing pathogenic effects such as insulin resistance (IR), diabetes, and alterations in host lipids. Indeed, mounting evidence supports hepatitis C as a metabolic disorder [5]. Hence, intervention against key host cellular factors critical for both HCV replication and viral pathogenesis may yield anti-hepatitis C therapies that both halt replication and abrogate other pathogenic effects of HCV, which might be further thought of as host-directed antiviral and antipathogenic therapies. Based on this assumption, we have previously conducted a functional genomic screen for host cellular factors supporting HCV replication using an HCV replicon system [6]. Among positive hits in our original screen was sucrose-non-fermenting protein kinase 1 (SNF1)/AMP-activated protein kinase (AMPK)-related protein kinase (SNARK), the fourth member of 14 mammalian AMPK-related kinases [7], which has been consistently found in other screens [8,9]. Although SNARK function is not well understood, SNARK heterozygote knockout mice displayed elevated serum triglyceride concentrations, hyperinsulinemia, glucose intolerance [10], and impaired contraction-stimulated glucose transport [11], implying that SNARK operates to maintain glucose and lipid homeostasis in a

manner analogous to AMPK, which was recently described to inhibit HCV replication [12].

Transforming growth factor (TGF)-β is a pleiotropic cytokine partaking in cell proliferation, differentiation, apoptosis, migration [13], and the major cytokine responsible for fibrosis in tissues including the liver. In HCV-infected persons, levels of TGF-β are elevated [14], and TGF-β was exhibited to promote the viral replication in a replicon model and was correlated with accelerated liver fibrosis in an *in vivo* model [15,16]. Intriguingly, a prior high-throughput mapping study of protein-protein interaction (PPI) identified an association of SNARK with SMADs [17], implying a direct link of SNARK to TGF-β signaling. Therefore, we sought to examine the significance and potential of SNARK as a therapeutic target in HCV replication and pathogenesis and its contribution to TGF-β signaling. We report that the phosphorylation and phosphotransferase activities of SNARK are required for HCV replication. Furthermore SNARK was demonstrated to enhance TGF-β signaling, and finally chronic HCV infection upregulated the expression of SNARK in patients. SNARK has pleiotropic functions including pro-TGF-β signaling activities in addition to the previously described AMPK-like properties. The finding of a reciprocal regulation between HCV and SNARK suggests that SNARK could be an effective host cellular target not only for an antiviral but also antipathogenic strategy.

Materials and methods

Compounds, antibodies, cells, and viruses

Metformin, TGF-β, and CsA were purchased from EMD chemicals USA (Gibbstown, NJ), Fitzgerald (North Acton, MA), and Sigma-Aldrich (St. Louis, MO), respectively. Antibodies to SNARK, FLAG, and β-actin were obtained from Sigma-Aldrich, and antibodies to HCV NS5A and phosphothreonine were obtained from BioFront Technologies (Tallahassee, FL) and Cell Signaling Technology (Danvers, MA), respectively. HuH7.5.1 and OR6 replicon cells were cultured as described previously [18], and HeLa cells were cultured in DMEM with 10% FBS. JFH1 virus infection was performed as described previously [19].

Further Materials and methods are described in the Supplementary Material section.

Results

Functional SNARK enhances HCV replication

To assess the contribution of SNARK to HCV replication, we first knocked down endogenous SNARK expression (Supplementary Fig. 1) with siRNAs in the Japanese fulminant hepatitis 1 (JFH1) virus infection system. HuH7.5.1 cells were transfected with SNARK-targeted siRNAs, which was followed by JFH1 infection. Reduced levels of *SNARK* mRNA were associated with impaired viral replication (Fig. 1A). We then constructed plasmids encoding the siRNA-resistant *SNARK* open reading frame (ORF) bearing synonymous mutations that are not recognized by *SNARK* siRNAs. The over expression of these siRNA-resistant SNARK proteins successfully rescued *SNARK* RNAi-impaired HCV replication (Fig. 1A, rSN-1 and rSN-7). We also tested the effects of SNARK knockdown and overexpression in the genotype 1 OR6 replicon system, and found that the decreased level of HCV RNA replication was also rescued by overexpression of siRNA-resistant forms of SNARK (Fig. 1B). Thus, SNARK was demonstrated to specifically support HCV replication in both a *bona fide* infection system and replicon model.

Next we sought to identify the function(s) responsible for SNARK's contribution to HCV replication. We introduced single

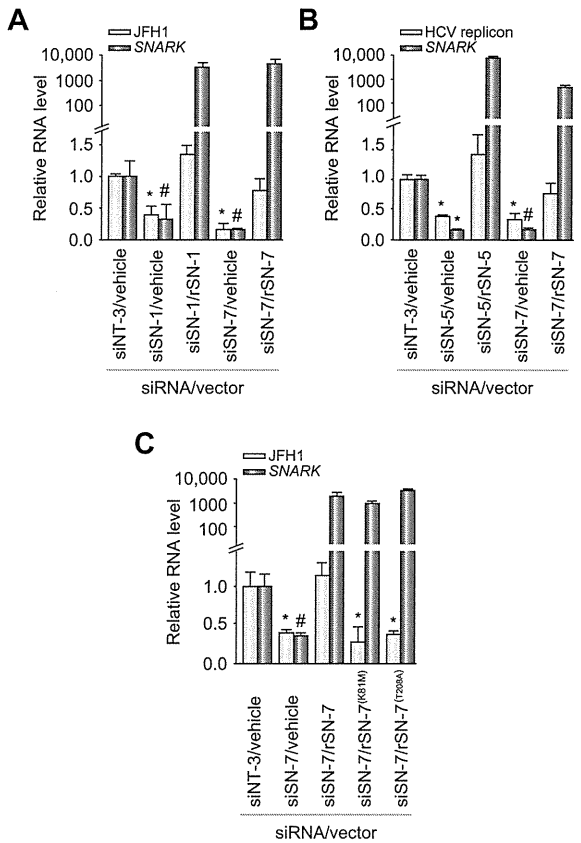

Fig. 1. SNARK supports HCV replication. (A) HuH7.5.1 cells were transfected with either non-targeting (siNT-3) or *SNARK*-targeted siRNAs (siSN-1 and siSN-7), followed by transfection of either empty or siRNA-resistant SNARK expression vectors (rSN-1 and rSN-7) 48 h later. The cells were infected with JFH1 on the next day and total RNA was harvested 48 h later. Relative JFH1 RNA and *SNARK* mRNA levels were quantified by real-time PCR analysis and normalized to *GAPDH*; $^{*}p$ <0.05 or $^{\#}p$ <0.01 *vs.* siNT-3 empty control. (B) OR6 replicon cells were transfected with siRNAs, followed by the transfection of empty or siRNA-resistant SNARK expression vectors 48 h later. Then total RNA was harvested 72 h later. Relative replicon RNA and *SNARK* mRNA levels were quantified by real-time PCR analysis and normalized to *GAPDH*. $^{*}p$ <0.01 or $^{\#}p$ <0.05 *vs.* siNT-3 empty control. (C) The rescue assay was conducted as described in (A). Here expression vectors of siRNA-resistant SNARK with either kinase-deficient mutation (rSN-7 K81M) or phosphorylation-deficient mutation (rSN-7 T208A) were used. Relative JFH1 RNA and *SNARK* mRNA levels were quantified by real-time PCR and normalized to *GAPDH*. $^{*}p$ <0.01 or $^{\#}p$ <0.05 *vs.* siNT-3 empty control.

mutations that abrogate either its phosphotransferase activity in the enzymatic pocket (K81M) or its phosphorylation at the phosphoacceptor site (T208A) in the siRNA-resistant *SNARK* ORF and overexpressed them in the rescue assay system used above with JFH1. In contrast to the rescue effects by wild type SNARK on viral replication, both functionally deficient mutants failed to recover impaired HCV replication by SNARK depletion (Fig. 1C and Supplementary Fig. 2). This result suggested that both the phosphorylation and kinase activities of SNARK are essential for its support of HCV replication.

SNARK phosphotransferase activity can be targeted

In a human hepatocarcinoma cell line, the kinase activity of SNARK was previously reported to be inhibited by metformin

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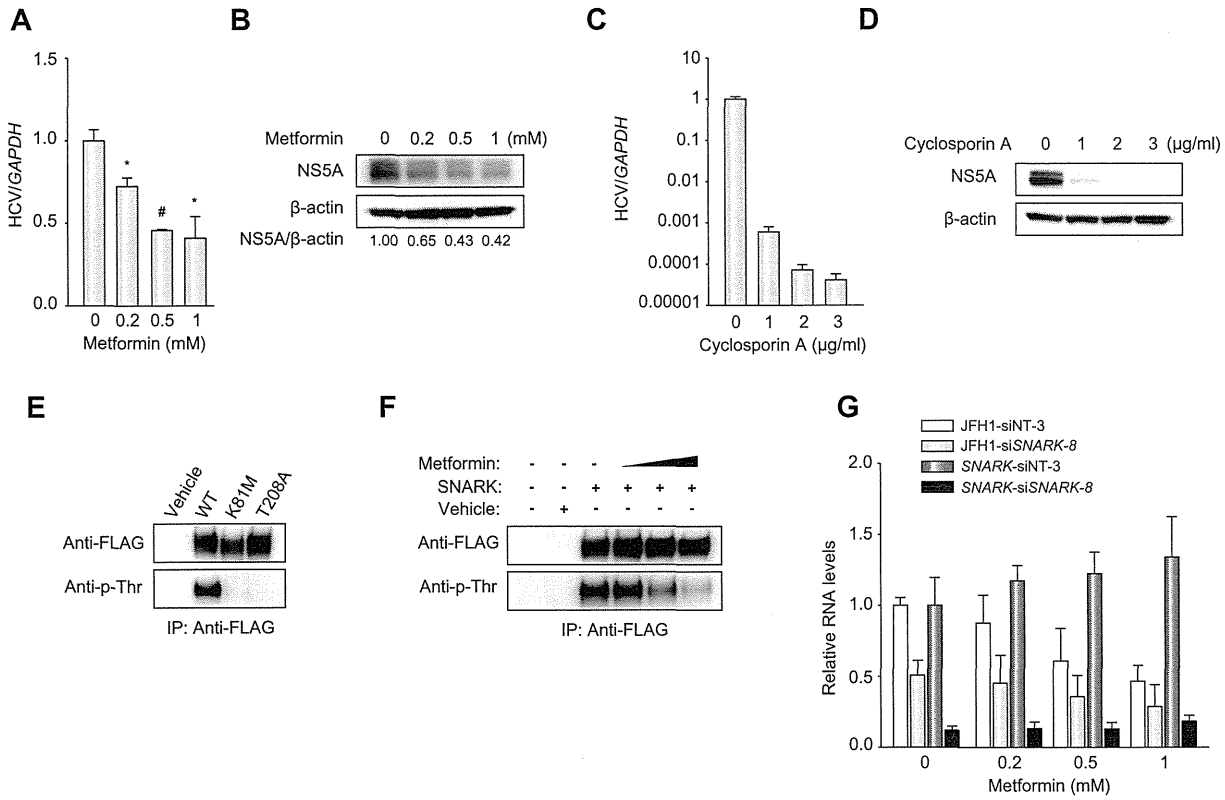

Fig. 2. Metformin suppressed HCV replication. HuH7.5.1 cells were infected with JFH1 and then treated with either metformin (A and B) or CsA (C and D) for 3 days. Densitometric values of NS5A normalized to those of β-actin were given as NS5A/β-actin. No significant cytotoxicity was observed by the compounds at the indicated concentrations. Relative HCV RNA level was quantified by real-time PCR and normalized to *GAPDH*; $^{*}p$ <0.05 or $^{\#}p$ <0.01 *vs.* untreated control. (E) In HuH7.5.1 cells either wild type or mutants (K81M or T208A) of FLAG-tagged SNARK were overexpressed and immunoprecipitated, followed by the detection with anti-FLAG or anti-phosphothreonine antibodies. (F) Wild type FLAG-tagged SNARK was overexpressed in the presence of metformin at 0.2, 0.5, and 1 mM in HuH7.5.1 cells and the levels of threonine phosphorylation were examined as indicated in (E). (G) HuH7.5.1 cells were transfected with either non-targeting (siNT-3) or *SNARK*-targeted (si*SNARK-8*) siRNAs. 48 hours later, cells were infected with JFH1 and treated with the indicated concentrations of metformin for 72 h. Relative mRNA levels for JFH1 or *SNARK* were quantified by real-time PCR and normalized to *GAPDH*.

[20], a well-known type 2 diabetes drug. In that setting, the kinase activity of SNARK was measured by incorporation of phosphate into SAMS peptide substrate, which was demonstrated to be significantly decreased by metformin treatment in the cell line. Here, we treated JFH1-infected HuH7.5.1 cells with metformin and observed moderate antiviral effects (Fig. 2A and B) with no cytotoxicity within the indicated dose range (data not shown). As a positive control, cyclosporin A (CsA) [21] strongly inhibited viral replication (Fig. 2C and D). To assess the phosphorylation level of SNARK subsequently, FLAG-tagged SNARK was overexpressed in HuH7.5.1 cells and immunoprecipitated for Western blot to monitor phosphothreonine levels [22]. While wild type SNARK was detected to be phosphorylated at threonine residue(s), neither K81M nor T208A mutant was (Fig. 2E), implying the autophosphorylation [23] and importance of threonine 208 as a phosphorylated site as reported [7]. Then we performed the assay using the wild type SNARK in the presence of metformin, which resulted in the reduced levels of phosphorylation dose-dependently (Fig. 2F). Here the data indicated that metformin suppressed SNARK phosphorylation, potentially interfering the phosphotransferase activity. The dose response of JFH1 to metformin was next examined when SNARK was knocked down by siRNAs. Metformin exerted dose-dependent antiviral effects in the cells transfected with non-targeting siRNAs, which was blunted by the siRNA-mediated reduction of SNARK expression (Fig. 2G). These data indicate that metformin's antiviral effect is mediated by inhibition of activated SNARK, bringing its full kinase activity, which may be a pharmacologic target for anti-HCV activity, and that metformin by itself could be a plausible component of a combination regimen targeting HCV.

SNARK is involved in TGF-β signaling

To investigate the possible roles of SNARK in viral pathogenesis based upon the induction of mRNA expression over the viral replication in cell culture (Supplementary Fig. 3), we also explored its involvement in downstream cellular signaling pathways. Intriguingly, SNARK appeared as an interactor with SMAD proteins in a high-throughput protein-protein interaction mapping study [17], which raised the distinct possibility that SNARK is involved in TGF-β signaling, the major profibrogenic pathway in the liver. Therefore, we first knocked down SNARK in HuH7.5.1 cells and assessed alterations in TGF-β signaling using an expression construct (PAI/L) encoding a luciferase reporter gene driven by promoter sequences of plasminogen activator inhibitor 1 (PAI-1), a transcriptional target of TGF-β [24]. siRNAs against *SNARK*

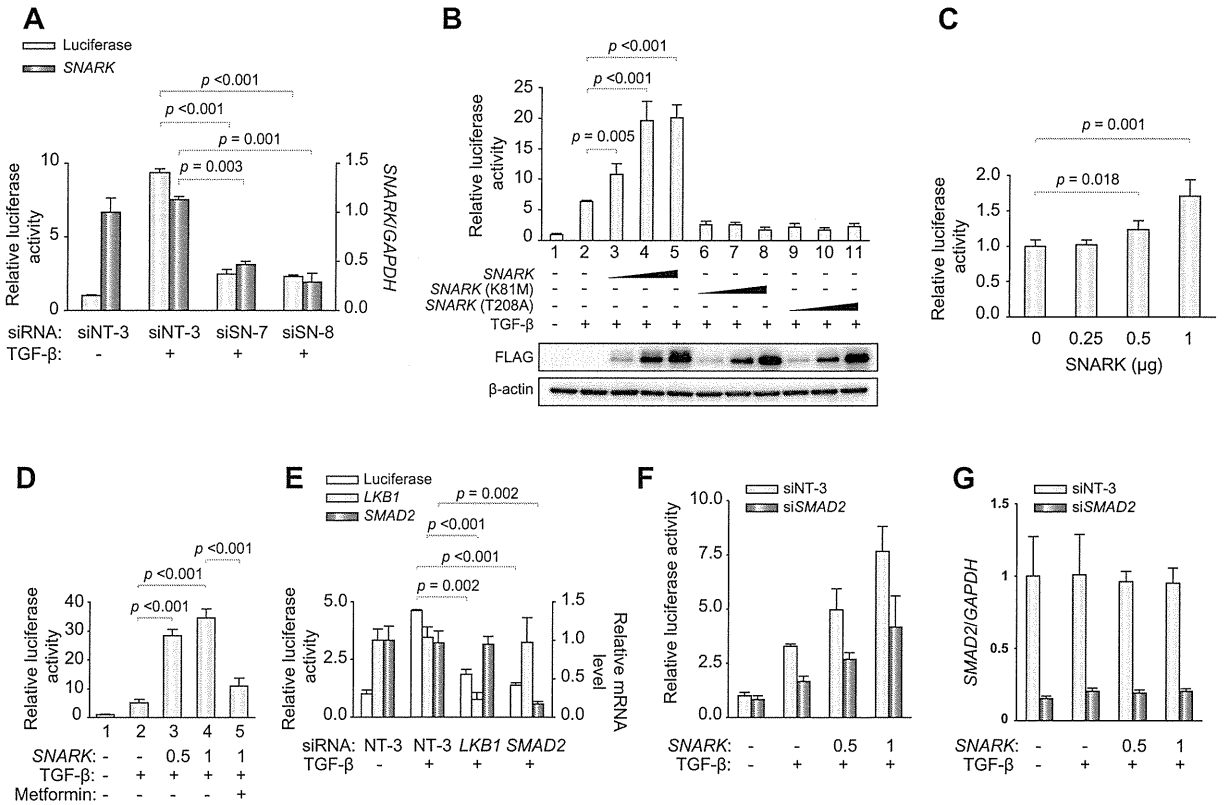

Fig. 3. SNARK accentuated TGF-β signaling. (A) HuH7.5.1 cells were transfected with siRNAs, which was followed by transfection of PAI/L reporter with pRL-TK 72 h later. On the next day, the cells were treated with TGF-β and lysed 24 hours later. The firefly and *Renilla* luciferase activities were measured and knockdown of *SNARK* was confirmed by real-time PCR with normalization to *GAPDH*. *P* Values were calculated as indicated. (B) Naïve HuH7.5.1 cells were transfected with either wild type (lanes 3–5) or mutant (lanes 6–11) SNARK expression plasmid at increasing doses (0.25, 0.5, and 1 μg) together with PAI/L reporter and pRL-TK. At 24 h post transfection, the cells were treated with TGF-β at 7 ng/ml for 20 h and lysed for dual luciferase assay. *P* Values were calculated as indicated. (C) HuH7.5.1 cells were transfected with increasing amount of wild type SNARK expression plasmid for dual luciferase assay as described in (A) without TGF-β. *P* Values were calculated as indicated. (D) HuH7.5.1 cells were transfected with increasing amount of SNARK expression plasmids (0.5 and 1 μg) together with PAI/L reporter and pRL-TK. On the next day, the cells were treated with TGF-β at 7 ng/ml and metformin at 1 mM (lane 5) for 20 h, and then lysed for dual luciferase assay. *P* Values were calculated as indicated. (E) HuH7.5.1 cells transfected with siRNAs against either *LKB1* or *SMAD2* for 72 h were transfected with PAI/L reporter and pRL-TK, which was followed by TGF-β treatment at 7 ng/ml for 20 h. Then the cells were lysed for dual luciferase assay and knockdown of targeted genes expression was confirmed by real-time PCR with normalization to *GAPDH*. *P* Values were calculated as indicated. (F) HuH7.5.1 cells transfected with siRNAs for 72 h were transfected with increasing amounts of wild type SNARK expression plasmid (0.5 and 1 μg) together with PAI/L reporter and pRL-TK. 24 h later, the cells were treated with TGF-β for 20 h, and then lysed for dual luciferase assay. (G) *SMAD2* knockdown levels were quantified by real-time PCR with normalization to *GAPDH*.

markedly reduced PAI/L luciferase activity and SNARK expression (Fig. 3A) in parallel, suggesting that SNARK is an important regulator of TGF-β signaling.

In order to elucidate the function of SNARK responsible for its contribution to TGF-β signaling, we assessed the effects of the overexpressed either wild type or mutant SNARK on TGF-β-stimulated PAI/L activity. In contrast to the dose-dependent increase of PAI/L activity by wild type SNARK, either kinase-dead K81M or unphosphorylated T208A mutant suppressed TGF-β-driven PAI/L activity (Fig. 3B). Moreover, the overexpression of SNARK in the absence of TGF-β moderately induced luciferase activity in HuH7.5.1 cells (Fig. 3C). These data demonstrate that both kinase activity and phosphorylation of SNARK are required for TGF-β signaling. Thereupon, in the same setting we treated HuH7.5.1 cells with metformin and found that SNARK-mediated stimulation of TGF-β signaling was inhibited by metformin (lane 5, Fig. 3D) though that was not the case in the absence of SNARK overexpression (Supplementary Fig. 5A) and the basal level of procollagen mRNA was not affected by metformin alone in HuH7.5.1 cells (Supplementary Fig. 5B), again underscoring that the kinase activity of SNARK is important for TGF-β signaling, and additionally raises the possibility that metformin may have utility as an anti-fibrotic agent in SNARK-facilitated pathogenesis.

We next examined regulators upstream and downstream of SNARK, depleting either liver kinase B1 (LKB1)/serine threonine kinase 11 (STK11), an upstream kinase of SNARK, or SMAD2, and assessed TGF-β-dependent PAI-1 luciferase activity. We found that knockdown of LKB1 abrogated PAI/L stimulation by TGF-β to the same extent as did SMAD2 knockdown (Fig. 3E). In addition, in contrast to luciferase activities in the presence of non-targeting siRNAs, overexpression of SNARK failed to rescue PAI/L activity in cells knocked down for either SMAD2 (Fig. 3F and G) or LKB1 (Supplementary Fig. 4). These data indicate that SNARK-mediated stimulation depends on SMAD2, and also that phosphorylation of SNARK by LKB1 and ensuing SNARK-mediated phosphorylation of downstream substrates, potentially in conjunction with SMAD2, are critical for TGF-β signaling.

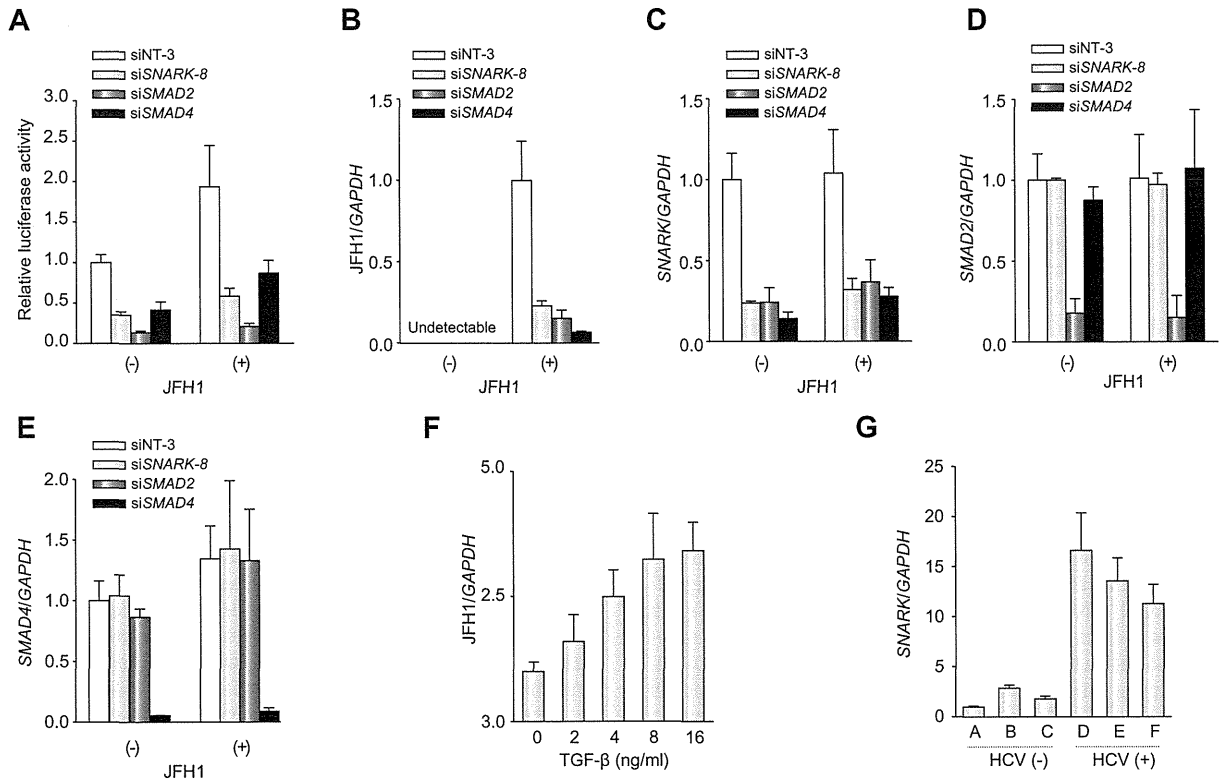

Fig. 4. Reciprocal regulation between SNARK and HCV in TGF-β signaling. (A) 48 hours after transfection of siRNAs, HuH7.5.1 cells were infected with JFH1, which was followed by transfection of PAI/L reporter 24 hours later and lysis 72 hours later for dual luciferase assay (A) and quantification of RNA levels for JFH1 (B), *SNARK* (C), *SMAD2* (D), and *SMAD4* (E) by real-time PCR with normalization to *GAPDH*. JFH1 RNA was not detected in the uninfected HuH7.5.1 cells (Undetectable, B). (F) 24 hours after the infection with JFH1, HuH7.5.1 cells were treated with TGF-β for 48 hours at indicated doses (0–16 ng/ml). Then cells were lysed and the viral RNA levels were measured by real-time PCR and normalized to *GAPDH*. (G) mRNA levels of *SNARK* in liver biopsies from patients were measured by real-time PCR and normalized to *GAPDH*. Patients A, B, and C were HCV-negative, and D, E, and F were infected with HCV. Details of patient characteristics are provided in Supplementary Table 2.

Reciprocal enhancement of TGF-β signaling and HCV infection via SNARK

Our data agree with the reported observation of TGF-β signaling elevated by HCV [14,25] and resultant proviral effects in a replicon cell line [15]. We therefore examined the effects of HCV replication on TGF-β signaling and immediate involvement of SNARK in the JFH1-HuH7.5.1 system. JFH1 replication (Fig. 4B) upregulated PAI/L luciferase activity, which was abolished by siRNA-mediated knockdown of SNARK (Fig. 4A and C), as was observed with the knockdown of either SMAD2 or SMAD4 (Fig. 4D and E). The data clearly demonstrate that SNARK expression plays a role in TGF-β signaling in JFH1 replication. Intriguingly, knockdown of either SMAD2 or SMAD4 led to the reduced expression of SNARK (Fig. 4C), implying converse regulation of SNARK by SMAD pathway. Next, the effects of enhanced TGF-β signaling on HCV replication were tested in this *bona fide* HCV infection system. JFH1-infected cells were treated with increasing quantities of TGF-β, and we observed that TGF-β enhanced viral replication in a dose-dependent fashion (Fig. 4F) with no cytotoxicity at the concentrations indicated (data not shown).

Lastly, SNARK expression was examined in human liver tissue to investigate its pathophysiological dynamics. The levels of *SNARK* mRNA were prominently elevated in HCV-infected patients in comparison to HCV-negative controls (Fig. 4G). These findings strongly suggest that HCV-mediated induction of SNARK facilitates both proviral and profibrogenic signaling of TGF-β, leading to reciprocal amplification of HCV and profibrogenic signals. Collectively, these factors could interact to accelerate hepatic fibrosis progression in HCV infection.

Discussion

SNARK is an AMPK-related kinase identified through our previous genome-wide RNAi screen as a host cellular cofactor for HCV replication [6]. Our present studies reveal an intersection of TGF-β-SMAD signaling, LKB1-AMPK-related kinase signaling, and viral replication, in which there is reciprocal stimulation. This convergence could well explain the relationship between HCV replication and its pathogenic effects observed *in vitro* and *in vivo*, providing strong support for the concept that the TGF-β signaling pathway is one of the key cellular pathways targeted by HCV to promote replication and may be therefore a future therapeutic target.

Growing numbers of host cellular cofactors for HCV replication have been discovered so far to support the viral lifecycle through interaction with viral proteins and alteration of host signaling pathways. Here we demonstrated that SNARK contributes to HCV replication through a reportedly proviral cytokine, TGF-β [15,26]. Simultaneously, the induction of SNARK by prolonged HCV replication in cell culture and patients demonstrates its reciprocal regulation by HCV. SNARK was transcriptionally upregulated in an NF-κB-dependent manner in a breast cancer cell line

[27] and was identified in a microarray analysis as the only kinase substantially induced in endothelial cells by tumor necrosis factor (TNF)-α, a well-known NF-κB activator [28]. Moreover, we and others reported that HCV infection activates NF-κB phosphorylation [29] and that NS5A stimulates NF-κB-dependent luciferase activity [30], respectively. Thus, SNARK expression may be transcriptionally induced by sustained HCV infection through virally-triggered NF-κB activation even though its basal expression may be quite low as illustrated by Western blot in HuH7.5.1 cells. Despite these low basal expression levels, the activation by infection of SNARK appears to enable further viral replication.

TGF-β is a pivotal cytokine and the central driver of hepatic fibrogenesis during HCV infection [31]. An increasing variety of proteins mediating TGF-β signaling have been described [32] and phosphorylation of SMAD2 and SMAD3 by non-TGF-βRI kinases such as Mps1 [33] and Rho/ROCK [34] also promoted TGF-β signaling. Therefore, SNARK associated with SMAD2 can be responsible for phosphorylation of SMAD2, enhancing SMAD-signaling. On the other hand, partial dependence of SNARK expression on SMADs observed in our knockdown experiments (Fig. 4C) may better explain reciprocal regulation between SNARK and TGF-β pathway. Possible modes of participation of SNARK in other phosphorylation-dependent processes in TGF-β signaling and TGF-β signaling-regulated SNARK expression, together with those in mediators in cross-talking pathways leading to epithelial-mesenchymal transition through cell dedifferentiation including Notch1, whose expression was mildly induced by SNARK overexpression in the presence of TGF-β in HuH7.5.1 cells (data not shown), remain an open subject for further investigation. On the other hand, SNARK alone was also capable of moderately inducing PAI-1 promoter-driven luciferase activity, underscoring its potential transcription-modulatory functions suggested by the nuclear localization of SNARK in HuH7.5.1 (Supplementary Fig. 1A and B), PLC/PRF/5, and HeLa cells [35], similarly to the HCV-activated transcription factor Elk1 [36], responsible for epidermal growth factor (EGF)-driven enhancement of PAI-1 expression [37].

Knockdown experiments in HuH7.5.1 cells demonstrate the importance of LKB1 in the TGF-β signaling pathway and in its promotion by SNARK, consistent with the decreased SMAD2 and TGF-β pathway activities in *Stk11*$^{-/-}$ mice, a model of Peutz-Jeghers syndrome [38]. It is tempting to conjecture critical roles of SNARK in HCV-induced liver disease in consideration of SNARK as a LKB1 signaling molecule directly activated by LKB1 in the TGF-β pathway. Currently, a wide variety of inhibitors of mediators in TGF-β/PAI-1 expression control are under clinical evaluation [39]. Thus SNARK, whose expression and activity are closely linked to TNF-α and TGF-β, activators of hepatic stellate cells [40], appears to be an attractive target for antifibrotic development.

Metformin has been widely used for the treatment of type 2 diabetes for 50 years [41], primarily decreasing hepatic glucose production [42] via AMPK activation [43]. Simultaneously, metformin was also revealed to exert multifaceted actions through AMPK-independent mechanisms targeting several kinases [44]. Indeed, the phosphotransferase activity of SNARK was inhibited by metformin in a human hepatocellular carcinoma cell line [20]. The phosphotransferase-dependent phosphorylation level of overexpressed SNARK was diminished by metformin in our immunoprecipitation assay as well, again indicating metformin-mediated inhibitory effects on SNARK phosphorylation partly via interference with its autophosphorylation. Hence it is rational that we observed an antiviral action of metformin against JFH1, and indeed a trial in Spain showed that the addition of metformin to standard anti-HCV treatment improved SVR [45], which suggests a possible productive application of metformin and SNARK inhibitors to the anti-HCV armamentarium. Furthermore, SNARK phosphotransferase activity-driven stimulation of TGF-β signaling in HuH7.5.1 cells allowed us to confirm the suppressive effects of metformin on TGF-β signaling accentuation by SNARK overexpression, implying SNARK as a target in HCV pathogenesis. Further studies to elucidate the mechanisms and consequences of inhibition of SNARK and metformin itself are warranted. Besides, since another pathogenic effect of HCV is insulin resistance, and type 2 diabetes is a risk factor for HCC [46], novel SNARK-mediated antiviral and antifibrotic properties of the antidiabetic metformin could offer an important and multifaceted agent for long term HCV disease management.

On the heels of development of anti-HCV agents targeting viral proteins, proviral host cellular cofactors have been discovered [3] and subsequent HTAs are emerging, best typified by cyclophilin (CyP) inhibitors [47], overcoming the drug resistance against virally-targeted inhibitors. In this study, we provide an example of a potential target for host-directed antiviral and antipathogenic therapies, which target a key host cellular cofactor involved not only in viral replication but also in viral pathogenesis. In fact, HCV modulates and depends on lipid metabolism enhancing lipogenesis for the establishment of efficient viral infection [48], and cholesterol-lowering statins were not only antiviral [49], but also effective in reducing steatosis and retarding fibrosis in viral and non-alcoholic fatty liver disease (NAFLD) patients [50], nicely exemplifying the notion above.

Taken together, our data overall suggest that SNARK is a novel host cellular factor for HCV replication and an additional mediator of TGF-β-SMAD signaling. Involvement of its activity as a kinase in proviral and pathogenic pathways positions SNARK as a potentially critical and druggable target for new therapies against hepatitis C.

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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.

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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.2013. 06.025.

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IL28B minor allele is associated with a younger age of onset of hepatocellular carcinoma in patients with chronic hepatitis C virus infection

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Abstract
Background IL28B polymorphisms were shown to be associated with a response to peg-interferon-based treatment in chronic hepatitis C (CHC) and spontaneous clearance. However, little is known about how this polymorphism affects the course of CHC, including the development of hepatocellular carcinoma (HCC). We evaluated the influence of IL28B polymorphisms on hepatocarcinogenesis in CHC patients.
Methods We genotyped the rs8099917 single-nucleotide polymorphism in 351 hepatitis C-associated HCC patients without history of IFN-based treatment, and correlated the age at onset of HCC in patients with each genotype.
Results Frequencies of TT, TG, and GG genotypes were 74.3 % (261/351), 24.8 % (87/351), and 0.9 % (3/351), respectively. The mean ages at onset of HCC for TT, TG, and GG genotypes were 69.9, 67.5 and 66.8, respectively. In multivariate analysis, IL28B minor allele (TG and GG genotypes) was an independent risk factor for younger age at onset of HCC ($P = 0.02$) in males ($P < 0.001$) with higher body mass index (BMI; $P = 0.009$). The IL28B minor allele was also associated with a lower probability of having aspartate aminotransferase-to-platelet ratio index (APRI) >1.5 (minor vs. major, 46.7 vs. 58.6 %; $P = 0.01$), lower AST (69.1 vs. 77.7 IU/L, $P = 0.02$), lower ALT (67.8 vs. 80.9 IU/L, $P = 0.002$), higher platelet count (12.8 vs. 11.2 $\times$ 10^4/μL, $P = 0.002$), and higher prothrombin time (79.3 vs. 75.4 %, $P = 0.002$).
Conclusions The IL28B minor allele was associated with lower inflammatory activity and less progressed fibrosis of the liver; however, it constituted a risk factor for younger-age onset of HCC in CHC patients.

Keywords rs8099917 · Hepatocarcinogenesis · Interferon-λ · Risk allele · Fibrosis

Abbreviations

AFP	α-Fetoprotein
APRI	Aminotransferase platelet ratio index
CHC	Chronic hepatitis C
GWAS	Genome-wide association study
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IL28B	Interleukin 28B
PCR	Polymerase chain reaction
peg-IFN	peg-Interferon
RIG- I	Retinoic acid-inducible gene-I
SNP	Single-nucleotide polymorphism
SVR	Sustained viral response
TLR3	Toll-like receptor 3

Introduction

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. Currently, patients with chronic

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hepatitis C (CHC) are treated with a combination of peg-interferon (peg-IFN) and ribavirin [2, 3]. Recently, HCV nonstructural 3/4A serine protease inhibitors combined with PEG-IFN and RBV were reported to achieve higher sustained viral response (SVR) rates in genotype 1 patients compared to conventional PEG-IFN/RBV. These triple therapies are considered to be the next standard of care for patients with CHC virus infection [4, 5].

Genetic variations near the interleukin 28B (IL28B) gene, encoding the type III IFN-λ3, were shown to be strongly associated with the response to peg-IFN and ribavirin treatment in patients with CHC [6–8] and also spontaneous clearance of HCV [9]. Host immune cells produce IFN and other cytokines in response to viral infection. In response to HCV, cellular sensors detect the double-stranded RNA via the retinoic acid-inducible gene-I (RIG-I) and toll like receptor 3 (TLR3) and activate a pathway to produce antiviral cytokines, including alpha and beta IFNs that trigger an antiviral response to eradicate the virus [10, 11].

Genetic polymorphisms of genes involved in innate immunities are likely to influence the strength and nature of this defense system [12]. Besides its antiviral properties, IFN-λ exhibits antitumor activity; in fact, several experimental studies in cell lines and in animal models demonstrated that the activation of type III IFN induces apoptosis [13] and antitumor activities [14–16]. Thus, this genetic factor is thought to influence the natural course of HCV infection, including the development of HCC. However, little is known about the influence of IL28B polymorphisms on hepatocarcinogenesis in patients with CHC.

In the present study, we examined the association between the rs8099917 single-nucleotide polymorphism (SNP) at the IL28B locus with the age at onset of HCC and other clinical findings in patients with CHC who had no history of receiving IFN-based treatment.

Materials and methods

Patients

The patients analyzed in the present study were derived from an HCV study cohort of the University of Tokyo Hospital. In this cohort, we enrolled the patients who visited the liver clinic at our institute between August 1997 and April 2009, and agreed to provide blood samples for human genome studies along with written informed consent according with the Declaration of Helsinki. All patients underwent laboratory blood tests at the time of enrollment in our cohort. The result of the blood tests were recorded with the information on alcohol consumption and BMI of each patient. The patients who were positive for hepatitis B surface antigen and had a history of biliary disease were excluded. All subjects in our cohort were Japanese, and this research project was approved by the ethics committees of the University of Tokyo (No. 400).

From this cohort, we examined the patients who had developed new-onset HCC and received initial therapy in our institute by January 31, 2010, and with available sample for genotyping. We excluded the patients with a history of receiving IFN-based treatment. Finally, 351 patients were enrolled for this study, and the association between the age at onset of HCC and the IL28B genotype was analyzed. Patient follow-up and Diagnosis of HCC was performed as previously described [17, 18].

IL28B genotyping

Human genomic DNA was extracted from the whole blood of each patient. Genotyping for the IL28B rs8099917 T/G polymorphism was performed by polymerase chain reaction (PCR) using the TaqMan predesigned SNP Genotyping Assay (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. Allele-specific primers were labeled with fluorescent dye (FAM or HEX) and used in the PCR reaction. Aliquots of the PCR products were genotyped using an allele-specific probe of the SNP on a real-time PCR thermocycler (MX3000P, Stratagene, La Jolla, CA). Samples were subjected to 50 cycles of denaturation for 15 s at 92 °C, annealing of primers for 30 s at 60 °C, and elongation for 30 s at 60 °C.

Study endpoint

We analyzed the relationship between the age at onset of HCC (the primary endpoint of this study) and host factors, including the IL28B genotypes, sex, BMI, alcoholic consumption, and HCV genotype. We also examined the relationship between IL28B genotypes and the clinical findings at the time of enrollment in our cohort (the secondary endpoint), such as the biochemical markers and presence of liver fibrosis. Liver biopsies were only available in a small number of patients (48); liver fibrosis was assessed using the aspartate aminotransferase platelet ratio index (APRI), and an APRI of >1.5 was classified as bridging fibrosis or cirrhosis (F stage 3–4) [19].

Statistical analysis

Continuous variables were presented as the mean ± standard deviation (SD) while categorical variables were expressed as frequencies (%). Categorical data were analyzed using the Chi square test, and stepwise logistic regression analyses were used to adjust the influence of IL28B genotype by other covariates such as sex, BMI (<25

or not), and alcoholic consumption (<50 g/day or not). For continuous data, the univariate associations were evaluated using the Student's t test or nonparametric Wilcoxon rank-sum test as appropriate. Since the age at onset of HCC (the primary endpoint of this study) satisfied the assumption of normal distribution (Kolmogorov–Smirnov test, $P > 0.05$), we used stepwise regression analysis to adjust the influence of IL28B genotype by sex, BMI (<25 or not), and alcoholic consumption (<50 g/day or not). All statistical analyses were two-sided, and the threshold of the reported P values for significance was accepted as <0.05. All statistical analyses were performed using R 2.13.1 software (http://www.r-project.org).

Results

Patient characteristics

Patient characteristics are shown in Table 1. Frequencies of the rs8099917 TT, TG, and GG genotype were 74.3 % (261/351), 24.8 % (87/351), and 0.9 % (3/351), respectively. The SNP genotype distribution was in Hardy–Weinberg equilibrium (P value was not significant). We defined the IL28 major genotype as homozygous for the major sequence (TT) and the IL28B minor genotype as homozygous (GG) or heterozygous (TG) for the minor sequence. The mean age at onset of the HCC patients was 69.3 years, and approximately 60 % were male. The mean age at the time of enrollment was 67.2 years and the follow-up period was 27.9 months in average.

Table 1 Clinical characteristics and genotype distributions in the study cohort ($n = 351$)

Parameter	Values
Mean age at onset of HCC, in years	69.26 ± 8.07
Mean age at the time of enrollment, in years	67.16 ± 8.32
Male sex	200 (57.0 %)
BMI >25	70 (20.0 %)
Alcohol consumption (>50 g/day)	75 (21.4 %)
IL28B genotype	
TT	261 (74.3 %)
TG	87 (24.8 %)
GG	3 (0.9 %)
T allele frequency	0.87
HCV genotype	
Genotype 1	240 (68.4 %)
Genotype 2	91 (25.9 %)
Not tested	20 (5.7 %)

Continuous variables were represented as the mean ± standard deviation (SD) and categorical variables were as number and frequencies (%)

Primary endpoint

Table 2 shows the age at onset of patients with HCC and the associations among IL28B genotypes, sex, BMI, alcohol consumption, and HCV genotype. The mean age at onset in patients with HCC for the IL28B major and minor genotypes were 69.88 ± 7.97 and 67.48 ± 8.17, respectively, and significantly higher in patients with the IL28B major genotype than in those with the minor genotype ($P = 0.02$). In multivariate analysis, the age at onset of HCC was significantly younger in patients with the IL28B minor genotype ($P = 0.02$, Fig. 1), independently of male sex ($P < 0.001$) and higher BMI ($P = 0.009$). The characters of HCC, such as sizes (2.56 vs. 2.40 cm, $P = 0.41$) or the numbers (1.94 vs. 2.23, $P = 0.54$) at diagnosis were not significantly different between IL28B major and minor genotypes. We also analyzed the interval between blood transfusion and the onset of HCC in 161 patients who have histories of blood transfusion which had been the major cause of HCV infection in Japan [20]. The mean interval between blood transfusion and the onset of HCC for the IL28B major and minor genotypes were 39.09 ± 9.99 and 38.86 ± 9.27 years, respectively ($P = 0.9$; data not shown).

Secondary endpoint

Table 3 shows the clinical findings and associations between the IL28B genotypes at the time of enrollment in our cohort. The IL28B major genotype was significantly associated with a higher probability of having an APRI >1.5 (58.62 vs. 46.67 %, $P = 0.01$; Fig. 2), a lower platelet count (11.15 vs. 12.80 × 10^4/μL, $P = 0.002$), a higher AST level (77.69 vs. 69.12 IU/L, $P = 0.02$), a higher ALT level (80.92 vs. 67.79 IU/L, $P = 0.002$), and a lower prothrombin time (75.40 vs. 79.27 %, $P = 0.002$) compared to the IL28B minor genotype after adjustment for sex, BMI, alcoholic consumption, and the age at enrollment of our cohort. A lower γ-GTP level was significantly associated with the IL28B major genotype in univariate analysis, and alcoholic consumption, sex, and age were stronger factors associated with the γ-GTP level. Thus, after adjustment for these factors, the IL28B genotype was not extracted as a significant factor associated with the γ-GTP level. Histological assessments of liver fibrosis were performed in 248 patients at the time of initial therapy. The prevalence of histologically proved liver cirrhosis (F4) was 65.6 % (118/180) in patients with major genotype and 51.5 % (35/68) in those with minor genotype. The prevalence of liver cirrhosis was significantly higher in patients with major genotype after adjustment for sex, BMI, alcoholic consumption, and the age at the time of initial therapy for HCC ($P = 0.045$, data not shown).

Table 2 Factors associated with the age at onset of HCC

Variable	Mean	Standard deviation (SD)	*P* value	
			Univariate	Multivariate[a]
IL28B genotype			0.02	0.02
Major (TT)	69.88	7.97		
Minor (TG/GG)	67.48	8.17		
Sex			<0.001	<0.001
Male	67.94	8.48		
Female	71.02	7.16		
BMI			0.01	0.009
>25	66.87	9.11		
≤25	69.86	7.70		
Alcohol consumption			0.11	–
>50 (g/day)	67.78	9.37		
≤50 (g/day)	69.67	7.65		
HCV genotype			0.29	–
Genotype 1	69.65	7.59		
Genotype 2	68.22	8.79		

[a] Stepwise regression analysis for the age at onset of HCC (the dependent variable) using IL28B genotype, sex, BMI, alcohol consumption, and HCV genotype as independent variables

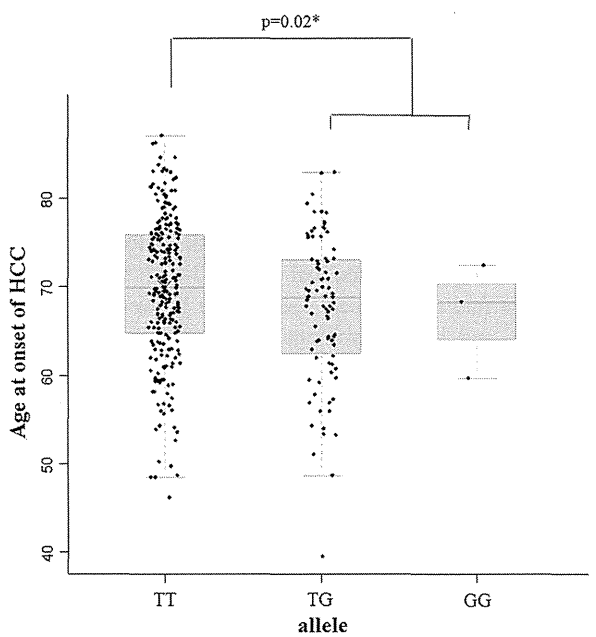

Fig. 1 *Box and whisker and dot plot* distributions of the age at onset of HCC in each genotype. The mean age at onset of HCC for the IL28B major and minor genotypes were 69.88 ± 7.97 and 67.48 ± 8.17, respectively, and was significantly higher in patients with the IL28B major genotype than in those with the minor genotype ($P = 0.02$). *P values after adjustment for sex, BMI, and alcoholic consumption

Discussion

In the present study, we evaluated the association between the IL28B polymorphism and the age at onset of HCC in patients with CHC. The IL28B minor genotype was significantly associated with younger age at onset of HCC with well known risk factors for the development of HCC such as male gender and higher BMI [21] without prior IFN-based treatment. Our previous study analyzing a susceptibility locus for HCV-induced HCC using a genome-wide association study (GWAS) could not detect the significant association between IL28B genotypes and the development of HCC in a cross-sectional distribution analysis between patients with and without HCC in more than 3,000 samples [22]. Also, IL28B alleles were not identified as a susceptibility locus for HCV-induced HCC in another GWAS study [23]. The cross-sectional distribution analyses may have underestimated the susceptibility to HCC because it could not take into consideration the future development of HCC and the duration after the past onset of HCC. Moreover, although GWAS would provide an effective and unbiased approach for revealing risk alleles for genetically complex non-Mendelian disorders, the risk of multiple comparisons made in a GWAS have resulted in reports of false positive results (Type 1 errors), and if the correction is overly conservative or the power is inadequate, false negative results (Type 2 errors) [24–26]. The relation between IL28B polymorphism and the susceptibility to HCC is still controversial. A previous study from Japan reported that the rs8099917 TT genotype was associated with a lower incidence of HCC even in non-responders to IFN based treatment [27] that was in agreement with the present study. Another study from Italy evaluating the association between genome frequency and the presence of cirrhosis due to hepatitis C, hepatitis B, alcohol use, and other factors also showed a higher prevalence of the IL28B minor allele in patients with HCC

Table 3 Associations between the IL28B genotype and clinical findings at the time of enrollment in our cohort

Variable	Mean/proportion (standard deviation; SD)		*P* values	
	Major (TT)	Minor (TG/GG)	*P* value	Adjusted *P* value[¶]
APRI >1.5[a]	58.62 % (52.38–64.66)	46.67 % (36.07–57.69)	0.07	0.01[‖]
Platelet count ($\times 10^4/\mu L$)	11.15 (5.00)	12.80 (5.43)	0.01	0.002**
AST (IU/L)	77.69 (45.14)	69.12 (38.16)	0.12	0.02**
ALT (IU/L)	80.92 (60.45)	67.79 (41.78)	0.17	0.002**
T.B (mg/dL)	0.90 (0.40)	0.83 (0.39)	0.02	–
Alb (g/dL)	3.69 (0.46)	3.71 (0.46)	0.9	–
ALP (IU/L)[b]	236.4 (81.75)	216.4 (58.96)	0.08	0.11**
γGTP (IU/L)[c]	76.83 (65.34)	87.23 (42.92)	0.005	–
PT (%)[d]	75.40 (13.36)	79.27 (13.13)	0.02	0.002**

[¶] Adjusted for sex, BMI, alcoholic consumption, and the age at enrollment (independent variables). The dependent variables of each *P* values are the items in the leftmost fields of corresponding rows (the proportion of having APRI >1.5, platelet count, AST, ALT and so on)

[‖] *P* value by stepwise logistic regression analysis

** *P* value by stepwise regression analysis

[a] Odds ratio (95 % CI) for major allele was 1.88 (1.13–3.11), and 95 % confidence interval (CI) of each proportion is parenthesized for this outcome

[b] Missing in 115 patients

[c] Missing in 112 patients

[d] Missing in 4 patients

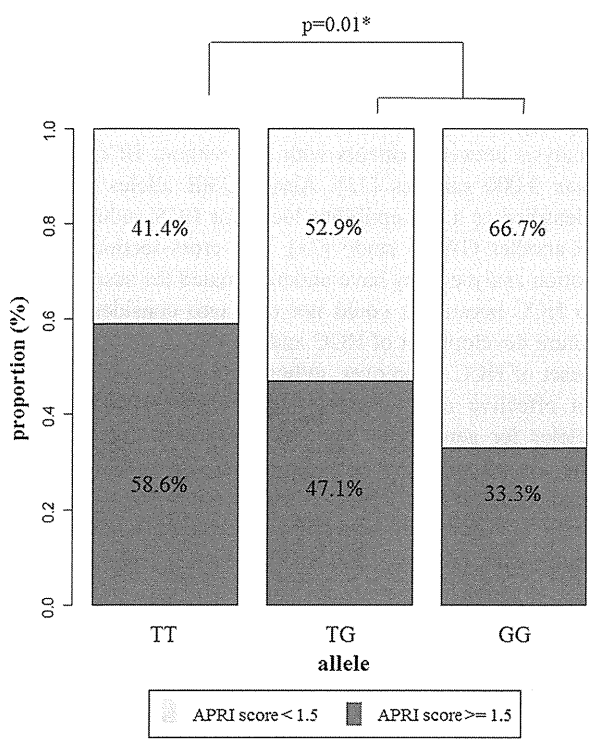

Fig. 2 *Bar plot* the proportion of having an AST-to-platelet ratio (APRI) score >1.5 in each allele. **P* values after adjustment for sex, BMI, alcoholic consumption, and the age at enrollment

compared to those without HCC [28]. However, other studies showed no relation between IL28B polymorphism and the susceptibility to HCC [29–32]. Some studies have reported the HCV genotype 1 as a risk factor associated with HCC in patients who had CHC [33–35]; however, we could not find a significant association between the HCV genotype and hepatocarcinogenesis in the present study. Our data showed no relationship between the duration of HCV infection in the patients with a history of blood transfusion. The mean age of blood transfusion was not significantly different between patients with major and minor genotypes (28.99 in major genotype vs. 27.60 in minor genotype, $P = 0.18$). Moreover, older age at HCV infection was reported to be associated with more rapid disease progression [36]. Thus, the difference in the duration of HCV infection may have little effect on the result of the present study. The IL28B genotype may have a critical role in the onset of HCC. Moreover, only about 45 % of all patients in the present study have the history of blood transfusion; hence, further analysis with larger samples may be indicated.

Previous studies evaluating patients with chronic HCV infection showed severer histological inflammatory activity and fibrosis, as well as higher ALT levels and APRI scores in patients homozygous for the IL28B major alleles [29, 32, 37, 38]. Similarly, in the present study, the IL28B