HCV-related HCC (12), and HBV-related HCC (12). Both miR-122 and miR-22 were significantly elevated in patients with acute or chronic HBV infection compared to other case types (Fig. 6A and B) and were more strongly correlated (Fig. 6C).

DISCUSSION

In this study, expression levels of miR-122 and miR-22 were correlated with each other, as well as with markers of HBV infection, including HBsAg and HBV DNA titers (Fig. 2). Circulating levels of both microRNAs were also higher in patients who were positive for HBeAg. Although this suggests that these microRNAs may be up-regulated in cells infected with HBV, it will be necessary to compare serum and liver microRNA level to confirm this, as many other factors may influence circulating microRNA levels.

A notable result of this study is the strong linear association between miR-122 and serum HBsAg levels

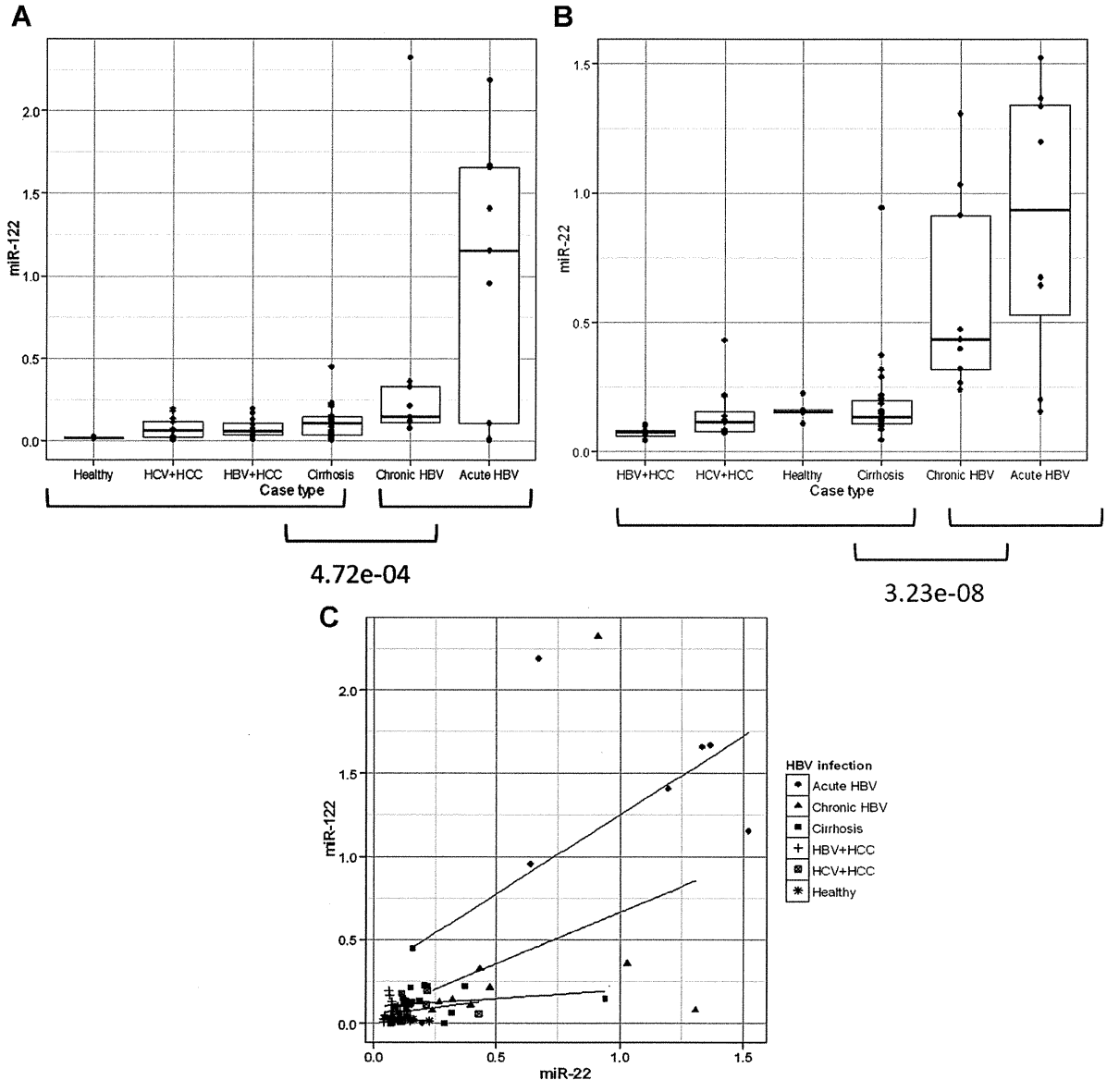

Fig. 6. miR-122 and miR-22 levels by case type. To examine the association of serum miR-122 and miR-22 with HBV infection, expression levels of miR-122 (**A**) and miR-22 (**B**) were compared among healthy controls, patients with acute or chronic HBV infection, liver cirrhosis, and HCC associated with either HBV or HCV. Both miR-122 and miR-22 were significantly higher in patients with acute or chronic HBV infection compared to patients with other case types, including patients with HBV-associated HCC. **C**: miR-122 and miR-22 also appear to be more strongly correlated in patients with acute or chronic HBV infection than in healthy controls or patients with cirrhosis or HCC.

(Table II; Fig. 2A). miR-122 has recently been shown to bind to a highly conserved HBV RNA sequence and negatively regulates viral gene expression and replication [Qiu et al., 2010; Chen et al., 2011]. Loss of miR-122 expression has also been shown to enhance HBV replication indirectly through cyclin G1-modulated p53 activity [Wang et al., 2011]. If miR-122 suppresses HBV replication, an inverse relationship between HBsAg titer and miR-122 levels might be expected, but instead a strong positive correlation was observed in this study. Although the reason for higher levels of miR-122 in patients with high HBsAg production is unclear, the innate immune response in liver cells against HBV replication may potentially induce higher expression of miR-122, which might be reflected in serum levels. Another possibility is that HBV might evade miR-122 suppression by sequestering and excreting miR-122 within the massively over-produced HBsAg particles in serum, in which case serum levels might be proportional to HBsAg levels but may not reflect miR-122 levels in the liver. It will be necessary to compare matched serum and liver miR-122 levels to address this issue.

In contrast to miR-122 levels, miR-22 expression was most strongly correlated with ALT and AST levels (Fig. 2; Table II). As it is known that miR-122 is expressed primarily or exclusively in hepatocytes [Mariana et al., 2002], the higher levels of miR-122 might reflect liver cell damage caused in the course of chronic hepatitis, and the same may be true for miR-22. Tissue-specificity of miR-22 is less clear, although it appears to be strongly expressed in hepatocellular carcinoma cell lines [Landgraf et al., 2007]. However, the fact that the levels of miR-22 are more strongly associated with ALT levels than miR-122 suggests that miR-122 is more likely to be over-expressed in liver cells infected with HBV. In this sense, miR-22 might be a better marker of liver injury than miR-122, although the lack of correlation of miR-22 with inflammatory activity complicates this association. Therefore, miR-122 and miR-22 may reflect different aspects of HBV infection and disease progression. miR-122 and miR-22 were expressed more strongly in acute and chronic HBV infection than in healthy controls or in patients with cirrhosis or HCC, suggesting an association with HBV infection, but notably miR-22 expression was comparatively higher in chronic HBV infection than miR-122 (Fig. 6). Measuring expression levels of one or both of these microRNAs may aid in assessment of disease severity [Waidmann et al., 2012].

In this study, miR-122 and miR-22 levels were both associated with HBV replication and liver injury. This suggests the need for a more systematic approach to examining multiple microRNAs under various chronic hepatitis B conditions and possibly in HBV-associated hepatocellular carcinoma. Further study is needed to establish a system to evaluate various disease conditions or prognoses in chronic HBV infection using microRNA biomarkers. It may also be of interest to determine the mechanism underlying the strong linear correlation between HBsAg and miR-122 levels to improve understanding of HBV virology.

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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,

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

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

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

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

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

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

Materials and methods

Ethics statement

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

Study population

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

SNP genotyping

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

Statistical analysis

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

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

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

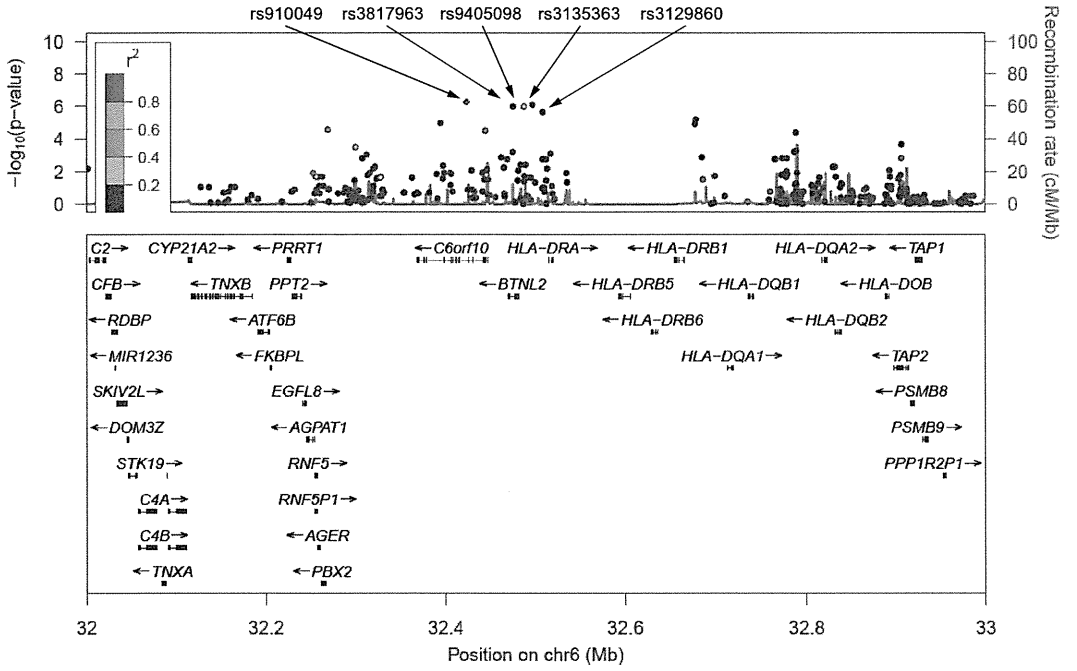

Fig. 1. Regional association plot at 6p21.3. (Upper panel) p Values of genotyped SNPs (circle) and imputed SNPs (cross) are plotted (as $-\log_{10}$ p value) against their physical position on chromosome 6 (NCBI Build 36). The p value for rs910049 at GWAS is represented by a purple diamond. Estimated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

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

Software

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

Results

Genome-wide association study for HCV-induced liver cirrhosis

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

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Table 2. Summary of GWAS and replication analyses.

SNP	Stage	Allele (1/2)	Gene	Liver cirrhosis				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].

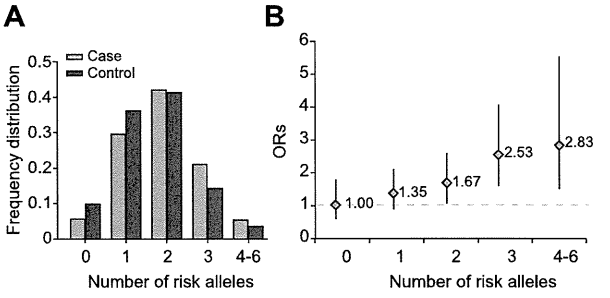

Fig. 2. Cumulative effects of liver cirrhosis risk alleles. (A) Frequency distribution divided by risk allele numbers (rs910049, rs3135353, and *HLA-DQA0601*) among liver cirrhosis (light blue bars) and chronic hepatitis C (dark blue bars) patients. (B) Plot of the increase odds ratio (OR) for liver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

Research Article

Discussion

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

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

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

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Conflict of interest

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

Authors' contributions

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

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Supplementary data

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

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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

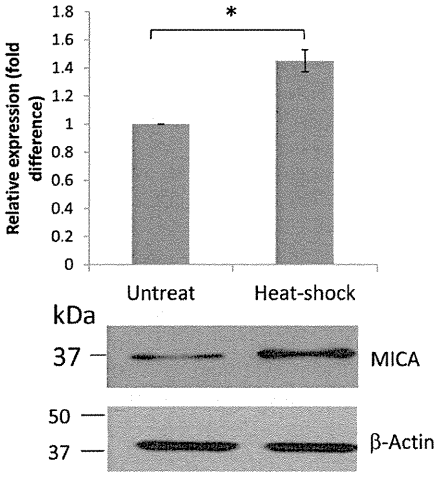
To investigate whether these genetic variations would affect the binding affinity of some transcription factors, we had conducted the electrophoretic mobility shift assay (EMSA) using the nuclear extract of HLE human hepatocellular carcinoma cells. Since MICA is a stress-inducible protein [21], we first 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.
doi:10.1371/journal.pone.0061279.t002

a



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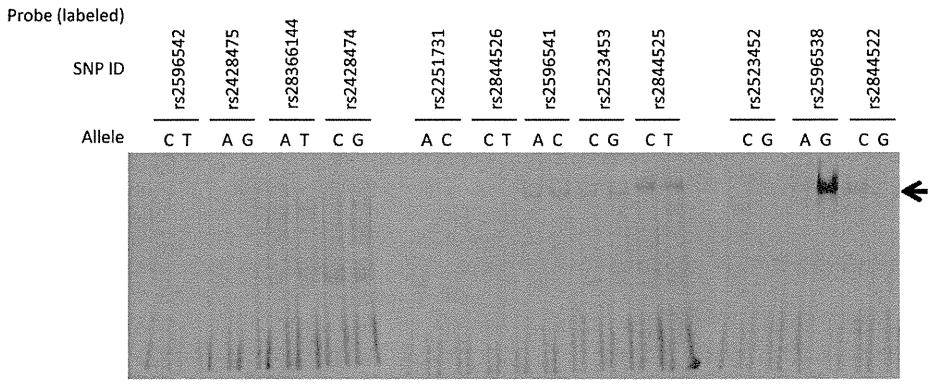

c

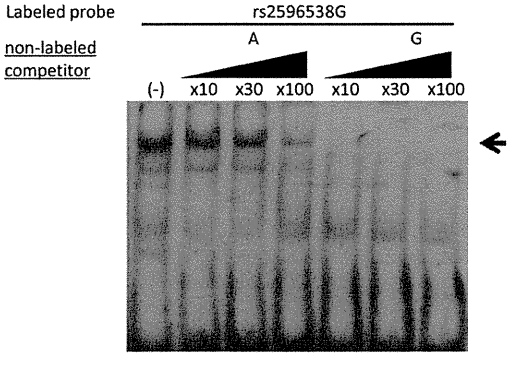

Figure 1. SNP rs2596538 affects the binding affinity of nuclear proteins. (**A**) Real-time quantitative PCR (upper) and Western blotting (lower) of MICA before and after heat shock treatment in HLE cells. *B2M* and β-actin are served as internal and protein loading control. (**B**) EMSA using 31 bp 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.

doi:10.1371/journal.pone.0061279.g001

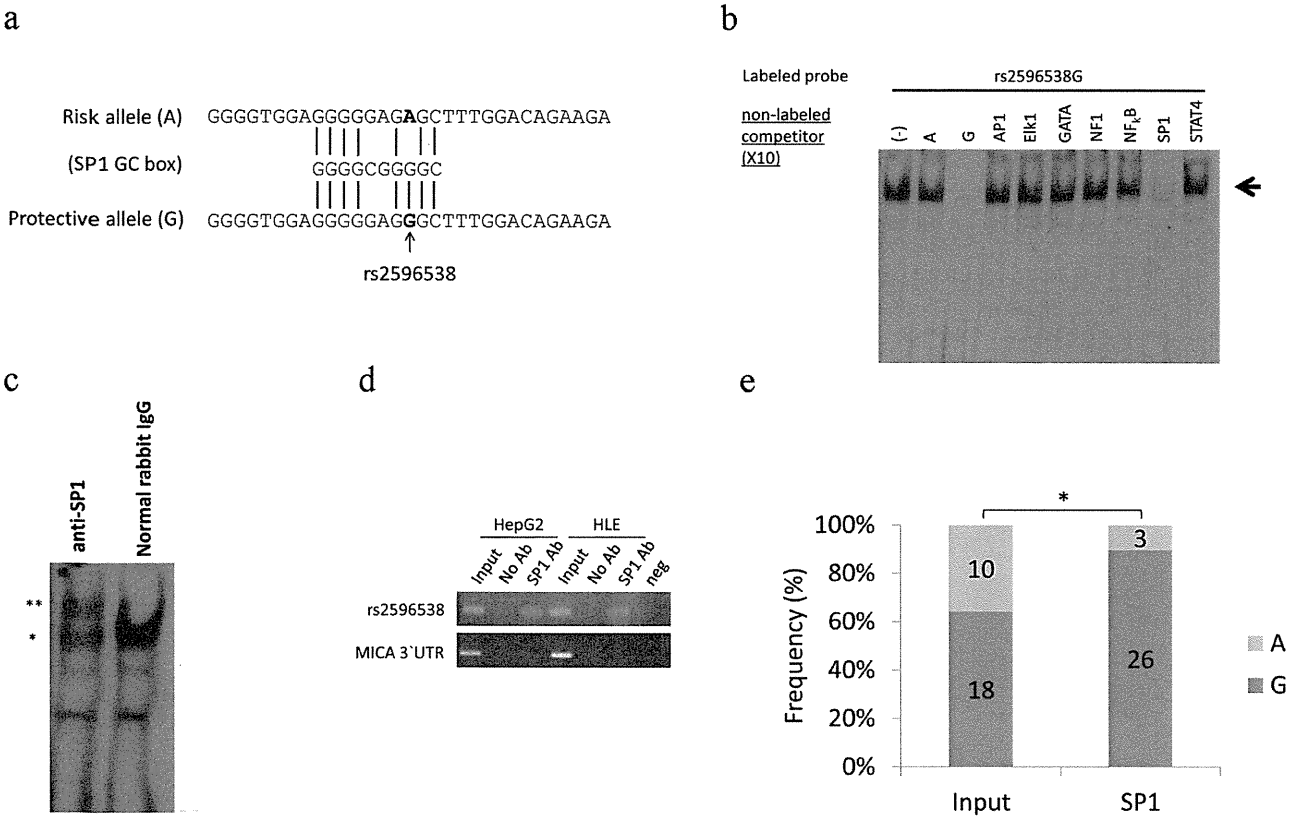

Figure 2. Binding of transcription factor SP1 to G allele of SNP rs2596538. (**A**) Multiple alignment of a GC box and DNA sequence of A or G probe of SNP rs2596538 used in EMSA. (**B**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat treated HLE cells. Non-labeled consensus 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.
doi:10.1371/journal.pone.0061279.g002

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).

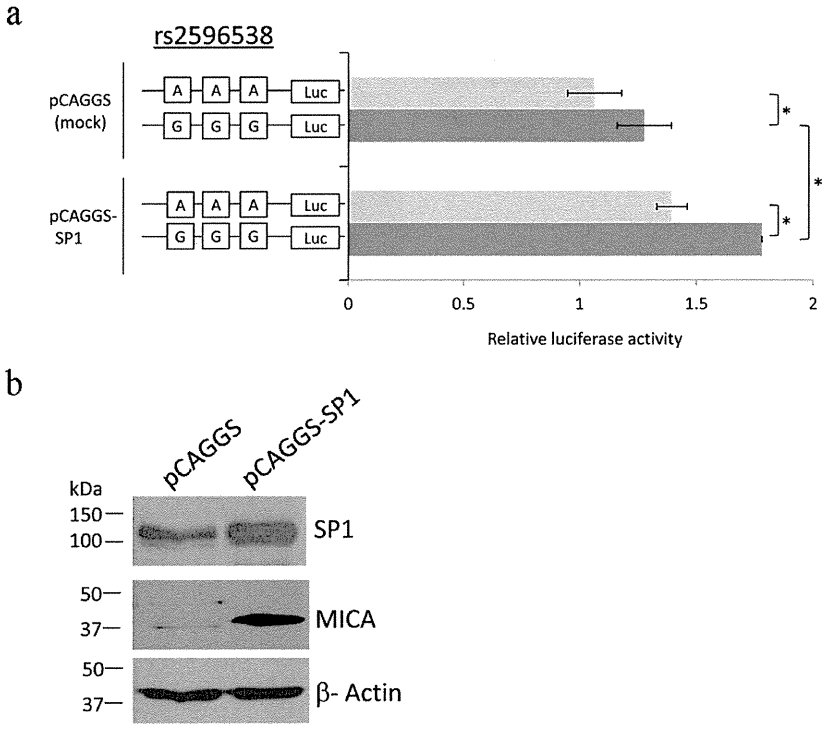

Figure 3. Transcriptional regulation of MICA by SP1 through genomic region including SNP rs2596538. (**A**) Reporter assay using constructs including 3 copies of 31 bp DNA fragment flanking SNP rs2596538. Reporter constructs are transfected into HLE cells with pRL-TK and pCAGGS or pCAGGS-SP1 vector. 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.
doi:10.1371/journal.pone.0061279.t003

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

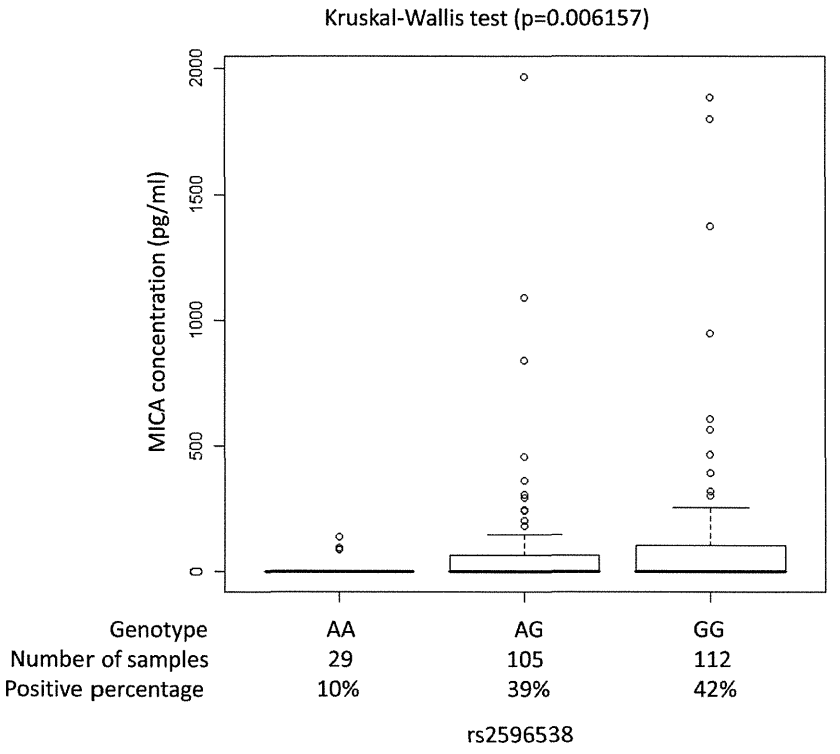

Figure 4. Association between the soluble MICA levels and SNP rs2596538 genotype. The samples were classified into 3 groups according to rs2596538 genotype. The sMICA levels measured by ELISA are indicated in y-axis. The numbers of samples and the proportion of sMICA positive subjects from each group 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

sequencing of *MICA* promoter region from 50 randomly selected HCV-HCC patients.
(TIF)

Table S1 Characteristics of samples and methods used in this study.
(DOCX)

Table S2 The sequences of each oligo used in the EMSA and ChIP assay.
(DOCX)

Table S3 Copy number variation between HCV-HCC and control samples.
(DOCX)

Author Contributions

Conceived and designed the experiments: PHYL YN KM. Performed the experiments: PHYL YU VK. Analyzed the data: PHYL YU CT. Contributed reagents/materials/analysis tools: KK NK DM KC MK. Wrote the paper: PHYL KM.

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PLOS | GENETICS

A Nonsynonymous Polymorphism in *Semaphorin 3A* as a Risk Factor for Human Unexplained Cardiac Arrest with Documented Ventricular Fibrillation

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Abstract

Unexplained cardiac arrest (UCA) with documented ventricular fibrillation (VF) is a major cause of sudden cardiac death. Abnormal sympathetic innervations have been shown to be a trigger of ventricular fibrillation. Further, adequate expression of *SEMA3A* was reported to be critical for normal patterning of cardiac sympathetic innervation. We investigated the relevance of the semaphorin 3A (*SEMA3A*) gene located at chromosome 5 in the etiology of UCA. Eighty-three Japanese patients diagnosed with UCA and 2,958 healthy controls from two different geographic regions in Japan were enrolled. A nonsynonymous polymorphism (I334V, rs138694505A>G) in exon 10 of the *SEMA3A* gene identified through resequencing was significantly associated with UCA (combined P = 0.0004, OR 3.08, 95%CI 1.67–5.7). Overall, 15.7% of UCA patients carried the risk genotype G, whereas only 5.6% did in controls. In patients with *SEMA3A*^{I334V}, VF predominantly occurred at rest during the night. They showed sinus bradycardia, and their RR intervals on the 12-lead electrocardiography tended to be longer than those in patients without *SEMA3A*^{I334V} (1031±111 ms versus 932±182 ms, P = 0.039). Immunofluorescence staining of cardiac biopsy specimens revealed that sympathetic nerves, which are absent in the subendocardial layer in normal hearts, extended to the subendocardial layer only in patients with *SEMA3A*^{I334V}. Functional analyses revealed that the axon-repelling and axon-collapsing activities of mutant *SEMA3A*^{I334V} genes were significantly weaker than those of wild-type *SEMA3A* genes. A high incidence of *SEMA3A*^{I334V} in UCA patients and inappropriate innervation patterning in their hearts implicate involvement of the *SEMA3A* gene in the pathogenesis of UCA.

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Introduction

Unexpected sudden death in healthy individuals remains a daunting problem. Unexplained cardiac arrest with documented ventricular fibrillation (UCA) including idiopathic ventricular fibrillation (IVF) is defined as spontaneous VF that is not associated with a known structural or electrical heart disease. IVF is diagnosed in up to 10% of survivors of out-of-hospital cardiac arrest [1].

Many reports have documented the role of abnormal sympathetic innervations as a trigger of VF [2–6]. Sympathetic innervation of the heart is determined during development by chemoattractive and chemorepulsive factors. Semaphorins, members of a conserved family of both secreted and integral membrane