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].

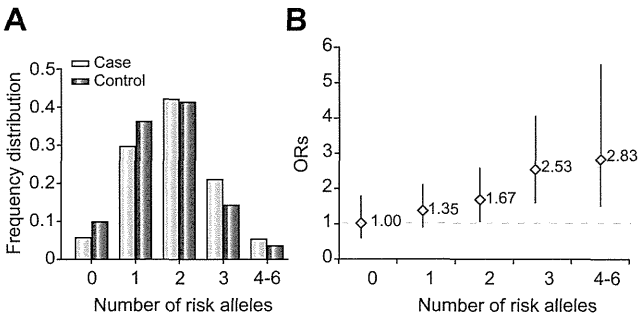

Fig. 2. Cumulative effects of liver cirrhosis risk alleles. (A) Frequency distribution divided by risk allele numbers (rs910049, rs3135353, and *HLA-DQA0601*) among liver cirrhosis (light blue bars) and chronic hepatitis C (dark blue bars) patients. (B) Plot of the increase odds ratio (OR) for liver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

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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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Perihepatic lymph node enlargement is a negative predictor of liver cancer development in chronic hepatitis C patients

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Abstract
Background Perihepatic lymph node enlargement (PLNE) is a common ultrasound finding in chronic hepatitis C patients. Although PLNE is considered to reflect the inflammatory response to hepatitis C virus (HCV), its clinical significance remains unclear.
Methods Between December 2004 and June 2005, we enrolled 846 chronic hepatitis C patients in whom adequate ultrasound examinations had been performed. PLNE was defined as a perihepatic lymph node that was at least 1 cm in the longest axis by ultrasonography. We analyzed the clinical features of patients with PLNE and prospectively investigated the association between PLNE and hepatocellular carcinoma (HCC) development.
Results We detected PLNE in 169 (20.0 %) patients. Female sex, lower body mass index (BMI), and HCV serotype 1 were independently associated with the presence of PLNE. However, there were no significant differences in liver function tests, liver stiffness, and hepatitis C viral loads between patients with and without PLNE. During the follow-up period (mean 4.8 years), HCC developed in 121 patients. Unexpectedly, patients with PLNE revealed a significantly lower risk of HCC development than those without PLNE ($p = 0.019$, log rank test). Multivariate analysis revealed that the presence of PLNE was an independent negative predictor of HCC development (hazard ratio 0.551, $p = 0.042$). In addition, the sustained viral response rate in patients who received interferon (IFN) therapy was significantly lower in patients with PLNE than in patients without PLNE.
Conclusions Patients with PLNE had a lower risk of HCC development than those without PLNE. This study may provide new insights into daily clinical practice and the pathophysiology of HCV-induced hepatitis and hepatocarcinogenesis.

Keywords Perihepatic lymph node enlargement · Chronic hepatitis C · Hepatocarcinogenesis

Abbreviations

ALT	Alanine aminotransferase
AFP	α-Fetoprotein
PLNE	Perihepatic lymph node enlargement
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
IFN	Interferon
LN	Lymph node
US	Ultrasonography

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and chronic hepatitis C virus (HCV)

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infection is a major cause of HCC in the United States, southern European countries, and Japan [1, 2]. The host immune responses to HCV are often not strong enough to completely clear the infection, resulting in chronic stimulation of the antigen-specific immune response. Accumulating basic and clinical lines of evidence indicate that a sustained inflammatory reaction in the liver is the major contributing factor to HCC development [3–5].

Inflammatory processes in organs frequently lead to hyperplasia of regional lymph nodes (LNs). Perihepatic LN enlargement (PLNE) is a common finding in patients with chronic hepatitis, especially in patients with hepatitis C [6, 7]. Some studies have revealed that PLNE in chronic hepatitis C patients was associated with a higher HCV viral load, [7] higher histological grade of hepatic inflammation and fibrosis [8, 9], and higher CD8 lymphocyte level in the blood [10]; therefore, PLNE is considered to reflect an inflammatory response to HCV. However, such associations as those noted above are inconsistent among studies [7–15], and the precise mechanism and clinical relevance of PLNE are not fully understood. Furthermore, to our knowledge, there have been no studies designed to investigate the association between PLNE and hepatocarcinogenesis. To clarify whether we should pay attention to the risk of HCC development in patients with PLNE is very important, because we encounter such situations very often.

The purpose of this study was to reevaluate the clinical relevance of PLNE and to carry out a prospective assessment of the association between PLNE and HCC development. To elucidate these matters, we investigated a well-characterized chronic hepatitis C cohort in which we previously reported the utility of performing transient elastography for risk assessment of HCC development [16]. In the present study, we prospectively assessed the association between PLNE and HCC development in chronic hepatitis C patients in that cohort.

Patients and methods

Patients and screening for perihepatic LN enlargement

As described previously [16], we enrolled 866 chronic hepatitis C patients, excluding those with HCC or a past history of it, who visited the University of Tokyo Hospital between December 2004 and June 2005. All patients were positive for HCV-RNA and showed at least a transiently elevated serum alanine aminotransferase (ALT) level. Patients with concomitant hepatitis B virus surface antigen positivity, patients with uncontrollable ascites, patients on interferon (IFN) therapy, and patients who visited only for consultation purposes were excluded from this study.

Each subject was screened for HCC with ultrasonography (US) at or immediately after the first visit. At the same time, we surveyed the presence of PLNE with US. The US examination was performed using the SSD-2000 (Aloka, Tokyo, Japan). To identify LNs the following criteria were used, according to a previous report [17]: one or more masses with an ovoid shape and less echogenic than liver parenchyma, separated from adjacent organs and vessels by a clear-cut cleavage on repeated transverse, sagittal, and oblique scans. LNs were searched for near the trunk of the portal vein, hepatic artery, celiac axis, superior mesenteric vein, and pancreas head. Furthermore, we used Doppler US to differentiate LNs from vessels.

We defined PLNE as an LN that was at least 1 cm in the longest axis. There were two reasons for this definition. First, we preliminarily investigated the prevalence of PLNE with US in 465 healthy subjects who had had medical check-ups and did not have liver disease or other underlying causes of LN enlargement. We found that only 15 (3.2 %) of these subjects had a perihepatic LN larger than 1 cm in the longest axis (unpublished data). Second, when an LN was smaller than 1 cm, it was sometimes difficult to distinguish the LN from other structures. If two or more LNs were detected with US, we determined PLNE to be present based on the length of the largest LN. In 20 of the 866 patients in the present study, adequate visualization of the liver hilum was not achieved with US because of severe obesity or excessive meteorism. Therefore, we analyzed the association between PLNE and the subsequent incidence of HCC in 846 patients.

HCV RNA was measured using Amplicore HCV version 2.0 (Roche, Tokyo, Japan), and the HCV serotype was examined using a serotyping assay (SRL, Tokyo, Japan). We also monitored IFN therapy and responses during the follow-up period. A sustained virological response (SVR) was defined as undetectable HCV-RNA at least 24 weeks after the end of therapy. All blood tests were performed at the time of US examination.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Patient follow up

Patients were followed up every 3–6 months at the outpatient clinic, when blood tests (including tumor markers) and US were carried out. Contrast-enhanced computed tomography (CT) was performed when HCC was suspected, based on US, and/or if the serum α-fetoprotein (AFP) level showed an abnormal increase. HCC was diagnosed by dynamic CT, and hyperattenuation in the arterial phase with washout in the late phase was considered a definite sign of HCC [18]. When a diagnosis of HCC was ambiguous, ultrasound-guided tumor biopsy was

performed and a pathologic diagnosis was made based on the Edmondson and Steiner criteria. Time to HCC occurrence was defined as the interval between the date of the first US screening and the diagnosis of HCC. Patients were censored at the time of death without HCC development, the last visit when lost to follow up, or the end of the study period. The last observation in this study was made on December 31, 2010. Thus, the time of observation was extended from that of our previous study, which was censored on May 31, 2008 [16].

Transient elastography

Transient elastography was performed using Fibroscan (Echosens, Paris, France) as described previously [16].

Statistical analysis

Data were expressed as means ± standard deviation (SD) unless otherwise indicated. Categorical variables were compared by χ^2tests, whereas continuous variables were compared by the unpaired Student's *t*-test (parametric) or the Mann–Whitney *U*-test (non-parametric). Multivariate logistic regression analysis was used to identify factors that were independently associated with the presence of PLNE. Cumulative HCC incidence was estimated using the Kaplan–Meier method, and the difference between groups was assessed with the log-rank test. In the analysis of risk factors for hepatocarcinogenesis, we tested the following variables in univariate analysis and multivariate Cox proportional hazard regression analysis: age, sex, platelet count, serum albumin concentration, total bilirubin concentration, ALT and aspartate aminotransferase (AST) levels, higher AFP concentration (>10 ng/ml), prothrombin activity, heavy alcohol drinking (alcohol intake >80 g/day), BMI, higher liver stiffness measurement (LSM) (>10 kPa), HCV serotype, HCV viral load (>100 kIU/ml), IFN treatment, achievement of SVR, and presence of PLNE. A *p* value of less than 0.05 on a two-tailed test was considered significant. Data processing and analysis were performed using StatView (ver. 5.0; SAS Institute, Cary, NC, USA) and SPSS (ver. 14.0; SPSS, Chicago, IL, USA) software.

Results

Patient profiles

We detected PLNE in 169 of 846 (20.0 %) patients with chronic hepatitis C. A representative ultrasound image is shown in Fig. 1. The mean (±SD) length of the longest axis was 1.7 (±0.5) cm (range 1.0–3.5 cm). The clinical features of patients with and without PLNE are summarized in Table 1. The proportion of females was significantly higher in the PLNE-positive group than in the PLNE-negative group (63.3 vs. 52.9 %), and BMI was slightly but significantly lower in the PLNE-positive group than in the PLNE-negative group (21.9 ± 2.6 vs. 22.5 ± 2.9). The proportion of HCV serotype 1 patients was higher in the PLNE-positive group than in the PLNE-negative group, with borderline significance. There was a tendency of a higher serum ALT level in the PLNE-positive group, but the difference was without statistical significance. There were no significant differences in other liver function test results, or in liver stiffness and hepatitis C viral load between the two groups. Multivariate logistic regression analysis using the factors of sex, serum ALT, BMI, and HCV serotype revealed that female sex, lower BMI, and HCV serotype 1 were independently associated with the presence of PLNE (Table 2).

Incidence of HCC

The mean follow-up period was 4.8 years, constituting a total observation of 4,021 person-years. During the observation period, 70 (8.3 %) patients were lost to follow up: 15 (8.8 %) patients in the PLNE-positive group and 55 (8.1 %) patients in the PLNE-negative group. There were no patients in whom an enlarged perihepatic LN turned out to be caused by other underlying diseases including metastasis of HCC. The SVR rate in patients who received IFN therapy during the follow-up period was significantly lower in the PLNE-positive group compared with that in the PLNE-negative group [7/34 (20.6 %) vs. 93/172

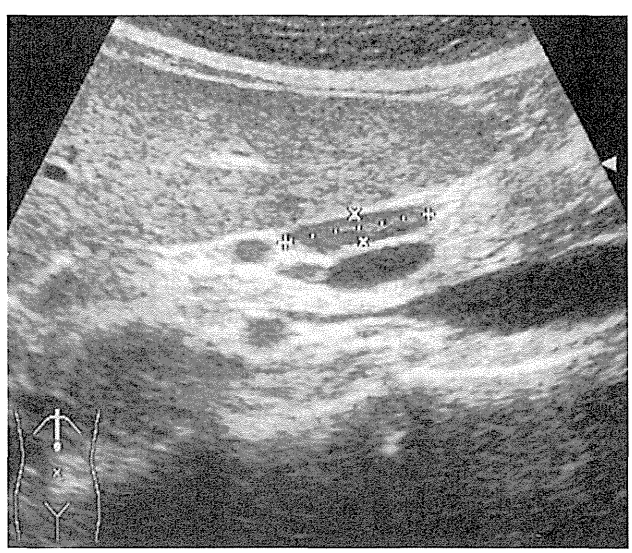

Fig. 1 Representative ultrasound image of enlarged perihepatic lymph node (LN) in a patient with chronic hepatitis C

Table 1 Clinical features of patients with and without PLNE

Variable	PLNE-positive group ($n = 169$)	PLNE-negative group ($n = 677$)	p value
Age (years)	62.4 ± 10.1 (29–83)	62.4 ± 11.5 (17–89)	0.58
Male, n (%)	62 (36.7)	319 (47.1)	0.018
Serum albumin (g/dl)	4.0 ± 0.4 (2.8–4.8)	4.0 ± 0.4 (2.5–5.0)	0.93
Total bilirubin (mg/dl)	0.8 ± 0.3 (0.3–2.1)	0.9 ± 0.5 (0.3–4.6)	0.23
AST (IU/l)	52 ± 34.1 (17–223)	50 ± 33.8 (9–286)	0.16
ALT (IU/l)	57 ± 48.7 (4–374)	53 ± 45.2 (2–503)	0.10
Platelet count ($\times 10^4/\mu l$)	16.1 ± 6.6 (2.1–42.2)	16.1 ± 6.7 (3.2–43.6)	0.89
Prothrombin time (%)	86.0 ± 12.1 (50.3–100.0)	85.7 ± 12.4 (38.9–100.0)	0.88
AFP (ng/ml)	22.0 ± 67.9 (1–592)	13.4 ± 37.1 (1–563)	0.61
BMI (kg/m^2)	21.9 ± 2.6 (14.4–28.7)	22.5 ± 2.9 (15.1–29.8)	0.007
Liver stiffness (kPa)	10.9 ± 7.8 (2.8–42.2)	12.0 ± 10.0 (2.5–75.0)	0.59
Alcohol consumption >80 g/day, n (%)	6 (3.6)	25 (3.7)	0.82
HCV viral load (kIU/ml)	549 ± 646 (5–5000)	651 ± 842 (5–5000)	0.48
HCV serotype 1, n (%)	146 (86.3)	538 (79.5)	0.053
Patients who received IFN, n (%)	34 (20.1)	172 (25.4)	0.18
Patients who achieved SVR, n (%)	7 (4.1)	93 (13.7)	0.0009

PLNE perihepatic lymph node enlargement, *AST* aspartate aminotransferase. *ALT* alanine aminotransferase, *AFP* α-fetoprotein, *BMI* body mass index, *HCV* hepatitis C virus

Table 2 Factors associated with the presence of PLNE: multivariate analysis

Variable	Odds ratio (95 % confidence interval [CI])	p value
Male sex	0.667 (0.464–0.936)	0.024
ALT level (per 1 IU/l)	1.003 (0.999–1.006)	0.10
BMI (per 1 kg/m^2)	0.919 (0.864–0.978)	0.017
HCV serotype 1	1.64 (1.02–2.66)	0.043

(54.1 %), $p = 0.0005$]. This finding was consistent with previous reports [12, 19].

By the end of the follow-up period, HCC had developed in 121 patients (3.0 % per 1 person-year). The cumulative incidence rates of HCC at 3 and 5 years estimated by the Kaplan–Meier method were 8.9 and 13.7 %, respectively. We then assessed the incidence of HCC stratified by the presence of PLNE. Unexpectedly, the PLNE-positive group revealed a significantly lower incidence of HCC than the PLNE-negative group ($p = 0.019$, log-rank test) (Fig. 2). The cumulative incidence rates at 3 and 5 years were 3.6 and 8.2 %, respectively, in the PLNE-positive group, and 10.1 and 15.1 % in the PLNE-negative group. These results indicate that patients with PLNE have a lower risk of HCC development despite having a lower SVR rate with IFN therapy.

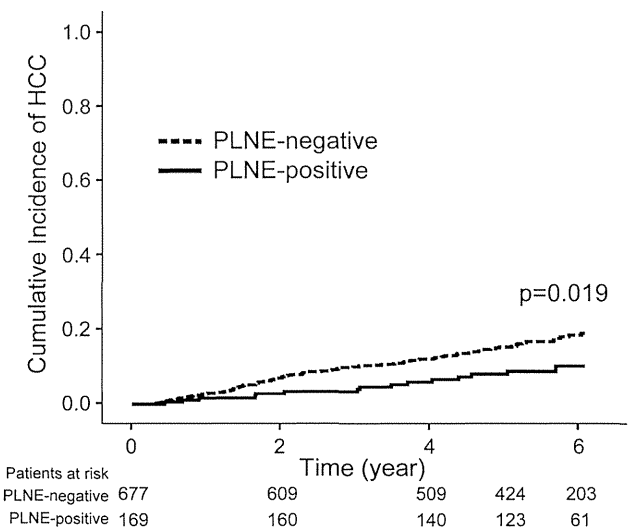

Fig. 2 Cumulative incidence of hepatocellular carcinoma (*HCC*) development stratified by the presence of perihepatic lymph node enlargement (*PLNE*)

Risk analyses

We analyzed the risk factors for HCC development. In the univariate analyses, older age, male sex, lower serum albumin concentration, higher total bilirubin concentration, higher AST level, higher ALT level, lower prothrombin activity, lower platelet count, heavy alcohol drinking, higher BMI, LSM greater than 10 kPa, AFP level greater than 10 ng/ml, HCV serotype 1, not receiving IFN, not achieving SVR, and absence of PLNE were significant risk factors for HCC (Table 3). As we had reported previously, a higher LSM (i.e., greater than 10 kPa) was a strong predictor of HCC development [hazard ratio (HR) 15.4, 95 % confidence interval (CI) 8.6–27.0, $p < 0.0001$)]. Multivariate proportional hazard regression analyses

Table 3 Risk factors for HCC development: univariate and multivariate analyses

Variable	Univariate analysis		Multivariate analysis	
	Hazard ratio (95 % CI)	*p* value	Hazard ratio (95 % CI)	*p* value
Age (per 1 year age)	1.07 (1.05–1.09)	<0.0001	1.04 (1.01–1.06)	0.002
Male sex	1.45 (1.02–2.08)	0.041	1.49 (1.02–2.17)	0.039
Platelet count (per 10^4/μl)	0.852 (0.823–0.882)	<0.0001	0.965 (0.926–1.005)	0.089
Total bilirubin (per 1 mg/dl)	1.88 (1.45–2.45)	<0.0001	0.825 (0.567–1.2)	0.32
Serum albumin level (per 1 g/dl)	0.12 (0.084–0.17)	<0.0001	0.441 (0.263–0.739)	0.002
AST level (per 1 IU/l)	1.01 (1.007–1.014)	<0.0001	1.002 (0.991–1.013)	0.71
ALT level (per 1 IU/l)	1.004 (1.002–1.007)	0.002	1.0 (0.991–1.013)	0.94
AFP level >10 ng/ml	6.76 (4.69–9.8)	<0.0001	1.9 (1.22–2.97)	0.005
Prothrombin time (per 1 %)	0.973 (0.966–0.979)	<0.0001	0.989 (0.976–1.001)	0.072
Alcohol consumption >80 g/day	2.73 (1.43–5.24)	0.002	3.53 (1.76–7.09)	0.0004
BMI (per 1 kg/m^2)	1.09 (1.03–1.16)	0.006	1.09 (1.01–1.17)	0.025
Liver stiffness >10 kPa	15.4 (8.6–27.0)	<0.0001	4.41 (2.24–8.7)	<0.0001
HCV serotype 1	1.76 (1.03–3.03)	0.04	1.36 (0.774–2.38)	0.29
HCV-RNA >100 kIU/ml	1.36 (0.87–2.13)	0.18	1.24 (0.781–1.97)	0.36
Patients treated with IFN	0.44 (0.262–0.75)	0.002	0.59 (0.315–1.11)	0.1
Patients with SVR	0.175 (0.055–0.549)	0.003	0.621 (0.169–2.28)	0.47
Presence of PLNE	0.53 (0.31–0.91)	0.02	0.551 (0.31–0.978)	0.042

HCC hepatocellular carcinoma, *IFN* interferon, *SVR* sustained viral response

revealed that older age, male sex, lower serum albumin concentration, AFP level greater than 10 ng/ml, heavy alcohol drinking, higher BMI, LSM greater than 10 kPa, and absence of PLNE were independent risk factors for HCC (Table 3). These results suggest that the presence of PLNE is an independent negative predictor of HCC development in chronic hepatitis C patients.

Subgroup analysis of non-obese patients

To further rule out the possibility that obesity acted as a confounder in the association between the presence of PLNE and HCC development, we reanalyzed the contribution of PLNE to HCC development in a subgroup of non-obese patients, defined as those with BMI <25 kg/m^2 ($n = 695$), because we could clearly visualize the liver hilum in such individuals. As shown in Fig. 3, the PLNE-positive group had a significantly lower incidence of HCC than the PLNE-negative group even in the non-obese subgroup ($p = 0.02$). Thus, we further confirmed that the presence of PLNE was negatively associated with HCC development independently of obesity.

Significance of the size of perihepatic LNs

To examine the significance of the size of perihepatic LNs, we divided patients with PLNE into two groups: a smaller LN group (longest axis of LN 1 cm to 2 cm, $n = 122$) and a larger LN group (longest axis of LN ≥2 cm, $n = 47$).

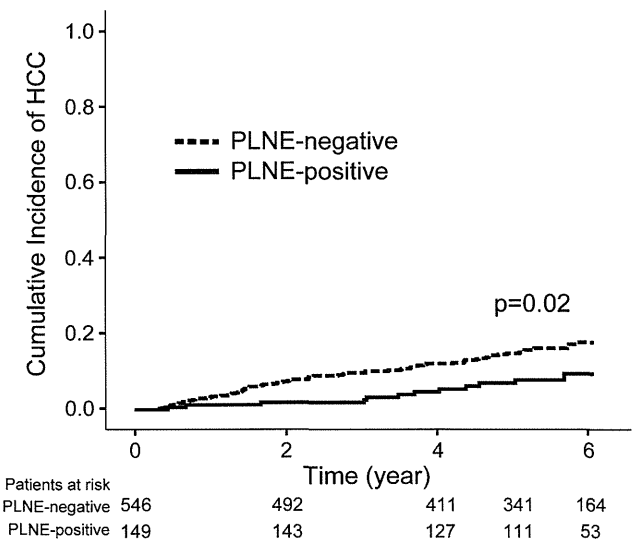

Fig. 3 Cumulative incidence of HCC development stratified by the presence of PLNE: subgroup analysis of non-obese patients (body mass index [BMI] <25 kg/m^2)

The characteristics of each group are summarized in Table 4. The proportion of male patients and the LSM value tended to be higher in the larger LN group than in the smaller LN group, but the difference was not statistically significant for either factor. There were no significant differences in other factors between the two groups. Furthermore, there was no significant difference in HCC incidence rates between the two groups (Fig. 4), although the larger LN group revealed a slightly higher incidence of

Table 4 Comparison of clinical features between patients with small perihepatic lymph nodes (LNs) and those with large perihepatic LNs

Variable	LN size 1 cm to <2 cm, (n = 122)	LN size ≥2 cm (n = 47)	p value
Age (years)	62.7 ± 10.4 (29–83)	61.5 ± 9.2 (32–77)	0.32
Male, n (%)	40 (32.8)	22 (46.8)	0.092
Serum albumin (g/dl)	4.0 ± 0.3 (3.0–4.8)	4.0 ± 0.4 (2.8–4.8)	0.96
Total bilirubin (mg/dl)	0.8 ± 0.3 (0.3–1.6)	0.8 ± 0.4 (0.3–2.1)	0.98
AST (IU/l)	53 ± 36.0 (17–223)	50 ± 28.7 (17–181)	0.83
ALT (IU/l)	56 ± 49.0 (4–374)	59 ± 48.2 (6–308)	0.48
Platelet count ($\times 10^4/\mu l$)	16.3 ± 6.2 (2.1–36.4)	15.4 ± 7.4 (4.8–42.2)	0.25
Prothrombin time (%)	86.7 ± 11.7 (57.4–100.0)	84.2 ± 13.0 (50.3–100.0)	0.27
AFP (ng/ml)	13.0 ± 27.7 (1–168)	45.3 ± 118.6 (1–592)	0.19
BMI (kg/m^2)	21.8 ± 2.6 (16.8–28.0)	22.0 ± 2.6 (14.4–28.7)	0.75
Liver stiffness (kPa)	10.2 ± 7.1 (2.8–37.4)	12.5 ± 9.3 (4.2–42.2)	0.064
Alcohol consumption >80 g/day, n (%)	5 (4.1)	1 (2.1)	0.54
HCV viral load (kIU/ml)	658 ± 788	504 ± 582	0.17
HCV serotype 1, n (%)	107 (87.7)	39 (83.0)	0.58
Patients who received IFN, n (%)	24 (19.6)	10 (21.2)	0.98
Patients who achieved SVR, n (%)	5 (4.1)	2 (4.3)	0.99

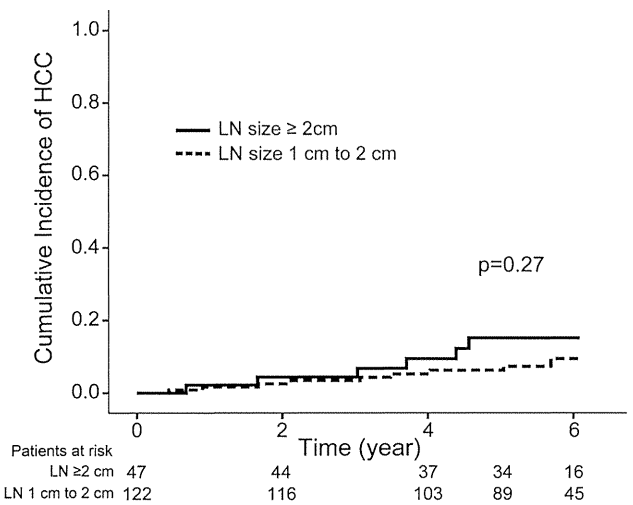

Fig. 4 Cumulative incidence of HCC development in patients with PLNE stratified by the size of perihepatic LNs: i.e., smaller (longest axis of LN 1 to <2 cm) and larger (longest axis of LN ≥2 cm)

HCC. These results suggest that the size of perihepatic LNs in chronic hepatitis C patients may not be clinically as important as the presence of PLNE itself.

Discussion

Although PLNE is a common finding in patients with chronic hepatitis C, its clinical significance has remained unclear. In the present study, we reevaluated the clinical relevance of PLNE in a large cohort of chronic hepatitis C patients. We found, by prospective analysis, that patients with PLNE had a lower risk of HCC development than those without PLNE. To our knowledge, this is the first study reporting a negative association between the presence of PLNE and HCC development.

Before we started this study, we expected that patients with PLNE would have a higher risk of HCC development, based on previous reports showing positive associations between PLNE and liver inflammation and fibrosis [8, 13–15]. However, in our study, neither inflammatory markers, such as serum AST and ALT levels, nor fibrosis markers, such as the platelet count and LSM, had statistically significant associations with the presence of PLNE. On the contrary, patients with PLNE revealed a significantly lower risk of HCC development. One possible explanation for this result is that obesity may affect the ability of US to detect perihepatic LNs, although patients with severe obesity were excluded from the study. To rule out the effect of confounders, especially obesity, we performed multivariate analysis and subgroup analysis of non-obese patients, and the results showed that the presence of PLNE was an independent negative predictor of HCC development. Additionally, of the 846 patients enrolled in this study, 175 patients underwent abdominal computed tomography (CT) within one year from the date of the US examination. The concordance rate for the diagnosis of PLNE between CT and US in these patients was 91.4 % (160/175). Therefore, we consider that the diagnostic accuracy of US for PLNE was acceptable in this study.

Although the mechanism of PLNE in patients with hepatitis C is still unknown, hyperplasia of regional LNs is generally considered to reflect inflammatory responses in the adjacent organs. The volume of perihepatic LNs has been reported to significantly decrease after antiviral

therapy, especially in patients with an SVR, supporting the hypothesis that PLNE reflects the inflammatory response to HCV [19–21]. In fact, PLNE was reported to be associated with CD8 lymphocyte counts in the peripheral blood [10]. Furthermore, HCV-specific IFN-γ production and proliferative responses of T cells were found most commonly in perihepatic LNs rather than in liver tissue or in the peripheral blood, indicating that there was ongoing T-cell activation in perihepatic LNs [22]. Our results, taken together with these previous reports, suggest that the presence of PLNE may reflect an adequate host immune response to HCV. T-cell immunity is very important in the control of HCV infection and in the prevention of hepatocarcinogenesis [23–25], and a T-cell response that is too weak may accelerate hepatocarcinogenesis, as seen in patients co-infected with HCV and human immunodeficiency virus [26, 27]. Thus, a weak T-cell response may be one explanation of the higher risk of HCC development in patients without PLNE. On the other hand, too strong an anti-HCV T-cell response may induce hepatocellular damage and lead to subsequent hepatocarcinogenesis [28], so patients with larger perihepatic LNs may have a slightly higher tendency to develop HCC. However, from the present type of observational study, we cannot evaluate a causal relationship between PLNE and hepatocarcinogenesis, so further studies are needed to clarify this point.

As mentioned above, several studies have shown that PLNE was positively associated with the degree of liver inflammation or fibrosis [8, 13–15], but, in the present study we could not find such associations, except for slight serum ALT elevation. However, because of ethical concerns regarding the performance of liver biopsy, we did not assess liver histology, so we cannot conclude whether or not PLNE is really associated with liver inflammation and fibrosis. Of note, the reported relationships of PLNE to liver function tests and liver inflammation and fibrosis are inconsistent among studies [7–15]. One reason may be that these findings were based on relatively small samples. Another reason is that there is a lack of established criteria for the diagnosis of PLNE. The lack of definite criteria may also contribute to the wide variation in the prevalence of PLNE among studies (from 20 to 100 %) [8–10, 21]. We defined PLNE as an LN that was at least 1 cm in the longest axis, and this definition was based on the report by Grier et al. [21] and our preliminary investigation in healthy subjects. Some studies have used more detailed measurements of LNs with calculations of node volume and shape [8, 10, 20]. These methods are certainly more accurate in terms of the assessment of nodal volume, but may be too complicated in the clinical setting, as discussed by Grier et al. [21]. We used a simpler method, because our study included a large number of patients and was conducted to examine the significance of PLNE in daily clinical practice. Admittedly, a more detailed method would be appropriate to elucidate more clearly the involvement of PLNE in the pathophysiology of hepatitis and hepatocarcinogenesis.

In the present study, female sex, lower BMI, and HCV serotype 1 were independently associated with the presence of PLNE. Soresi et al. [29] also reported that PLNE was observed significantly more often in female patients than in male patients with chronic hepatitis C. Although we cannot clarify the mechanism underlying this association, this finding may be interesting from the point of view of gender differences in immune systems and hepatocarcinogenesis. In the study by Soresi et al., BMI in patients with PLNE tended to be lower than that in patients without PLNE, although the difference was not statistically significant [29], and this finding may be in line with our present results. Regarding BMI in patients with chronic HCV infection, an anti-HCV specific immune response was reportedly associated with lower BMI through the expression of adiponectin, one of the major adipokines [30]. Thus, the active immune response to HCV in patients with lower BMI might cause PLNE. Recent studies have reported that obesity and obesity-induced dysregulation of adipokines play important roles in hepatocarcinogenesis [31–33], so the examination of adipokine expression may help to explain the relationship of PLNE to BMI and hepatocarcinogenesis.

Another important finding in our study was that the SVR rate in patients who received IFN therapy was significantly lower in patients with PLNE than in patients without PLNE. This finding is consistent with previous reports [12, 19]. Although the proportion of individuals with HCV serotype 1 was higher in our patients with PLNE than in patients without PLNE, subgroup analysis of the patients with HCV serotype 1 also revealed a significantly lower SVR rate in patients with PLNE than in patients without PLNE (data not shown). Therefore, further analyses are planned to clarify the relationship of PLNE to HCV serotype and response to IFN therapy.

In conclusion, the presence of PLNE is an independent negative predictor of HCC development in chronic hepatitis C patients. This study may provide new insights into daily clinical practice and the pathophysiology of HCV-induced hepatitis and hepatocarcinogenesis.

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Identification of a Functional Variant in the *MICA* Promoter Which Regulates *MICA* Expression and Increases HCV-Related Hepatocellular Carcinoma Risk

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Abstract

Hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma (HCC) in Japan. We previously identified the association of SNP rs2596542 in the 5′ flanking region of the *MHC class I polypeptide-related sequence A* (*MICA*) gene with the risk of HCV-induced HCC. In the current study, we performed detailed functional analysis of 12 candidate SNPs in the promoter region and found that a SNP rs2596538 located at 2.8 kb upstream of the *MICA* gene affected the binding of a nuclear protein(s) to the genomic segment including this SNP. By electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay, we identified that transcription factor Specificity Protein 1 (SP1) can bind to the protective G allele, but not to the risk A allele. In addition, reporter construct containing the G allele was found to exhibit higher transcriptional activity than that containing the A allele. Moreover, SNP rs2596538 showed stronger association with HCV-induced HCC ($P = 1.82 \times 10^{-5}$ and OR = 1.34) than the previously identified SNP rs2596542. We also found significantly higher serum level of soluble MICA (sMICA) in HCV-induced HCC patients carrying the G allele than those carrying the A allele ($P = 0.00616$). In summary, we have identified a functional SNP that is associated with the expression of MICA and the risk for HCV-induced HCC.

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Introduction

Hepatocellular carcinoma (HCC) is one of the common cancers in the world. It is well-known to be associated with the chronic infection of Hepatitis B (HBV) and Hepatitis C (HCV) viruses. In Japan, nearly 70% of HCC patients are infected with HCV [1]. The annual rate of developing HCC among patients with HCV-related liver cirrhosis in Japan is estimated to be about 4–8 percent [2]. Recent analyses have identified various genetic factors that are related with viral induced liver diseases [3–5]. In our previous two-stage genome-wide association study (GWAS) using a total number of 1,394 cases and 5,486 controls, a SNP rs2596542 located on chromosome 6p21.33 was shown to be significantly associated with HCV-induced HCC ($P = 4.21 \times 10^{-13}$ and OR = 1.39) [6]. This SNP is located within the class I major histocompatibility complex (MHC) region and is at about 4.8 kb upstream of *MHC class I polypeptide-related sequence A* (*MICA*) gene. We also identified that the risk A allele of SNP rs2596542 was strongly associated with the low expression of soluble MICA (sMICA) in the serum of HCV-related HCC patients [6].

MICA is a membrane protein which is up-regulated in various tumor cells and also induced in response to various cellular stresses such as infection, hypoxia, and heat shock [7]. It is an important component of the innate immune response, as MICA can bind to the NKG2D receptor and subsequently activate natural killer (NK) cells, CD8+ cells, and γδ T cells [8,9]. Moreover, membrane MICA can be shed by metalloproteinases, including MMP9, ADAM10, and ADAM17, and secreted into serum as a soluble form [10,11]. Since these metalloproteinases are often activated in HCC, the expressions of both membrane-bound MICA and sMICA are increased [12,13]. SNP rs2596542 was found to be associated with the progression from chronic hepatitis C (CHC) to HCC and also with serum sMICA level. Hence, both rs2596542 and sMICA would be possible prognostic biomarkers for CHC patients. However, their underlying molecular mechanisms were not fully elucidated so far.

We hypothesize that *MICA* variations could affect sMICA level by either one or both of the following two possible mechanisms: (1) the genetic variation(s) in the coding region affecting the protein stability and (2) the transcriptional regulation. Previously, variable

numbers of tandem repeats (VNTRs) in exon 5 of *MICA* were identified to affect MICA subcellular localization and serum MICA level [14]. The exon 5 of *MICA* encodes the transmembrane domain and the insertion of an extra G nucleotide in the domain would result in a premature stop codon that would generate MICA protein without a transmembrane domain and subsequently affect sMICA level [14]. However, our previous results indicated that MICA VNTR was not significantly associated with the sMICA level or HCC risk [6]. Therefore, in the current study, we have tried to investigate whether the *MICA* variations would affect the *MICA* transcription in the liver cancer cells. Through the functional analysis of genetic variations in the *MICA* promoter region, we here report a causative SNP rs2596538 that increases the binding affinity of the transcription factor Specificity Protein 1 (SP1) and the risk of progression of the disease.

Materials and Methods

Samples and genotyping

DNA samples for direct sequencing (50 HCV-related HCC cases), imputation analysis (721 HCV-related HCC cases and 5,486 HCV-negative controls), and serum samples for sMICA ELISA (246 HCV-related HCC) were obtained from BioBank Japan [15,16]. Genotyping of SNPs from 1,394 HCC patients and measurement of sMICA expression by ELISA were performed in the previous study [6]. Genotyping of SNP rs2596542 in 1,043 CHC was performed previously in RIKEN using Illumina HumanHap610-Quad BeadChip [17]. All CHC subjects had abnormal levels of serum alanine transaminase for more than 6 months and were positive for both HCV antibody and serum HCV RNA. The SNP rs2596542 in liver cirrhosis samples without hepatocellular carcinoma from BioBank Japan (n = 420) and the University of Tokyo (n = 166) were genotyped using Illumina HumanHap610-Quad BeadChip or invader assay [18]. All subjects were either subjected to liver biopsy or diagnosed by non-invasive methods including hepatic imaging, biochemical data, and the presence/absence of clinical manifestations of portal hypertension [18]. The samples used in the current project were listed in Table S1. Case samples with HBV co-infection were excluded from this study. The subjects with cancers, chronic hepatitis B, diabetes or tuberculosis were excluded from non-HCV controls. All subjects were Japanese origin and provided written informed consent. This research project was approved by the ethical committees of the University of Tokyo and RIKEN.

Imputation study

The imputation study was performed by using a hidden Markov model programmed in MACH [19] and haplotype information from 1000 genomes database [20]. The imputation results were confirmed by direct DNA sequencing in 50 randomly selected samples.

Cell culture

Human liver cancer cell lines HLE and HepG2 were purchased from JHSF (Osaka, Japan) and ATCC. These cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum. Cells were cultured at 37°C with 5% CO_2.

EMSA

HLE cells were grown in 15 cm culture plate until they reached 95% confluency. The plate was then sealed with parafilm and immersed in a water bath at 42.5°C for 1.5 hours [21]. Nuclear extracts from these cells were prepared according to the standard protocol [22]. EMSA was carried out using DIG Gel Shift Kit, 2nd Generation (Roche) according to the manufacturer's instructions. The sequences of the 12 probes were listed in the Table S2. In brief, 30 fmol of labeled probes were hybridized with 5 μg nuclear extract for 15 minutes at room temperature. The mixtures were then loaded into a 6% TBE gel, separated by electrophoresis at 4°C and transferred onto a nylon membrane. The membrane was then hybridized with anti-digoxigenin-AP antibody and developed by CSPD solution. For competition study, nuclear extracts were incubated with non-labeled oligonucleotides first before adding labeled probe. For supershift assay, SP1 antibody (SC-59X, Santa Cruz Biotechnology) was added into the nuclear extract and incubated on ice for 30 minutes first before adding labeled probe. The mixtures were then separated by electrophoresis using 4% TBE gel. All EMSAs were repeated twice for reconfirmation of the results.

ChIP

The HLE cells (G allele homozygote) and HepG2 cells (heterozygote) were used in the ChIP assay. The plasmid pCAGGS-SP1 was transfected into both cells by using FuGENE6 Transfection Reagent (Roche). The ChIP assays were carried out using Chromatin Immunoprecipitation Assay Kit (Millipore) according to the manufacturer's protocol. In brief, the cells were treated with formaldehyde to crosslink DNA-protein complexes at 48 hours post-transfection. DNA-protein complexes were then sheared by sonication and immunoprecipitated by rabbit polyclonal anti-SP1 antibody (SC-59X, Santa Cruz Biotechnology). The resulting DNAs were analyzed by PCR (Table S2). In order to determine the binding specificity of SP1 to the SNP rs2596538 allele, the PCR products from HepG2 cells were further subcloned into pCR 2.1 vector and sequenced to assess G to A ratio in both input DNA and immunoprecipitant.

Dual luciferase reporter assay

Three copies of 31 bp DNA fragments equivalent to the EMSA oligonucleotides of SNP rs2596538 were cloned into pGL3-promoter vector (Promega). The plasmids were co-transfected with pCAGGS-SP1 and pRL-TK plasmids (Promega) into HLE cells by FuGENE6 Transfection Reagent (Roche). The pCAGGS-SP1 plasmid provided the expression of transcription factor SP1, and pRL-TK plasmid served as internal control for transfection efficiency [23]. The cells were lysed at 48 hours post-transfection, and relative luciferase activities were measured by Dual Luciferase Assay System (Toyo B-Net).

Western blotting

Cancer cell lysates were prepared by using pre-chilled RIPA buffer, and 25 μg of each lysate was loaded into the gel and separated by SDS-PAGE. Western blotting was performed according to the standard protocol. Rabbit anti-MICA antibody (ab63709, abcam: 1/1000) and rabbit anti-SP1 antibody (17-601, Upstate Biotechnology: 1/500) were used in the experiment.

Statistical analysis

The case-control association was analyzed by Student's *t*-test and Fisher's exact test as appropriate. The association of allele dependent sMICA expression was studied by Kruskal-Wallis test using R statistical environment version 2.8.1. The LD and coefficients (D' and r^2) were calculated by Haploview version 4.2 [24].

Table 1. Association of rs2596542 with the progression from CHC to LC and HCC.

	Case MAF	Control MAF	P*	OR	95% C.I.
LC vs CHC	0.3797	0.3442	0.04842	1.166	1.01–1.35
HCC vs LC	0.4012	0.3797	0.20296	1.094	0.95–1.26

MAF, minor allele frequency; OR, odds ratio for minor allele. C.I., confidence interval. SNP rs2596542 was analyzed in 1,043 chronic hepatitis C (CHC), 586 liver cirrhosis without hepatocellular carcinoma (LC) and 1,394 HCV-induce hepaticellular carcinoma patients (HCC). *calculated by Armitage trend test.
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Results

Analyses of SNP rs2596542 in HCV-infected patients at different disease stages

Since the development of HCC consists of multiple steps, we investigated the role of SNP rs2596542 with disease progression. SNP rs2596542 was genotyped in patients at three different disease categories of CHC (chronic hepatitis C) without liver cirrhosis (LC) or HCC, LC without HCC, and HCC. The statistical analysis indicated that SNP rs2596542 was significantly associated with disease progression from CHC to LC with P-value of 0.048 and odds ratio of 1.17 (Table 1). The risk allele frequency among HCC patients (40.1%) was higher than that among LC patients (38.0%), but the association was not statistically significant (P-value of 0.203 and odds ratio of 1.09). These results suggested the involvement of *MICA* with both liver fibrosis and hepatocellular carcinogenesis.

HCV-HCC risk is not associated with MICA copy number variation

A previous report has indicated the deletion of the entire *MICA* locus in 3.2% of Japanese population [25] and this deletion was shown to be associated with the risk of nasopharyngeal carcinoma (NPC), especially in male [26]. To identify the functional SNP that may affect *MICA* mRNA expression, we analyzed the relation between the *MICA* copy number variation (CNV) and the HCC susceptibility. We quantified this CNV by real-time PCR in 375 HCV-related HCC patients and 350 HCV-negative controls. As shown in Table S3, we found no difference in the copy numbers between HCC cases and controls, indicating that this CNV is unlikely to be causative genetic variation for the risk of HCC.

Direct sequencing of 5′ flanking region of *MICA*

We then focused on the variations in the 5′ flanking region of the *MICA* gene which may be associated with its promoter activity. We had conducted direct DNA sequencing of the 5-kb promoter region which included the marker SNP rs2596542 using genomic DNAs of 50 HCC subjects and identified 11 SNPs showing strong linkage disequilibrium with the marker SNP rs2596542 ($D'>0.953$ and $r^2>0.832$) (Fig. S1, Table 2).

Allele specific binding of nuclear protein to genomic region including SNP rs2596538

To investigate whether these genetic variations would affect the binding affinity of some transcription factors, we had conducted the electrophoretic mobility shift assay (EMSA) using the nuclear extract of HLE human hepatocellular carcinoma cells. Since MICA is a stress-inducible protein [21], we first treated the cells with heat shock treatment at 42°C for 90 minutes and confirmed significant induction of MICA expression as shown in Fig. 1a. Then we performed EMSA using 24 labeled-oligonucleotides corresponding to each allele of the 12 candidates' SNPs. The results of EMSA demonstrated that an oligonucleotide corresponding to a G allele of SNP rs2596538 exhibited stronger binding affinity to a nuclear protein(s) than that to an A allele (Fig. 1b). We then confirmed the specific binding of nuclear proteins to the G allele by competitor assay using non-labeled oligonucleotides (Fig. 1c). The self (G allele) oligonucleotides inhibited the formation of DNA-protein complex in a dose-dependent manner, but the non-self (A allele) oligonucleotides showed no inhibition effect. Taken together, some nuclear protein(s) in hepatocellular carcinoma cells would interact with a DNA fragment including the G allele of SNP rs2596538.

Table 2. Linkage disequilibrium between 11 candidate SNPs and SNP rs2596542.

SNP ID	Relative position[a]	A1	A1 frequency	D′	r^2
rs2596542	−4815	A	0.36		
rs2428475	−4788	G	0.36	1	1
rs28366144	−4586	T	0.36	1	1
rs2428474	−4387	G	0.39	1	0.88
rs2251731	−4045	A	0.39	1	0.88
rs2844526	−3703	C	0.38	1	0.918
rs2596541	−3572	A	0.38	1	0.918
rs2523453	−3285	G	0.38	1	0.918
rs2544525	−3259	C	0.38	1	0.918
rs2523452	−2870	G	0.34	0.953	0.832
rs2596538	−2778	A	0.34	0.953	0.832
rs2844522	−2710	C	0.34	0.953	0.832

Note: Direct DNA sequence of 5-kb promoter region of MICA from 50 HCV-HCC subjects. D' and r^2 were calculated by comparing the genotypes of these SNPs to the marker SNP rs2596542 by Haploview. A1, minor allele; [a]Relative position to exon 1 of the *MICA* gene.
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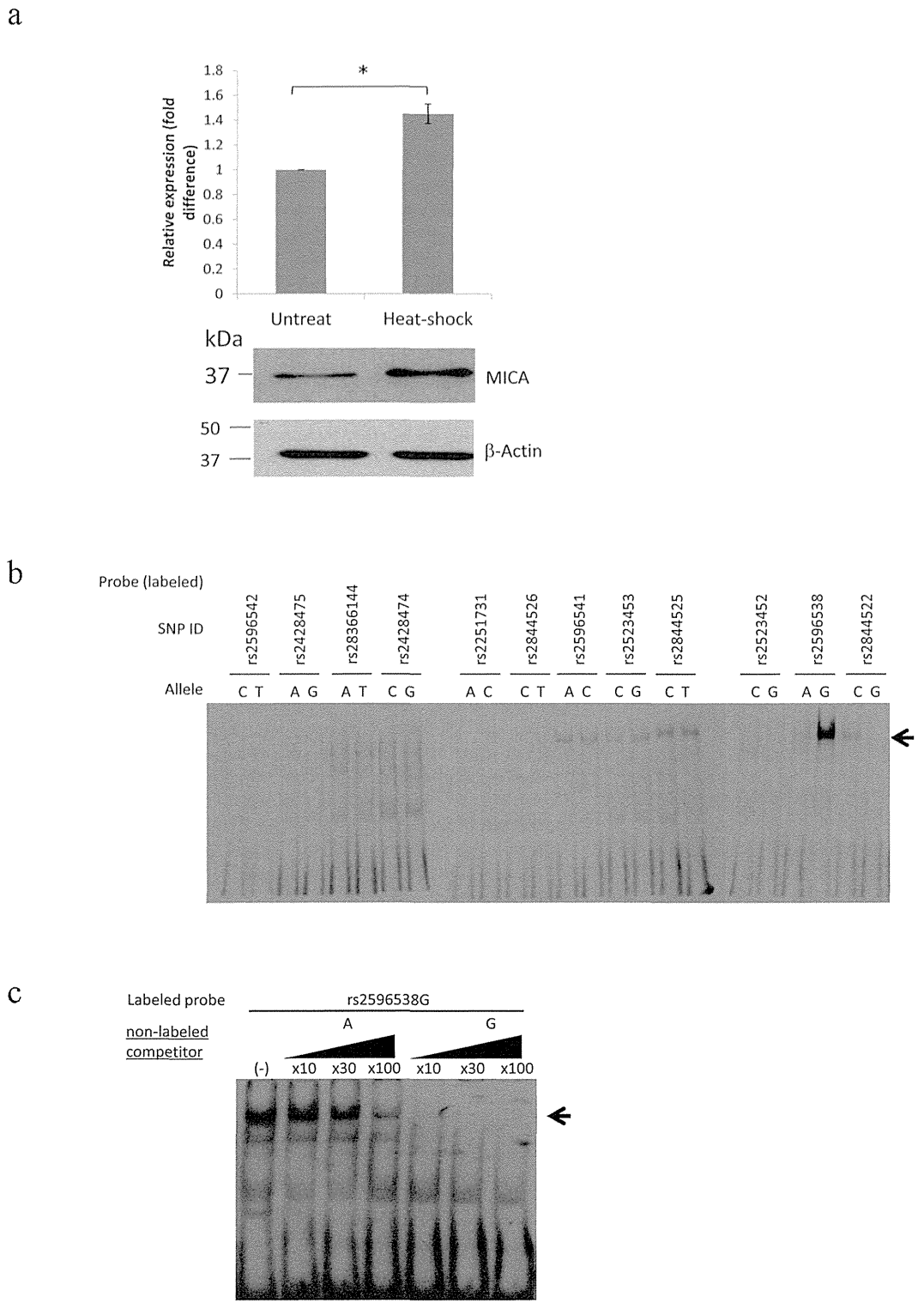

Figure 1. SNP rs2596538 affects the binding affinity of nuclear proteins. (**A**) Real-time quantitative PCR (upper) and Western blotting (lower) of MICA before and after heat shock treatment in HLE cells. *B2M* and β-actin are served as internal and protein loading control. (**B**) EMSA using 31 bp labeled probes flanking each SNP located within the 4.8 kb region upstream of *MICA* transcription start site. A black arrow indicates the shifted band specific to G allele of SNP rs2596538. (**C**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat treated HLE cells. Non-labeled A or G allele of SNP rs2596538 at different concentrations are used as competitors. Pointed arrow indicates shifted band. *$P<0.05$ by Student's *t*-test.

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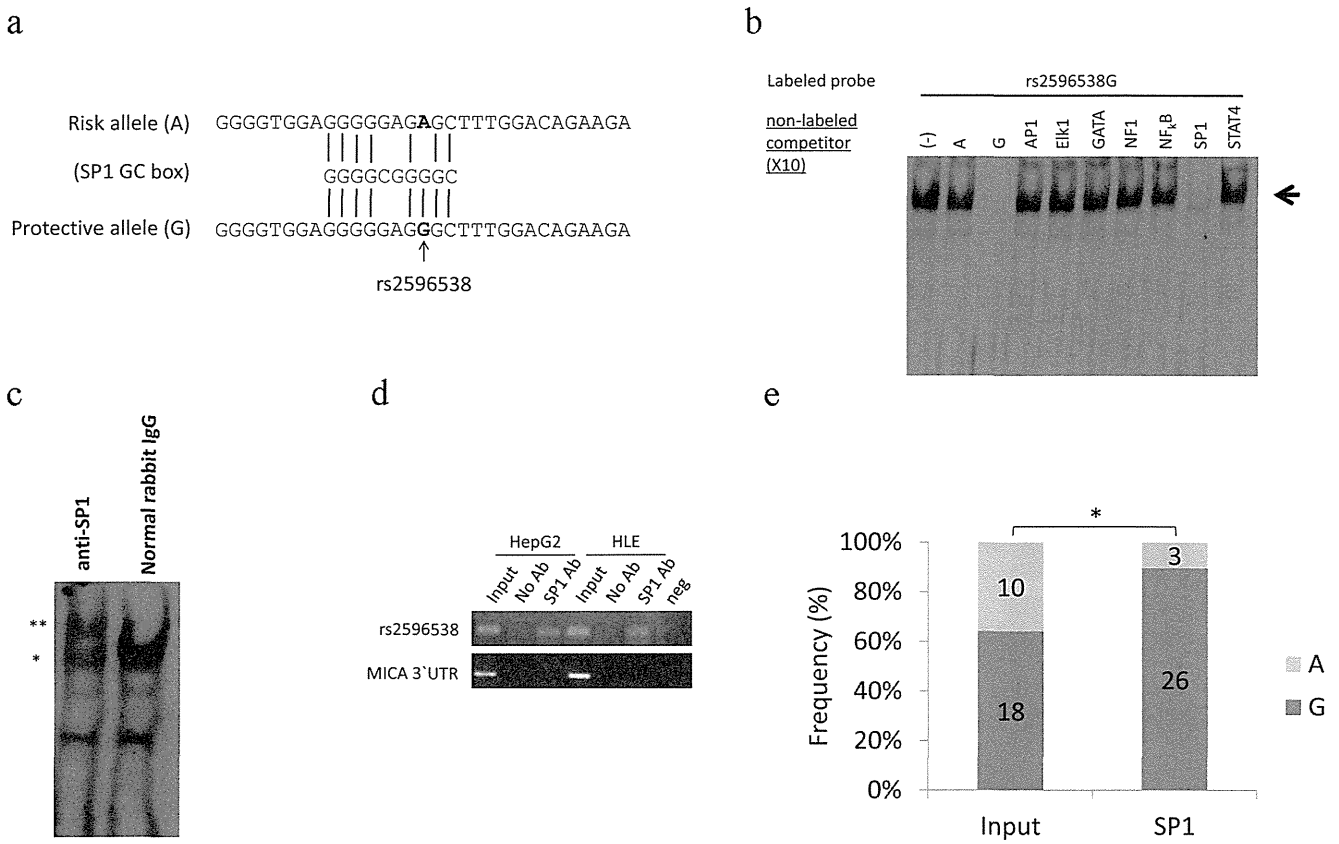

Figure 2. Binding of transcription factor SP1 to G allele of SNP rs2596538. (**A**) Multiple alignment of a GC box and DNA sequence of A or G probe of SNP rs2596538 used in EMSA. (**B**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat treated HLE cells. Non-labeled consensus oligonucleotides of seven transcription factors are used as competitors. Pointed arrow indicates shifted band. (**C**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat shock treated HLE cells in the presence of anti-SP1 antibody or normal rabbit IgG. Asterisks on the left side indicate the shifted (*) and super-shifted bands (**). Normal rabbit IgG serves as a negative control. (**D**) ChIP assay using HepG2 and HLE cell lines were ectopically expressed with SP1 protein. DNA-protein complex was immunoprecipitated with anti-SP1 antibody followed by PCR amplification using a primer pair flanking SNP rs2596538. DNAs precipitated without antibody are served as a negative control. PCR primers flanking the 3' UTR region of *MICA* are served as a negative control. (**E**) Genotype distribution at SNP rs2596538 in PCR fragment amplified from the input genomic DNA and DNA-protein complex immunopurified from HepG2 cells by using anit-SP1 antibody. *$P<0.05$ by Student's *t*-test.
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SNP rs2596538 regulates the binding of SP1

Since *in silico* analysis identified a putative GC box in a protective G allele but not in a risk A allele (Fig. 2a), the transcription factor SP1 might preferentially bind to the G allele. Base on this information, we further performed competitor assay using non-labeled oligonucleotides (Table S2) and found that among seven tested oligonucleotides, only SP1-consensus oligonucleotides could effectively inhibit the binding of the nuclear protein(s) to the labeled G allele (Fig. 2b). In addition, we identified that the addition of anti-SP1 antibody caused a supershift of a band corresponding to the DNA-protein complex while control IgG did not cause the band shift (Fig. 2c). This result clearly indicated that the SP1 protein is very likely to be a component of the DNA-protein complex.

Furthermore, we performed chromatin immunoprecipitation (ChIP) assay to confirm the binding of SP1 to this genomic region *in vivo*. We had used two cell lines with different genetic backgrounds at SNP rs2596538 locus: HLE cells carrying the only G allele, while HepG2 cells harboring both A and G alleles. After the introduction of SP1 expression vector (pCAGGS-SP1) into these cell lines, the cell extracts were subjected to ChIP assay using anti-SP1 antibody (Fig. 2d). Subsequent PCR experiments indicated that SP1 bound to a genomic fragment containing the G allele of SNP rs2596538 *in vivo*, while 3' UTR region of *MICA* (negative control) was not immunoprecipitated with anti-SP1 antibody. To further evaluate the binding ability of SP1 to each allele *in vivo*, we sub-cloned the DNA fragment that amplified from genomic DNA of HepG2 cells before and after immunoprecipitation by anti-SP1 antibody. The subsequent sequencing results showed that 26 out of 29 tested clones contained the G allele, demonstrating the preferential binding of SP1 to the G allele (Fig. 2e).

SP1 over-expression preferentially up-regulates MICA expression at G allele

To further investigate the physiological role of the interaction between SP1 and this genomic region, we performed reporter gene assay. Three copies of 31-bp DNA fragments flanking the candidate functional SNP rs2596538 were subcloned into the multiple cloning sites of the pGL3 promoter vector. The relative luciferase activity of the plasmid including the G allele was significantly higher than that including the A allele (Fig. 3a). Furthermore, over-expression of SP1 in the cells could significantly enhance the luciferase activity of the G-allele vector, while the enhancement of the A-allele vector was relatively modest (Fig. 3a).

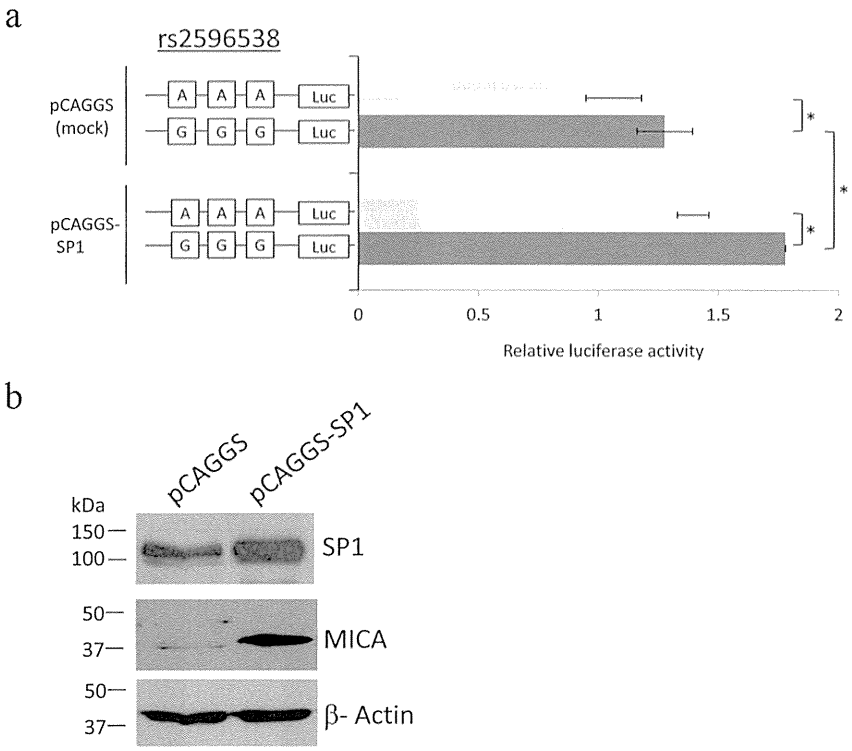

Figure 3. Transcriptional regulation of MICA by SP1 through genomic region including SNP rs2596538. (**A**) Reporter assay using constructs including 3 copies of 31 bp DNA fragment flanking SNP rs2596538. Reporter constructs are transfected into HLE cells with pRL-TK and pCAGGS or pCAGGS-SP1 vector. The value of relative luciferase activity was calculated as the firefly luciferase intensity divided by the renilla luciferase intensity. The data represent the mean ± SD value of 4 independent studies. (*$P<0.05$, Student's *t*-test) (**B**) MICA expression in HLE cells after transfection with pCAGGS or pCAGGS-SP1 vector. β-actin is served as a protein loading control.
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We also evaluated the effect of ectopically expressed SP1 on the MICA expression in HLE cells. Western-blot analysis showed that MICA protein expression was significantly increased after the SP1 over-expression (Fig. 3b). These results provided a strong evidence that the G allele has higher transcriptional potential that can be inducible by SP1.

Association of SNP rs2596538 with HCC risk and sMICA level in HCV-induced HCC patients

To further investigate the role of SNP rs2596538 in human carcinogenesis, we investigated the association of SNP rs2596538 with HCV-induced HCC in 721 HCV-HCC cases and 5,486 HCV-negative controls that had been genotyped using Illumina HumanHap610-Quad Genotyping BeadChip in our previous study [6]. We performed imputation analysis by using haplotype data from 1000 genome database [20] and found that an A allele of SNP rs2596538 was considered to be a risk allele for HCV-related HCC (Table 3, odds ratio = 1.343, $P = 1.82\times10^{-5}$). The functional SNP rs2596538 exhibited a stronger association with the HCC risk than the marker SNP rs2596542 (2.46×10^{-5}). We also analyzed the relationship between the SNP rs2596538 and the sMICA level among 246 HCV-induced HCC patients and found a significant association with the P-value of 0.00616 (Fig. 4). These results were concordant with our functional analyses in which the G allele exhibited a higher affinity to SP1 and revealed a higher transcriptional activity.

Table 3. Association of SNP rs2596542 and SNP rs2596538 with HCV-induced HCC.

SNP ID	Relative position[a]	A1	OR	*P* value
rs2596542	−4815	A	1.339	2.46×10^{-5}
rs2596538	−2778	A	1.343	1.82×10^{-5}

Note: Genotype data of 721 HCV-HCC cases and 5,486 HCV-negative controls were imputed using 1000 genomes as reference. A1, risk allele; OR, odds ratio for the risk allele calculated by considering the protective allele as a reference.
[a]Relative position to exon 1 of the *MICA* gene.
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Discussion

Approximately 160 million people (2.35% of the worldwide population) are estimated to have HCV infection [27]. Since HCV carriers have an increased risk to develop liver cirrhosis and subsequent HCC [28,29], the prediction of cancer risk is especially important for CHC patients. In our previous study, we have identified that SNP rs2596542 located in the upstream of *MICA* gene was significantly associated with the risk of HCC development among CHC patients as well as the serum level of sMICA [6]. In this study, we found that the genetic variant at SNP rs2596538 strongly affected the binding affinity of SP1. Over-expression of SP1 remarkably induced *MICA* expression in cells carrying the G allele that has a higher affinity to the SP1 binding. These findings are concordant with higher serum sMICA level among HCC patients with the G allele at SNP rs2596538. SP1 is a

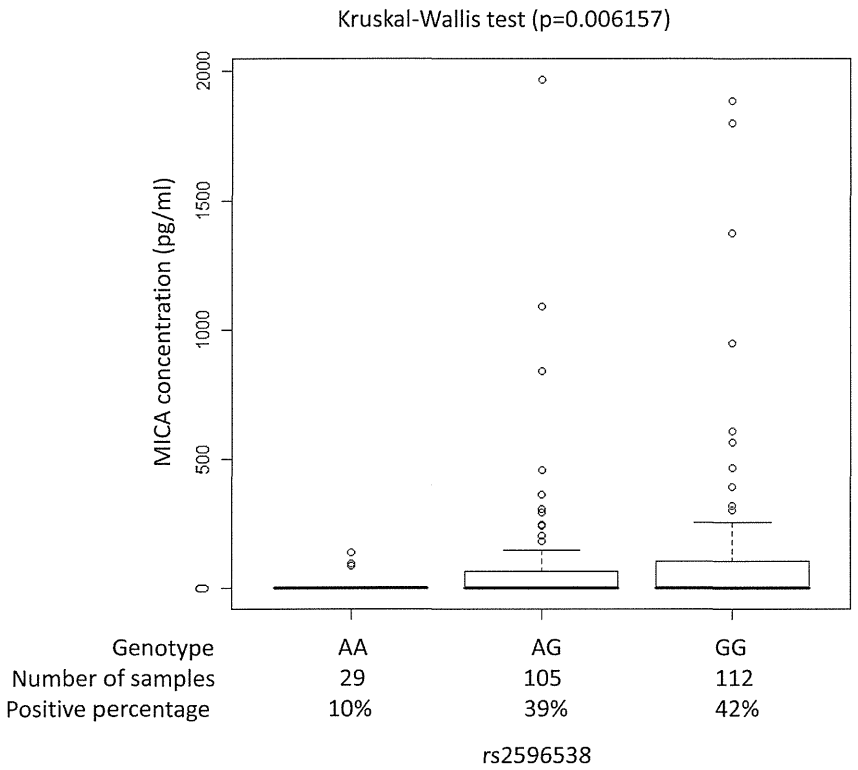

Figure 4. Association between the soluble MICA levels and SNP rs2596538 genotype. The samples were classified into 3 groups according to rs2596538 genotype. The sMICA levels measured by ELISA are indicated in y-axis. The numbers of samples and the proportion of sMICA positive subjects from each group are shown in x-axis. The percentage of the positive sMICA expression in each group are AA = 10%, AG = 39%, and GG = 42%. Statistical significance was determined by Kruskal-Wallis test.
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ubiquitously expressed transcription factor which binds to the GC-rich decanucleotide sequence (GC box) and activates the transcription of various viral and cellular genes [30,31]. Phosphorylation of SP1 was shown to be induced by HCV core protein and exhibited higher binding affinity to the promoter region of its downstream targets [32]. From our previous study, we showed a significant difference of sMICA expression between non-HCV individuals and CHC patients. This indicated that sMICA expression was induced after HCV infection [6]. Hence, we here propose the following hypothesis. After HCV infection, the virus core protein enhances the SP1 phosphorylation in hepatocytes, and the phosphorylated SP1 binds to the DNA segment corresponding to the G allele of SNP rs2596538 and then induces *MICA* expression. The membrane-bound MICA (mMICA) serves as a ligand for NKG2D to activate the immune system and results in the elimination of viral-infected cells by NK cells and CD8+ T cells [8,9]. Eventually, HCV-infected individuals with higher MICA level may cause stronger immune response to the infected cells and hence result in a reduced risk for HCC progression. Moreover, the mMICA is then shed by metalloproteinases that are often over-expressed in cancer tissues and convert mMICA to sMICA. This resulted in a significantly increase of sMICA level in the serum of HCV infected patients.

In contrast to HCV-induced HCC, our group had previously identified that higher sMICA level was associated with poor prognosis in HBV-induced HCC patients [33]. Such an opposite effect of *MICA* would be attributable to the difference in downstream pathway between HBV and HCV. HBV virus encodes hepatitis B virus X protein (HBx) that is pathogenic and promotes tumor formation. It had been reported that HBx protein was associated with an elevated expression of MT1-MMP, MMP2, and MMP3 [34,35]. HBx was also shown to transactivate MMP9 through ERKs and PI-3K-AKT/PKB pathway and suppress TIMP1 and TIMP3 activities [36,37]. The activation of metalloproteinases would induce the shedding of mMICA into sMICA, which promotes the tumor formation through the inhibitory effect of sMICA on NK cells. This can explain why high sMICA expression is a marker of poor prognosis for HBV-induced HCC. On the other hand, HCV infection was not associated with metalloproteinases activation, although the expression of sMICA was shown to be proportional to mMICA level. Therefore individuals with high MICA expression are likely to activate natural killer cells and CD8+ T cells to eliminate virus infected cells.

SP1 was previously identified as a transcriptional regulator of both *MICA* and *MICB* [7,9,38]. A polymorphism in the *MICB* promoter region was found to be associated with *MICB* transcription level [7]. To our knowledge, this is the first report showing that *MICA* transcription is directly influenced by functional variant. Moreover, this functional SNP is significantly associated with HCV-induced HCC. Our findings provide an insight that *MICA* genetic variation is a promising prognostic biomarker for CHC patients.

Supporting Information

Figure S1 Pairwise LD map between marker SNP and 11 candidates SNP. Black color boxes represent regions of high pairwise r^2 value. The LD was determined by direct DNA