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Research Paper

Genetics Variants and Serum Levels of MHC Class I Chain-related A in Predicting Hepatocellular Carcinoma Development in Chronic Hepatitis C Patients Post Antiviral Treatment

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ABSTRACT

Background/aims: The genome-wide association study has shown that MHC class I chain-related A (MICA) genetic variants were associated with hepatitis C virus (HCC) related hepatocellular carcinoma. The impact of the genetic variants and its serum levels on post-treatment cohort is elusive.
Methods: MICA rs2596542 genotype and serum MICA (sMICA) levels were evaluated in 705 patients receiving antiviral therapy.
Results: Fifty-eight (8·2%) patients developed HCC, with a median follow-up period of 48·2 months (range: 6–129 months). The MICA A allele was associated with a significantly increased risk of HCC development in cirrhotic non-SVR patients but not in patients of non-cirrhotic and/or with SVR. For cirrhotic non-SVR patients, high sMICA levels (HR/CI: 5·93/1·86–26.38·61, $P = 0·002$) and the MICA rs2596542 A allele (HR/CI: 4·37/1·52–12·07, $P = 0·002$) were independently associated with HCC development. The risk A allele or GG genotype with sMICA > 175 ng/mL provided the best accuracy (79%) and a negative predictive value of 100% in predicting HCC.
Conclusions: Cirrhotic patients who carry MICA risk alleles and those without risk alleles but with high sMICA levels possessed the highest risk of HCC development once they failed antiviral therapy.

1. Introduction

Hepatitis C virus (HCV) infection is one of the leading causes of hepatocellular carcinoma (HCC) worldwide. Successful HCV eradication reduces the risk of HCC in patients with all stages of liver disease (Yu et al., 2006a, 2006b; Huang et al., 2014; Morgan et al., 2013). Preexisting liver cirrhosis before treatment has been recognized the most critical factor for HCC in patients receiving anti-viral therapy (Huang et al., 2014). Recent meta-analysis has demonstrated an incidence of 1·05% per person-year for HCC development in patients with advanced liver disease, even if they achieved a sustained virological response (SVR) (Omata et al., 2010). Beyond the determinants of viral eradication and liver cirrhosis, several simple biochemical markers, such as α-fetoprotein (AFP) (Asahina et al., 2013), alanine transaminase (ALT) (Asahina et al., 2013), *r*-glutamyltransferase (*r*-GT) (Huang et al., 2014), and the aspartate aminotransferase (AST)-to-platelet ratio index (APRI) (Yu et al., 2006b), have been used to predict HCC occurrence in the post-treatment cohort.

Notably, HCC remains in a substantial proportion of non-cirrhotic patients who have successfully eradicated HCV by antiviral therapy (Huang et al., 2014). In addition to the impact of virus and fibrogenesis, host genetics play a role in HCV-related hepatocarcinogenesis. Interleukin 28B (IL-28B) genetic polymorphisms are by far the most important genetic determinant for anti-HCV treatment efficacy (Huang et al., 2012, 2013a, 2013b). These polymorphisms also influence liver-related clinical outcomes, including HCC development (Noureddin et al., 2013). A

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; APRI, the aspartate aminotransferase-to-platelet ratio index; AFP, α-fetoprotein; CHC, chronic hepatitis C; EGF, epidermal growth factor; HCV, hepatitis C virus; IL-28B, interleukin-28B; MICA, MHC class I chain-related A; PNPLA3, patatin-like phospholipase domain-containing 3; SNP, single-nucleotide polymorphism.

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genome-wide association study (GWAS) demonstrated that the single nucleotide polymorphism (SNP) rs2596542 of MHC class I chain-related A (MICA) and its serum level (sMICA) were associated with HCV-related HCC in a cross sectional study (Kumar et al., 2011). Genetic variants of epidermal growth factor (EGF) at rs4444903 are also associated with HCC (Abu Dayyeh et al., 2011; Tanabe et al., 2008). In addition, alcoholic cirrhotic patients who harbor the rs738409 GG genotype of patatin-like phospholipase domain-containing 3 (PNPLA3) are at increased risk of HCC (Guyot et al., 2013). Notably, the role of the host genetics in HCC development in chronic hepatitis C (CHC) patients after antiviral therapy has rarely been explored. Herein, we conducted a longitudinal follow-up study with a well characterized HCV cohort who had received anti-viral therapy and determined the association of the abovementioned candidate SNPs and sMICA with HCC development after weighing potential confounders, including viral eradication and preexisting cirrhosis.

2. Methods

CHC patients receiving anti-viral therapy were consecutively recruited as a prospective follow-up cohort at one tertiary hospital and two core regional hospitals from 2002 to 2012. All the participants received peginterferon alpha-2a or peginterferon alpha-2b plus ribavirin. Patients were excluded if they were co-infected with HIV or hepatitis B virus infection, exhibited alcohol abuse (≥20 g daily) or had evidence of HCC before, during or within 6 months after antiviral therapy. Patients with or without an SVR, defined as seronegativity of HCV RNA throughout a 24-week post-antiviral treatment follow-up period, were further evaluated for the risk of HCC development. Serum HCV RNA was detected using qualitative real-time polymerase chain reaction (PCR) (COBAS AMPLICOR Hepatitis C Virus Test, ver. 2.0; Roche, Branchburg, NJ, USA, detection limit: 50 IU/mL) or quantification branched DNA assay (Versant HCV RNA 3.0, Bayer, Tarrytown, New Jersey, USA; quantification limit: 615 IU/mL) if evaluated before 2011. The HCV genotypes were determined using the Okamoto method before 2011 (Okamoto et al., 1993). After 2011, both the HCV RNA and genotype were detected using real-time PCR assay (Real Time HCV; Abbott Molecular, Des Plaines IL, USA; detection limit: 12 IU/mL) (Vermehren et al., 2011). The definition of cirrhosis was based on liver biopsies, which were performed within 6 months before starting antiviral therapy, and liver histology was graded and staged according to the scoring system described by Knodell and Scheuer (Scheuer, 1991). The post-treatment follow-up strategy was based on cirrhotic status and treatment outcome, as previously described (Huang et al., 2014). Briefly, patients were followed every 3 months if they were cirrhotic or did not have an SVR and every 6 to 12 months if they were non-cirrhotic and had an SVR. The diagnosis of HCC was confirmed by histology or by imaging and laboratory evidence, in accordance with the American Association for the Study of Liver Diseases (Bruix & Sherman, 2011) and Asian Pacific Association for the Study of the Liver (Omata et al., 2010) guidelines. All patients provided written informed consent. The institutional review board at the participating hospital approved the protocols, which conformed to the guidelines of the International Conference on Harmonization for Good Clinical Practice.

2.1. Genetic Testing and sMICA Measurement

Four candidate single nucleotide polymorphisms (SNPs), including MICA rs2596542, IL-28B rs8099917, EGF rs4444903 and PNPLA3 rs738409, were selected in the current study. The IL-28B rs8099917 and PNPLA3 rs738409 genotypes were determined using methods that were previously described (Yu et al., 2011; Lawitz et al., 2014; Huang et al., 2015a). SNP rs2596542 of MICA and SNP rs4444903 of EGF were determined byABI TaqMan® SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) using the following pre-designed commercial genotyping assays (ABI Assay ID: C__27301153_10 and C__27031637_10, respectively). Briefly, PCR primers and two allelic-specific probes were designed to detect specific SNP target. The polymerase chain reaction (PCR) assays were performed in 96-well microplates with an ABI 7500 real-time PCR. Allele discrimination was achieved by detecting fluorescence using System SDS software version 1.2.3. All allele and genotype frequencies were in with Hardy-Weinberg equilibrium. sMICA levels before treatment were measured by sandwich enzyme-linked immunosorbent assay by DuoSet MICA eELISA kits (R & D Systems, Minneapolis, MN, USA).

2.2. Statistical Analyses

Frequency was compared between groups using the χ (Yu et al., 2006b) test, the Yates correction, or Fisher's exact test. Group means (presented as the mean ± standard deviation) were compared using analysis of variance and Student's *t*-test or the nonparametric Mann-Whitney test when appropriate. The aspartate aminotransferase (AST)-to-platelet ratio index (APRI), representing the severity of liver

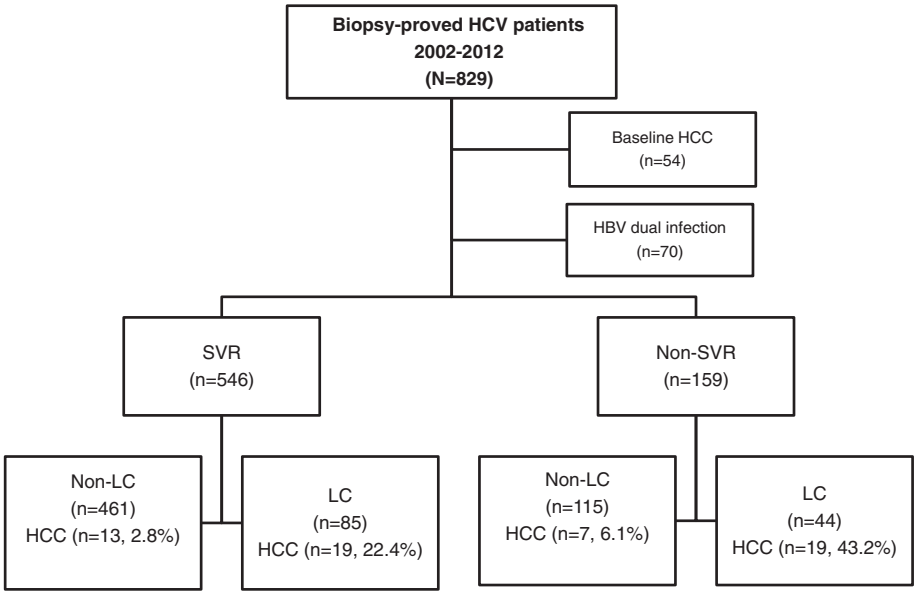

Fig. 1. Flow chart of the patients.

Table 1
Factors associated with the development of HCC of the entire cohort.

	All patients (n = 705)	Non HCC (n = 647)	HCC (n = 58)	P value	HR	C.I.	P value
Age (years, mean ± SD)	52.2 ± 11.4	51.6 ± 11.4	58.5 ± 8.3	<0.001	1.04	1.004–1.067	0.03
Male gender, n (%)	369 (52.3)	337 (52.1)	32 (55.2)	0.65			
Body weight (kg, mean ± SD)	65.8 ± 11.9	65.7 ± 11.9	67.2 ± 12.0	0.36			
Liver cirrhosis, n (%)	129 (18.3)	91 (14.1)	38 (65.5)	<0.001	4.75	2.631–8.569	<0.001
Non-SVR, n (%)	159 (22.6)	133 (20.6)	26 (44.8)	<0.001	1.83	1.061–3.169	0.03
DM, n/N (%)	88/701 (12.6)	78/643 (12.1)	10/58 (17.2)	0.26			
Platelet count (×10 (Huang et al., 2014) u/L, mean ± SD)	170 ± 63	175 ± 62	120 ± 38	<0.001	0.993	0.987–0.998	0.01
Ferritin (ng/mL, mean ± SD)	364 ± 385	358 ± 387	424 ± 350	0.22			
GOT (IU/L,mean ± SD)	98 ± 59	96 ± 58	118 ± 61	0.006			
GPT (IU/L,mean ± SD)	144 ± 94	144 ± 95	146 ± 85	0.86			
α-fetoprotein (ng/mL, mean ± SD)	16.7 ± 48.9	13.5 ± 31.6	51.8 ± 128.9	<0.001			
r-GT (U/L,mean ± SD)	63 ± 55	60 ± 52	95 ± 72	0.001	1.005	1.002–1.008	0.001
APRI (IU/L, mean ± SD)	2.07 ± 1.91	1.69 ± 1.56	2.80 ± 1.75	<0.001			
HCV genotype 1, n/N (%)	403/700 (57.6)	369/642 (57.5)	34/58 (58.6)	0.87			
HCV RNA (log IU/mL, mean ± SD)	5.42 ± 0.98	5.43 ± 0.98	5.32 ± 0.90	0.44			
MICA rs2596542 A allele, n (%)	360 (51.1)	329 (50.9)	31 (53.4)	0.71			
IL-28B rs8099917 TT genotype, n/N (%)	582/668 (87.1)	538/611(88.1)	44/57 (77.2)	0.02			
EGF rs4444903 GG genotype, n/N (%)	345/697 (49.5)	312/639 (48.8)	33/58(56.9)	0.24			
PNPLA3 rs738409 GG genotype, n/N (%)	67/660 (10.2)	62/603 (10.3)	5/5 (8.7)	0.72			

Note: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; MICA: MHC class I polypeptide-related chain A; sMICA: serum MICA level; EGF: epidermal growth factor; IL-28B: interleukin 28B; PNPLA3: patatin-like phospholipase domain-containing 3; r-GT: r-glutamyl transferase; SD: standard deviation; DM: diabetes mellitus; AST: aspartate aminotransferase; ALT: alanine aminotransferase; APRI: aspartate aminotransferase-to-platelet ratio index.HR: hazard ratio; CI: confidence intervals. Hazard ratio of HCC is for age (per year increase), liver cirrhosis(yes vs. no), SVR (no vs. yes), platelet (per ×10 (Huang et al., 2014) u/L, increase) and r-GT (per 1 U/L increase).

fibrosis, was determined by the following equation: (AST level/upper limit of normal range)/platelet counts (10^9/L) × 100 (Yu et al., 2006b). Kaplan–Meier analysis and the Log-rank test were performed by comparing the differences of the cumulative incidence of HCC between determinants. The risk factors independently associated with HCC development were evaluated using Cox regression analysis. Bonferroni multiple test correction was used to adjust the P value for the reduction the chances of obtaining false-positive results (type I errors) when multiple pairwise tests are performed on a single set of data. The area under the curve (AUC) was compared using receiver operating characteristic (ROC) analysis to determine the cut-off value for sMICA to be used for predicting HCC. Statistical analyses were performed using the SPSS 12.0 statistical package (SPSS, Chicago, IL, USA). All statistical analyses were based on two-sided hypothesis tests with a significance level of $p < 0{\cdot}05$.

3. Results

3.1. Patients

In total, 829 patients were recruited consecutively between 2002 and 2012. After excluding patients with underlying HCC (n = 54) and HBV dual infection (n = 70), 705 patients were enrolled for analysis. SVR was achieved in 546 patients (77·4%), whereas the remaining 159 (22·6%) patients failed anti-viral therapy. Fifty-eight (8·2%) patients developed HCC after antiviral therapy with a median follow-up period of 48.2 months (range: 6–129 months). The proportion of HCC was 2·8% (n = 13) and 22·4% (n = 19) in non-cirrhotic and cirrhotic patients with an SVR, respectively, and 6·1% (n = 7) and 43·2% (n = 19) in non-cirrhotic and cirrhotic patients without an SVR, (0·71%, 5·48%, 1·39%, and 12·2% per person-year, respectively) (Fig. 1).

3.2. Factors Associated With HCC Development in the Treatment Cohort

We first assessed the role of MICA rs2596542, IL-28B rs8099917, EGF rs4444903 and PNPLA3 rs738409 SNPs in HCC development in the entire cohort. The distribution of the SNPs of MICA rs2596542, EGF rs4444903 and PNPLA3 rs738409 did not differ between patients with or without HCC. However, patients without HCC had a significantly higher proportion of IL-28B rs809991 TT genotype compared with those with HCC (88·1% vs. 77·2%, P = 0·02). In addition, patient with HCC development were older and had a higher proportion of liver cirrhosis, a lower SVR rate, lower platelet counts, and higher levels of

Table 2
Basic characteristics, follow-up period and incidence of HCC development in cirrhotic and non-cirrhotic HCV patients who failed anti-viral therapy.

	Non-cirrhotic patients (n = 115)	Cirrhotic patients (n = 44)	P value
Age (years, mean ± SD)	52.0 ± 11.9	57.6 ± 7.7	0.001
Male gender, n (%)	57 (49.6)	18 (40.9)	0.33
Body weight (kg, mean ± SD)	66.4 ± 13.0	67.6 ± 10.0	0.57
DM, n/N (%)	18/114 (15.8)	10/44 (22.7)	0.31
Platelet count (×10 (Huang et al., 2014) u/L, mean ± SD)	172 ± 64	131 ± 71	0.001
Ferritin (ng/mL, mean ± SD)	407 ± 565	424 ± 292	0.86
GOT (IU/L, mean ± SD)	93 ± 11	119 ± 54	0.006
GPT (IU/L, mean ± SD)	137 ± 82	141 ± 69	0.77
r-GT (U/L, mean ± SD)	63 ± 49	90 ± 73	0.03
α-Fetoprotein (ng/mL, mean ± SD)	15.6 ± 21.0	59.8 ± 147.3	0.05
APRI (IU/L, mean ± SD)	1.63 ± 1.16	3.22 ± 2.83	<0.001
HCV genotype 1, n (%)	84 (73.0)	31 (70.5)	0.74
HCV viral loads (log IU/mL, mean ± SD)	5.88 ± 0.66	5.76 ± 0.48	0.27
MICA rs2596542 A allele, n (%)	53 (46.1)	21 (47.7)	0.85
IL-28B rs8099917 TT genotype, n/N (%)	85/111 (76.6)	30/42 (71.4)	0.51
EGF rs4444903 GG genotype, n/N (%)	61/114 (53.5)	18/43 (41.9)	0.19
PNPLA3 rs738409GG genotype, n/N (%)	17/107 (15.9)	8/42 (19.0)	0.64
sMICA (pg/mL)[a]	119.5 ± 389.4	136.3 + 220.6	<0.001
Follow-up period (months, mean ± SD)	52.5 ± 32.7	42.5 ± 29.2	0.08
Follow-up period (person-years)	503.5	155.7	—
HCC, n (%)	7 (6.1)	19 (43.2)	<0.001
HCC incidence (per person-year, %)	1.4	12.3	<0.001

Note: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; MICA: MHC class I polypeptide-related chain A; sMICA: serum MICA level; EGF: epidermal growth factor; IL-28B: interleukin 28B; PNPLA3: patatin-like phospholipase domain-containing 3; r-GT: r-glutamyl transferase; SD: standard deviation; DM: diabetes mellitus; AST: aspartate aminotransferase; ALT: alanine aminotransferase; APRI: aspartate aminotransferase-to-platelet ratio index.HR: hazard ratio; CI: confidence intervals.
[a] Available in 140 patients.

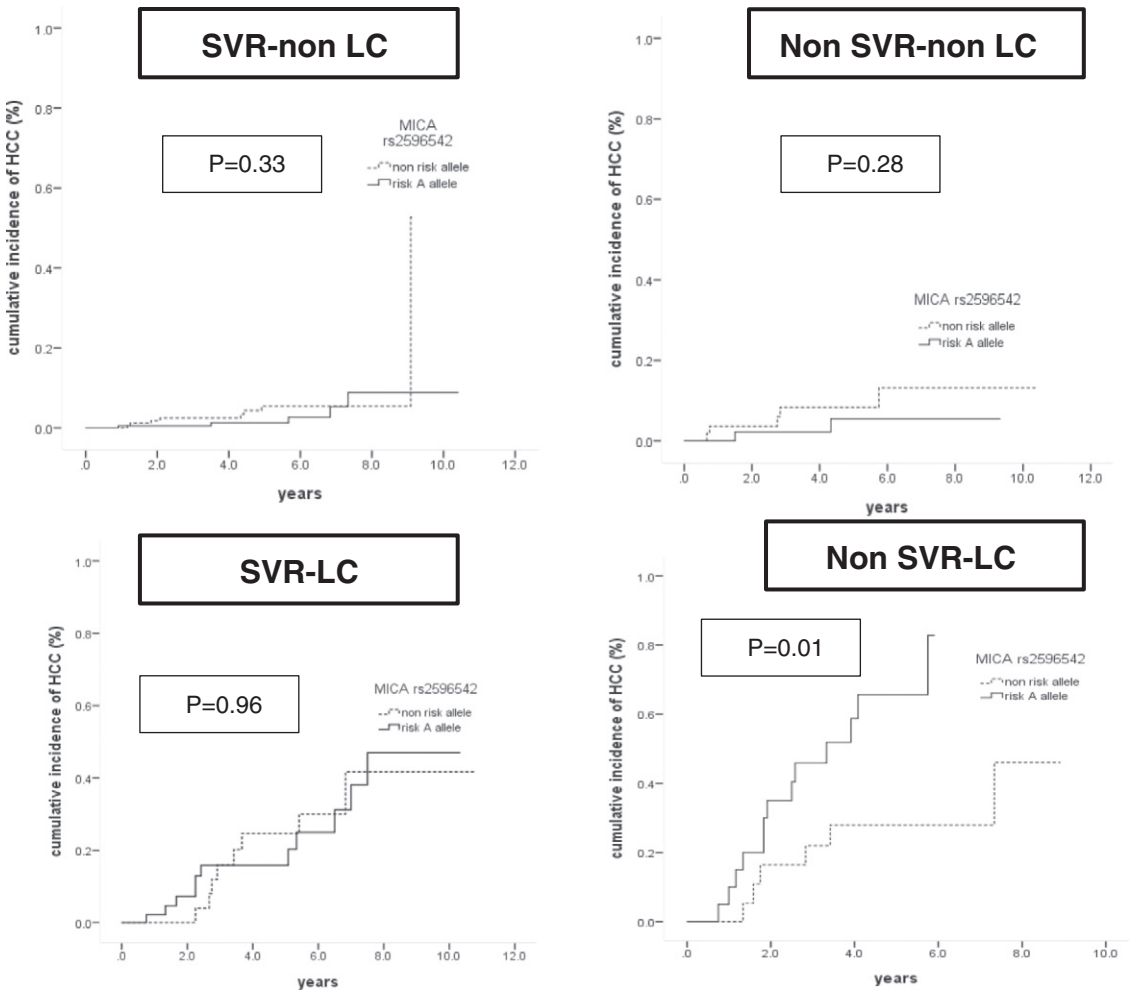

Fig. 2. Association of MICA rs2596542 genotype with HCC development stratified by the SVR and cirrhotic status. Risk allele: AA + AG genotype. Non-A allele: GG genotype.

AST, AFP and *r*-GT (Table 1). Cox-regression analysis revealed that the strongest factor independently associated with HCC in the treatment cohort was liver cirrhosis (hazard ratio [HR]/95% confidence intervals [CI]:4·75/2·631–8·569, $P < 0·001$) followed by non-SVR (HR/CI: 1·83/1·061–3·169, $P = 0·03$), old age (HR/CI: 1·04/1·004–1·067, $P = 0·03$), low platelet counts (HR/CI: 0·993/0·987–0·998, $P = 0·01$) and high *r*-GT levels (HR/CI: 1·005/1·002–1·008, $P = 0·001$). Although Kaplan–Meier analysis revealed that patients with IL-28B rs8099917 non-TT genotype had higher incidence of HCC compared with those with the TT genotype ($P = 0·015$) (Supplementary Fig. 1), the association of IL-28B genotype with HCC was not more significant in Cox-regression analysis.

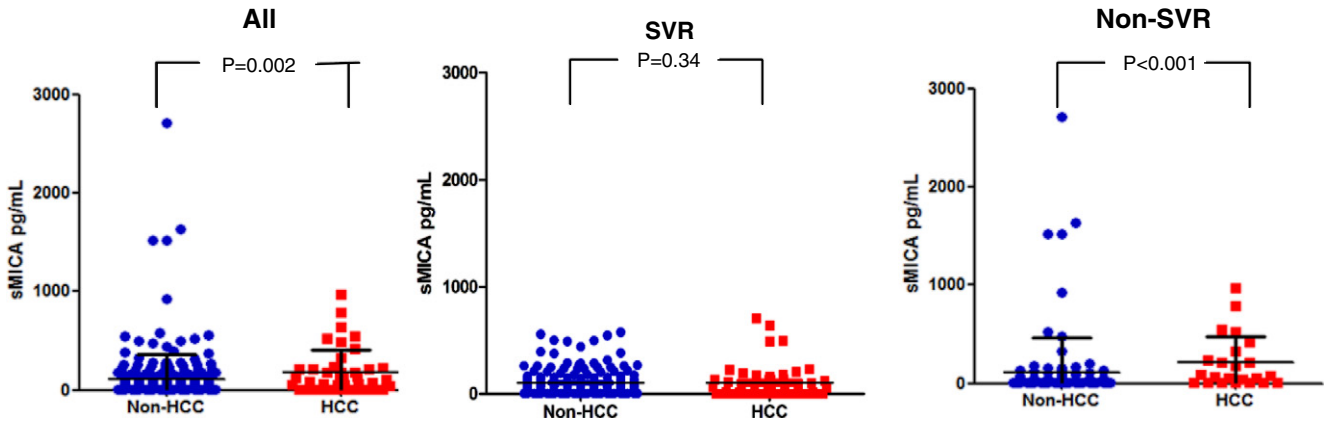

Fig. 3. sMICA levels in patients with or without HCC development stratified by SVR status.

Table 3
Basic factors associated with the development HCC in cirrhotic and non-cirrhotic HCV patients who failed antiviral therapy.

	Cirrhotic patients (n = 44)			Non-cirrhotic patients (n = 115)		
	Non-HCC (n = 25)	HCC (n = 19)	P value	Non-HCC (n = 108)	HCC (n = 7)	P value
Age (years, mean ± SD)	58.9 ± 8.3	55.8 ± 6.7	0.19	51.3 ± 11.7	61.1 ± 11.7	0.03
Male gender, n (%)	11 (44.0)	7 (36.8)	0.63	53 (49.1)	24(57.1)	0.72
Body weight (kg, mean ± SD)	68.0 ± 10.9	67.1 ± 9.1	0.75	66.5 ± 13.4	64.9 ± 6.3	0.76
DM, n/N (%)	7/25 (28.0)	3/19 (15.8)	0.47	18/107 (16.8)	0 (0)	0.59
Platelet count (×10 (Huang et al., 2014) u/L, mean ± SD)	147 ± 85	109 ± 40	0.06	176 ± 64	121 ± 50	0.03
Ferritin (ng/mL, mean ± SD)	339 ± 196	523 ± 356	0.07	410 ± 577	361 ± 367	0.83
GOT (IU/L, mean ± SD)	109 ± 43	131 ± 64	0.18	90 ± 50	137 ± 48	0.02
GPT (IU/L, mean ± SD)	128 ± 60	160 ± 77	0.13	137 ± 84	150 ± 59	0.67
r-GT (U/L, mean ± SD)	71 ± 50	115 ± 90	0.06	61 ± 49	88 ± 38	0.17
α-fetoprotein (ng/mL, mean ± SD)	19.7 ± 28.7	112.4 ± 213.4	0.02	13.8 ± 19.3	41.8 ± 29.4	0.046
APRI (IU/L, mean ± SD)	3.08 ± 3.39	3.40 ± 1.92	0.71	1.53 ± 1.07	3.22 ± 1.42	<0.001
HCV genotype 1, n (%)	16 (64.0)	15 (78.9)	0.28	80 (74.1)	4 (57.1)	0.39
HCV viral loads (log IU/mL, mean ± SD)	5.78 ± 0.46	5.73 ± 0.51	0.71	5.89 ± 0.66	5.81 ± 0.50	0.79
MICA rs2596542 A allele, n (%)	8 (32.0)	13 (68.4)	0.017	51 (47.2)	2 (28.6)	0.45
IL-28B rs8099917 TT genotype, n/N (%)	18/24 (75.0)	12/18 (66.7)	0.55	81/104 (77.9)	4/7 (57.1)	0.35
EGF rs4444903 GG genotype, n/N (%)	9/24 (37.5)	9/19 (47.4)	0.52	56/107 (52.3)	5/7 (71.4)	0.45
PNPLA3 rs738409GG genotype, n/N (%)	5/23 (21.7)	3/19 (15.8)	0.71	17/101 (16.8)	0/6 (0)	0.59
sMICA (pg/mL)[a]	59 ± 105	230 ± 285	0.03	119 ± 397	137 ± 229	0.14

Note: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; MICA: MHC class I polypeptide-related chain A; sMICA: serum MICA level; EGF: epidermal growth factor; IL-28B: interleukin 28B; PNPLA3: patatin-like phospholipase domain-containing 3; r-GT: r-glutamyl transferase; SD: standard deviation; DM: diabetes mellitus; AST: aspartate aminotransferase; ALT: alanine aminotransferase; APRI: aspartate aminotransferase-to-platelet ratio index.HR: hazard ratio; CI: confidence intervals.
[a] available in 140 patients.

3.3. Influence of Host Genetics in HCC Development in Patients Stratified by Liver Cirrhosis and Treatment Response

Given that preexisting liver cirrhosis and failing to achieve an SVR were the major determinants for HCC, we further analyzed the association of host genetics with HCC by stratifying patients based on the two factors. We observed that IL-28B rs8099917, EGF rs4444903 and PNPLA3 rs738409 genetic variants did not correlate with HCC development in each subgroup of patients stratified by cirrhotic and SVR status (Supplementary Figs. 2–4). However, the MICA rs2596542 genotype was associated with HCC development in cirrhotic non-SVR patients but not in the other 3 subgroups. Among the cirrhotic non-SVR patients, those with HCC development had a significantly increased proportion of the MICA rs2596542 A allele (68·4% vs. 32·0%, $P = 0·017$), and patients carrying the risk A allele had a significantly increased incidence of HCC development compared with those without (HR: 3·4, $P = 0·01$) (Fig. 2).

3.4. Association of Pretreatment sMICA Levels and Post-treatment HCC Development

Of the 302 patients with pretreatment sMICA available, those with HCC development had significantly increased sMICA levels compared with those without HCC development (180 ± 230 pg/mL vs. 107 ± 259 pg/mL, $P = 0·002$). Cox-regression analysis with sMICA as a co-variant revealed that the strongest factor independently associated with HCC was liver cirrhosis (HR/CI:8·02/3·474–18·528, $P < 0·001$) followed by non-SVR (HR/CI:2·11/1·112–3·994, $P = 0·02$), low platelet counts (HR/CI: 0·992/0·986–0·998, $P = 0·01$) and high sMICA levels (HR/CI: 1·001/1·000–1·002, $P = 0·008$). Although patients were stratified by the treatment outcome, patients who developed HCC had higher pretreatment sMICA compared with non-SVR patients without HCC development (210 + 271 pg/mL vs. 107 + 361 pg/mL, $P < 0.001$) but not in SVR patients (43 ± 168 vs. 107 ± 128, $P = 0·34$) (Fig. 3).

3.5. Impact of MICA SNP and sMICA on HCC Development in Non-SVR Patients

The basic characteristics, follow-up period and incidence of HCC development in cirrhotic and non-cirrhotic HCV patients who failed antiviral therapy were shown in Table 2. We further analyzed the effect of MICA SNP and sMICA on HCC development among non-SVR patients stratified by cirrhotic status. Among the non-cirrhotic, non-SVR patients, those who developed HCC were older and had lower platelet counts and higher levels of AST, AFP and APRI (Table 3). Cox regression analysis revealed that APRI was the single factor associated with HCC development (HR/CI:2·67/1·42–5.01, $P = 0·002$) in non-cirrhotic patients without an SVR (Table 4). Among cirrhotic patients, those with HCC had lower platelet counts, higher ferritin, significantly higher AFP levels and sMICA levels and a higher proportion of the MICA rs2596542 A allele (Table 3). Forty of 44 cirrhotic patients without an SVR had sMICA available. The best cut-off value of sMICA level in predicting HCC was 175·4 pg/mL (AUROC 0·70, $P = 0·002$). Compared with patients with low sMICA, patients with high sMICA levels (>175 pg/mL) were more likely to develop HCC in cirrhotic patients without an SVR (HR 4·3, $P = 0·001$) but not in non-cirrhotic or SVR patients (Supplementary Fig. 5). Cox regression analysis revealed that the factors independently associated with HCC development among cirrhotic patients without an SVR were high sMICA levels (HR/CI: 5·93 / 1·86–26.38·61, $P = 0·002$) and the MICA rs2596542 A allele (HR/CI: 4·37/1·52–12·07, $P = 0·002$).

Table 4
Cox regression analysis of risk factors associated with HCC development in HCV patients who failed antiviral therapy.

Variables	HR	95% CI	P value
Non-LC patients			
APRI			
Per 1 unit increased	2.67	1.42–5.01	0.002
LC patients			
MICA rs2596542 genotype			
Non-A allele	1		
A allele	4.37	1.52–12.07	0.008
sMICA			
< 175 ng/mL	1		
> 175 ng/mL	5.93	1.86–26.38	0.002

Note: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; APRI: aspartate aminotransferase-to-platelet ratio index; LC, liver cirrhosis; MICA, MHC class I polypeptide-related chain A; sMICA, serum MICA level; HR: hazard ratio.CI: confidence intervals. Variables with $P < 0.05$ in univariate analysis were put into analysis.

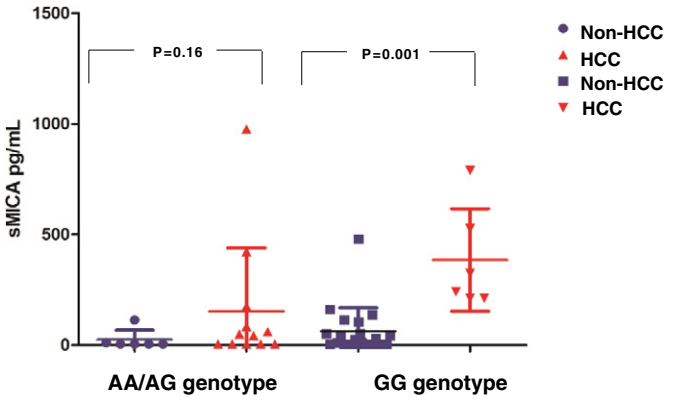

Fig. 4. sMICA levels of different MICA SNPs among LC non-SVR patients with or without HCC.

3.6. The Role of sMICA Levels in Specific MICA Genotypes on HCC Development

Compared with patients harboring the MICA rs2596542 GG genotype, sMICA levels were significantly reduced in those with the risk A allele (105 ± 234 pg/mL vs. 165 ± 210 pg/mL, $P = 0{\cdot}03$). Among patients with the risk A allele, sMICA levels did not differ between patients with or without HCC (153 + 285 pg/mL vs. 21 ± 41 pg/mL, respectively, $P = 0{\cdot}16$). Nevertheless, sMICA levels were significantly increased in HCC patients compared with non-HCC patients (385 ± 231 pg/mL vs. 77 ± 122 pg/mL, $P = 0{\cdot}001$) among those who carried the MICA rs2596542 GG genotype (Fig. 4).

Among the non-SVR cirrhotic patients with the GG genotype, sMICA levels were significantly increased in HCC subjects compared with non-HCC patients (100% vs. 6·7%, $P < 0{\cdot}001$). In contrast, there was no difference in the proportion of high sMICA levels between patients with and without HCC development if the patients were non-cirrhotic, exhibited an SVR, or harbored the risk A allele (Table 5).

3.7. Combined Effect of sMICA Levels and MICA rs2596542 Genetic Variants in Predicting HCC

sMICA levels and MICA rs2596542 SNP were the two independent factors associated with HCC development in cirrhotic non-SVR patients. We evaluated the combined effect of the two factors in predicting HCC in the subpopulation. As shown in Table 6, the risk A allele or GG genotype with sMICA > 175 ng/mL provided the best accuracy, at 79%, and a negative predictive value of 100% for predicting HCC. Nineteen of the 28 patients (67·9%) who carried the two risk factors developed HCC, with an annual incidence of 23·5%. In contrast, none of the 14 GG genotype carriers with sMICA < 175 ng/mL developed HCC after a median follow-up period of 58·9 months (range: 6–107 months). The incidence of HCC did not differ between patients with or without the risk factors, in terms of MICA SNP and sMICA, among the other three subpopulations (Fig. 5).

4. Discussion

Host genetic predispositions are associated with anti-HCV treatment efficacy, (Huang et al., 2012, 2013a, 2013b) HCV-related liver fibrosis, (Huang et al., 2015a; Urabe et al., 2013) clinical outcome (Noureddin et al., 2013) and HCC (Kumar et al., 2011; Abu Dayyeh et al., 2011; Tanabe et al., 2008; Guyot et al., 2013). However, whether host genetic variants play important roles in HCC development after anti-viral therapy is unclear. By testing the candidate SNPs in a large treatment cohort, we demonstrated that MICA rs2596542 genetic variants predicted HCC occurrence, and the influence was restricted to cirrhotic patients who failed antiviral therapy. Interestingly, we demonstrated that high sMICA was also predictive of HCC occurrence in the population. Most importantly, cirrhotic non-responders were at the highest risk for HCC development, with an annual incidence of 23·5%, if they carried the MICA A allele risk and had high pretreatment sMICA levels.

Preexisting liver cirrhosis is the most critical factor associated with HCC in CHC patients (Lee et al., 2014; Goto & Kato, 2015). Once cirrhosis has evolved, 1 to 4% of patients develop HCC per year (Goto & Kato, 2015). Although successful HCV eradication could reduce the risk of HCC occurrence by 75%, SVR patients remain at risk of HCC development with an average risk of 1·05% per person-year if they have advanced liver disease (Yu et al., 2006a; Morgan et al., 2013). As shown in the current study and other studies, cirrhosis carries a higher hazard risk ratio than failing viral eradication for HCC development in the treatment cohort (Lee et al., 2014; Goto & Kato, 2015). It is therefore imperative to identity the risk of HCC in patients with cirrhotic background with and without SVR.

MICA, a ligand for NKG2D, exerts its anti-tumor effect by activating natural killer cells and CD8 + T cells. A GWAS demonstrated that patients with HCV-related HCC had a higher rate of the MICA rs2596542 A allele (Kumar et al., 2011). The evidence was based on cross-sectional observations. However, whether genetic predisposition increased the long-term risk of HCC development is unclear. In the current study, we noticed that MICA SNP does not increase the risk of HCC development in patients who had successful viral eradication or mild liver disease. In contrast, among the cirrhotic non-responders who were on the extreme end of liver disease, patients who developed HCC had a significantly higher proportion of the MICA rs2596542 A allele. Carriers of the risk allele had a four-fold risk of HCC development after anti-HCV therapy.

Similar to previous study, we concordantly observed significantly reduced sMICA production in patients with the risk A allele compared with those with GG genotype (Kumar et al., 2011). The pathophysiological mechanism may be due to the potentially low production of membrane-bound MICA with the risk A allele in patients who respond to HCV infection, leading to poor or no activation of immune cells, including NK cells (Kumar et al., 2011; Goto & Kato, 2015). On the other hand, high expression of MICA is associated with a variety of malignancies, including melanoma, breast, colon and hepatocellular cancers (Goto & Kato, 2015; Kumar et al., 2012; Groh et al., 1999, 2002). This result is likely attributed to the fact that highly soluble MICA in the circulation down-regulated NKG2D expression in immune cells and disrupted NKG2D-mediated antitumor immunity. Recently, we also demonstrated

Table 5
Proportion of high sMICA levels[a] in patients with or without HCC stratified by MICA SNP.

MICA rs2596542% (n/N)	Risk A allele HCC	non-HCC	*P* value	GG genotype HCC	non-HCC	*P* value
All patients	24.0% (6/25)	7.5% (9/120)	0.02	58.8% (10/17)	22.9% (32/140)	0.003
SVR	27.3% (3/11)	9.7% (7/72)	0.12	37.5% (3/8)	33.8% (24/71)	1
Non LC	50.0% (1/2)	10.5% (4/38)	0.24	0% (0/1)	31.1% (14/45)	1
LC	22.2% (2/9)	8.8% (3/34)	0.28	42.9% (3/7)	38.5% (10/26)	1
Non SVR	21.4% (3/14)	4.2% (2/48)	0.07	77.8% (7/9)	11.6% (8/69)	<0.001
Non LC	0% (0/2)	4.9% (2/41)	1	33.3% (1/3)	13.0% (7/54)	0.37
LC	25.0% (3/12)	0% (0/7)	0.26	100% (6/6)	6.7% (1/15)	<0.001

[a] high sMICA levels: >175 pg/mL; SVR, sustained virological response; LC, liver cirrhosis; MICA,MHC class I polypeptide-related chain A.

Table 6
Accuracy of pretreatment sMICA and MICA SNP in predicting HCC in LC patients who failed anti-viral therapy.

MICA rs2596542 SNP & sMICA (ng/mL)	HCC n (%)	Non-HCC n (%)	*P* value	SEN %	SPE %	PPV %	NPV %	ACC %
A allele	13(68.4)	8 (32.0)	0.02	68	68	62	74	68
sMICA > 175	9 (50.0)	1 (4.5)	0.002	50	96	90	70	75
GG genotype AND sMICA > 175	6 (31.6)	1 (4.3)	0.03	32	96	86	63	67
A allele AND sMICA > 175	3 (16.7)	0 (0)	0.07	17	100	100	62	64
A allele OR sMICA > 175	19 (100)	9 (39.1)	<0.001	100	61	68	100	79

Note: MICA, MHC class I polypeptide-related chain A; sMICA, serum MICA level; SEN, sensitivity; SPE, specificity; PPV, positive predictive value; NPV, negative predictive value; ACC, accuracy.

that high sMICA levels were also associated with HCV-related HCC recurrence after curative treatment for HCC and antiviral therapy for HCV with and without SVR (Huang et al., 2015b). We observed that cirrhotic non-responders with high sMICA levels (>175 ng/mL) had a five-fold risk of HCC development compared with those with low sMICA levels.

Notably in the current study, among cirrhotic non-responders who harbored the low-risk MICA GG genotype, six of seven (85·7%) patients with the high sMICA levels developed HCC during a mean follow-up period of approximately 5 years. In contrast, none of the 14 patients with low sMICA levels developed HCC. Subsequently, we identified two risk factors associated with HCC development in cirrhotic non-responders, the carriage of A allele or GG genotype with high sMICA levels. Two-thirds of the cirrhotic non-responders who carried either factor developed HCC with an annual incidence of 23·5%. In contrast, the NPV of HCC development was up to 100% in the clinical setting after approximately 5 years of follow-up.

Patients with unfavorable EGF genetic polymorphisms have an increased risk of HCC in a Western cohort with advanced liver fibrosis (Abu Dayyeh et al., 2011). PNPLA3 genetic variants are associated with HCV-related liver fibrosis (Guyot et al., 2013; Huang et al., 2015a). However, there was no association between the SNP and HCV-related HCC (Singal et al., 2014). In the current study, we demonstrated that both EGF rs4444903 and PNPLA3rs738409 genotypes did not determine HCC development in the post-treatment Asian population, regardless of treatment response or liver fibrosis.

IL-28B genetic variants by far are the most powerful host genetic factors in predicting the HCV genotype 1 treatment efficacy of IFN-based therapy (Huang et al., 2012, 2013a, 2013b) and spontaneous clearance (Yu et al., 2013). However, its influence in liver fibrosis and hepatocarcinogenesis remains unclear (Noureddin et al., 2013; Kumar et al., 2012; Fabris et al., 2011). Patients with unfavorable IL-28B genotype had a higher likelihood of HCC development in univariate analysis,

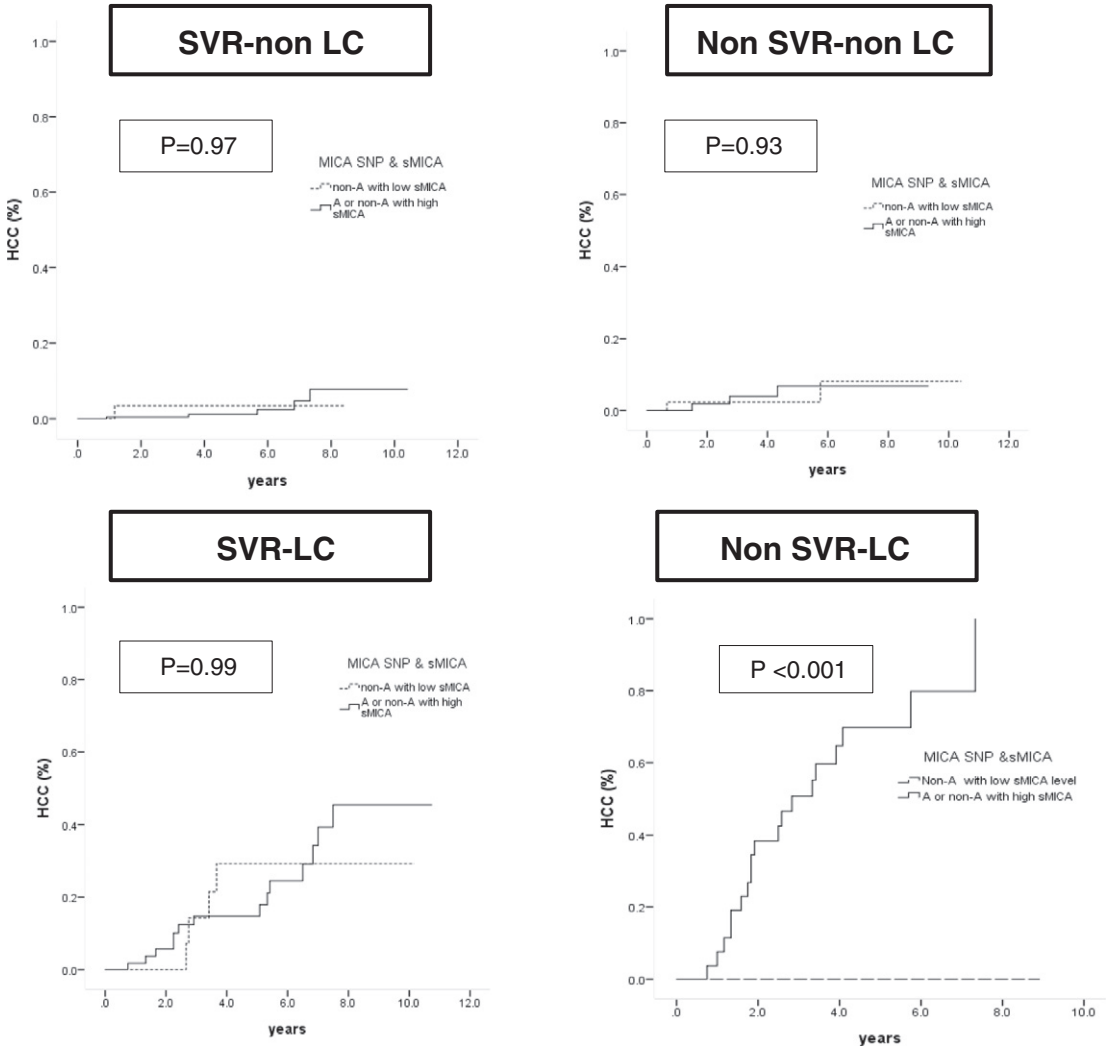

Fig. 5. HCC development in non-SVR LC patients with different MICA SNP and sMICA levels.

but the association became insignificant after weighing treatment responses. This finding may be attributed to the confounder of viral clearance. Patients with favorable IL-28B genotype were prone to have an SVR, which subsequently reduced the risk of HCC. Although patients were stratified by the SVR status, IL-28B genotype no longer influenced the HCC development in the cohort (data not shown).

Several potential confounders that may influence HCC occurrence were taken into account in the study. However, the limitation was that we failed to provide new masked associations between HCC development and host genetics during the follow-up period. In the era of direct antiviral agents (DAAs), the SVR rate could be achieved up to >95%. However, there are still some concerns. The DAA lacks anti-neoplasm and immune modulation effect as interferon does. Its impact on HCC occurrence or recurrence remains conflicting until now (Nault & Colombo, 2016). Secondly, a critical issue is that only a small proportion of patients could have access to DAA due to the unaffordability. There remains a huge gap between clinical efficacy and community effectiveness in the management of HCV infection. The current study demonstrated the important role of MICA SNP and sMICA in non-SVR patients, who represent the majority of untreated and persistent viremic population the real world. We believed that the current study would consistently provide important information regarding risk prediction and surveillance of HCC. In conclusion, non-responders who carried the MICA risk A allele or had high pretreatment sMICA levels have a high risk for HCC development after antiviral therapy. Combining the two surrogate markers greatly enhanced the predictive power in the high-risk population, which provides insight for closer follow-up strategies and re-treatment priority in the era of direct antiviral agents.

Author Contributions

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Manuscript drafting and critical revising: Chung-Feng Huang, Cing-Yi Huang, Ming-Lun Yeh and Ming-Lung Yu.

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Conflict of Interest

none.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.11.031.

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