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研究課題名（和文）HCV排除後肝発癌におけるmiRNA 発現と遺伝子多型に関する研究

研究課題名（英文）Study on miRNA expression and single nucleotide polymorphisms in hepatocellular carcinoma after HCV eradication

研究代表者

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研究成果の概要（和文）：肝細胞癌との関係が報告されている４つの遺伝子多型（MICA, DEPDC5, HCP5, PNPLA3)を717例のC型慢性肝疾患例について解析した．唯一MICAminor型を非発癌C型肝疾患例に比較してC型肝癌症例に多数検出した（p = 0.0185)。またMICA遺伝子多型は血液中のsoluble MICAタンパク量と相関していた.さらにサブグループについてMICA遺伝子多型の頻度を調べた結果,70歳以上の高齢者や血小板数低値のグループにおいてより顕著に肝癌発症例でのminor多型を多く認めた.以上よりC型慢性肝疾患例おける肝発癌にMICA遺伝子多型が関与していることを明らかにした.

研究成果の概要（英文）：Of the 4 SNPs analysed, only the MICA genotypes were significantly associated with development of HCC (p = 0.0185). The major (MA), hetero (HE), and minor (MI) genotypes occurred in 40%, 41%, and 19% of HCC patients and in 43%, 47%, and 10% of non-HCC patients, respectively. Interestingly, the MICA genotype was significantly correlated with soluble MICA protein levels. In patients older than 70 years, the MI genotype was significantly associated with HCC development. In addition, the MI genotype was related to HCC development when the platelet count range was 10-15 × 104/μL but not when the range was less than 10 or greater than 15 × 104/μL. Thus, polymorphisms in MICA, but not in DEPDC5, HCP5 or PNPLA3, are associated with HCC development in Japanese patients with chronic HCV infection, particularly older patients or patients with fibrosis.

研究分野：　医歯薬学

キーワード：　HCC SNP

１．研究開始当初の背景

Approximately 60-70 % of hepatocellular carcinoma (HCC) individuals are caused by hepatitis C virus (HCV) infection. Several reports showed that genetic backgrounds were closely related to HCC in patients with HCV. Interferon-based therapy could prevent or delay the development of HCC. In particular, HCC occurrence rate was significantly reduced in patients with sustained virological response (SVR). However, some of patients completed interferon/ribavirin (IFN/RBV) developed HCC after 5 years (Kobayashi S et al. 2007 Liver int 27; 186-191). Recently direct acting antivirals could achieve a high SVR rate, compared to Interferon-based therapy.

２．研究の目的

Although DAA-based therapies achieve a high SVR rate, HCC still develop in SVR patients. In this study, we aimed to clarify the mechanism of HCC development from SVR patients as well as to find out the risk factors of SVR HCC.

３．研究の方法

(1) *Patient*

This study was a cross-sectional analysis. A total of 717 patients were recruited at Osaka City University Hospital between December 2004 and December 2013. All patients had either a viral load of $> 10^5$ IU/mL according to the COBAS AMPLICOR HCV Monitor test, version 2.0 (Roche Diagnostics, Branchburg, NJ, USA), or a viral load of > 5 log copies/mL as determined by the COBAS TaqMan HCV test (Roche Diagnostics). HCC was diagnosed at the conclusion of the data collection in December 2013. All patients provided written informed consent, and all methods were carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. All experimental protocols were approved by the ethical committee of Osaka City University, Graduate School of Medicine (approval No. 1646).

The exclusion criteria included a history or evidence of a serious chronic or poorly controlled medical or psychiatric condition and infection with human immunodeficiency virus or hepatitis B virus. Patients with autoimmune liver diseases, primary biliary cirrhosis, and heavy alcoholic habits were also excluded.

Table 1. Comparison of the clinical characteristics of patients in the HCC and non-HCC groups[a]

	HCC (n=142)	Non-HCC (n = 575)	*p*-value
Age (years)	67 ± 9	59 ± 13	<0.0001
Sex (female/male)	42/100	326/249	<0.0001
HCV viral load (log copies/mL)	6.0 ± 1.0	6.2 ± 1.0	0.0718
Serotype (1/2/3)	98/20/0	435/112/2	0.55
CH/LC	54/88	487/88	<0.0001
AST (IU/L)	68.9±40.8	48.9±34.0	<0.0001
ALT (IU/L)	66.5±43.4	55.1±48.7	<0.0001
Platelets ($\times 10^4/\mu L$)	12.3 ± 6.1	16.7 ± 6.0	<0.0001
Albumin (g/dL)	3.8 ± 0.4	4.2 ± 1.8	<0.0001
PT%	88.0±17.5	99.1 ±16.2	<0.0001
AFP (ng/mL)	630.4 ± 4357.2	10.7±23.7	<0.0001
PIVKA-II (mAU/mL)	2097.6 ± 9991.9	23.1±42.9	<0.0001
IL28B rs8099917 (TT/TG/GG)[b]	96/34/1	391/143/1	0.5660
ITPA rs1127354 (CC/CA/AA)[b]	94/35/2	406/130/9	0.7309

[a]Continuous variables are shown as the mean ± SD.

[b]11 HCC and 30 non-HCC samples were lost to examine the *IL28B* and *ITPA* SNPs, respectively.

(2) *SNP genotyping*

We examined the genetic polymorphisms *IL28B* rs8099917 (T/G), *ITPA* rs1127354 (C/A), *DEPDC5* rs1012068 (T/G), *HCP5* rs2244546 (C/G), *MICA* rs2596542 (G/A), and *PNPLA3* rs738409 (C/G) in patients who consented to a genome analysis. Whole blood was collected from all patients and was centrifuged to separate the buffy coat. Genomic DNA was extracted from the buffy coat using a QIAamp® DNA Blood Midi Kit (QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, Germany). Genetic polymorphisms of SNPs were genotyped using (1) TaqMan SNP Genotyping Assays via a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and (2) direct sequencing. Approximately 10% of the samples were also randomly genotyped via direct sequencing to confirm the genotypes. A fragment of *MICA* was amplified via polymerase chain reaction (PCR). PCR was performed in a total volume of 20 µL with 1× Premix Ex Tag

(TaKaRa Bio Inc., Otsu, Shiga, Japan), 300 nM of each primer and 100 ng of genomic DNA. The PCR protocol was performed at 94°C for 10 min followed by 35 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products were sequenced bi-directionally using a BigDye Terminator v3.1 Cycle Sequencing Kit and an 3130XL Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Genotyping of *IL28B* and *ITPA* SNPs were performed as previously described[13]. Direct sequencing results were completely matched with TaqMan SNP genotyping assay. The ethical committee of our university permitted the genotyping analysis (approval No. 1871).

(3) *Measuring soluble MICA (sMICA) protein levels*

We randomly chose a group of 6 patients with each *MICA* genotype from either the HCC or non-HCC group for further analysis of soluble MICA levels. Serum was collected after the blood was allowed to clot. The clot was removed by centrifugation, and sMICA level in the resulting supernatants from 36 samples were quantified using a RayBio Human MICA ELISA Kit as described in the manufacturer's instructions (RayBiotech, Norcross, GA, USA). We assessed whether the soluble MICA levels decreased from the major to minor genotype in either group of HCC or non-HCC. We used the Kruskal-Wallis test for analysis.

4．研究成果

(1) *Patient profiles and treatment outcomes*

The genotype distributions of *MICA*, *DEPDC5*, *HCP5*, and *PNPLA3* SNPs in both the HCC and non-HCC groups were in Hardy-Weinberg equilibrium (HWE), as determined with the HWE test. The characteristics of the 717 patients with CHC (349 men and 368 women) are shown in Table 1. All patients were infected with HCV with a viral load > 5.0 copies/mL. Significant differences between the HCC (n = 142) and non-HCC groups (n = 575) were observed in age, sex, aspartate transaminase (AST), alanine transaminase (ALT), platelet count, percentage of prothrombin time (PT%), albumin, and AFP ($p < 0.0001$) but not in HCV viral load or the *IL28B* or *ITPA* SNP. The mean age of the patients with HCC was significantly greater than that of the patients without HCC (67 vs. 59 years old). Seventy percent of patients with HCC were male, a value significantly greater than the 43% observed among patients without HCC. AST and ALT levels were significantly higher in the HCC group than in the non-HCC group (69 ± 41 and 67 ± 43 IU/L vs. 49 ± 34 and 55 ± 49 IU/L, respectively). The platelet counts, PT%, and albumin levels in patients with HCC were significantly lower than those in patients without HCC (12.3 ± 6.1 × 10^4/μL, 88.0 ± 17.5% and 3.8 ± 0.4 g/dL vs. 16.7 ± 6.0 × 10^4/μL, 99.1 ± 16.2% and 4.2 ± 1.8 g/dL, respectively). Moreover, the mean levels of the tumour markers AFP and PIVKA-II were 630 ng/mL and 2,098 mAU/mL, respectively, in the HCC group, which were significantly higher than those in the non-HCC group (11 ng/mL and 23 mAU/mL, respectively).

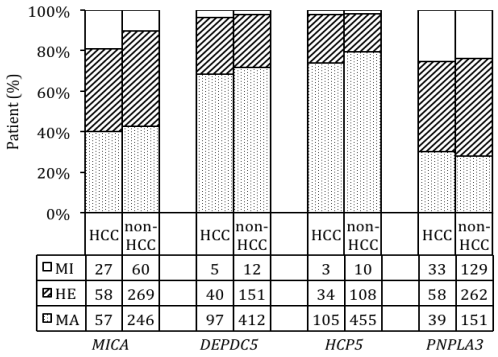
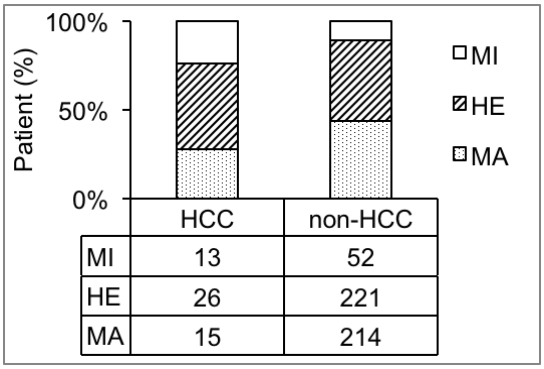


Figure 1: Genotypes of *MICA*, *DEPDC5*, *HCP5*, and *PNPLA3* in patients with or without HCC. The vertical axis shows the percentage of each genotype, and the data table shows the number of independent samples tested in each group.

(2) *Association between the risk allele of SNPs in 4 genes and the development of HCC in patients with CHC*

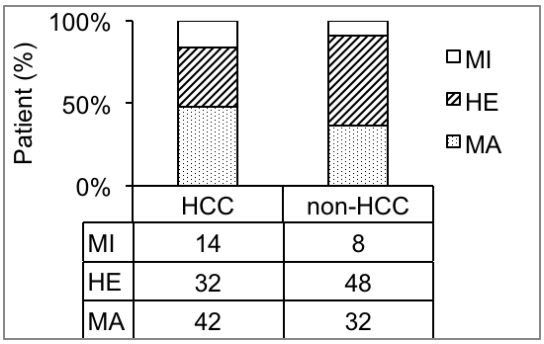
To investigate the association between the *MICA* SNP and HCC development, we genotyped 717 samples by using TaqMan SNP genotyping assays. Major (MA), hetero (HE), and minor (MI) genotypes were present in 57 (40%), 58 (41%), and 27 (19%) patients with HCC, respectively, and in 246 (43%), 269 (47%), and 60 (10%) patients without HCC, respectively, indicating a significant association between the *MICA* genotype and HCC development in patients with CHC (p = 0.0185, Fig. 1). The MI allele frequencies (MAFs) in the HCC and non-HCC groups were 0.394 and 0.338, respectively. In addition to the association of all 717 patients, a significant association also was observed in sub-cohorts of 541 chronic (p = 0.0057, Fig. 2a) and 176 cirrhosis patients (p = 0.0453, Fig. 2b).

(a)



	HCC	non-HCC
MI	13	52
HE	26	221
MA	15	214

(b)



	HCC	non-HCC
MI	14	8
HE	32	48
MA	42	32

Figure 2: *MICA* genotype and HCC development. A group of patients with background of CHC (a, n = 541) or cirrhosis (b, n = 176) is shown.

In addition to the *MICA* SNP, we also analysed three other reported HCV-related HCC SNPs. For the *DEPDC5* SNP, we found MA, HE, and MI genotypes in 97, 40, and 5 patients with HCC, respectively, and in 412, 151, and 12 patients without HCC, respectively (p = 0.5161); the MAFs were 0.18 and 0.15 for the HCC and non-HCC groups, respectively. For the *HCP5* SNP, we observed the MA, HE, and MI genotypes in 105, 34, and 3 patients with HCC, respectively, and in 455, 108, and 10 patients without HCC, respectively (p = 0.3668). Finally, the MA, HE, and MI *PNPLA3* genotypes were observed in 39, 58, and 33 patients with HCC, respectively, and in 151, 262, and 129 patients without HCC, respectively (p = 0.7466, Fig. 1).

(3) *Independent factors related to HCC development*

MICA SNP and variables with p values < 0.0001 in the univariate analysis (Table 1) were subjected to logistic regression analysis. These variables including age, sex, albumin, prothrombin time, AFP, AST, ALT, PIVKA-II concentration, platelets, and genotype of the *MICA* SNP were categorized and used to analyse associations with binary outcomes (HCC or non-HCC). Logistic regression analysis indicated that age (older than 65 years old), male sex, albumin ≤ 4 g/dL, prothrombin time ≤ 70%, AFP concentration ≥ 20 ng/mL, PIVKA-II concentration ≥ 40 mAU/mL, and minor genotype of the *MICA* SNP were independent factors that were significantly associated with HCC development.

(4) *The MICA SNP was correlated with soluble protein levels*

Because the MI genotype of the *MICA* SNP was associated with a high risk of HCC development, we assessed whether rs2596542 was correlated with MICA expression in patients with HCV-related HCC. sMICA levels were measured in 36 serum samples (n = 6 of each genotype from patients with or without HCC) by using ELISAs. The results showed that in the HCC samples, median protein levels of sMICA from patients with the MA, HE, and MI genotypes were 80, 50, and 0 pg/mL, respectively (Fig. 3). Although these protein levels tended to be to be higher in MA and HE, and lower in MI genotypes, they were not significantly different (p = 0.1284 by Kruskal-Wallis test). However, interestingly, in the non-HCC group, the sMICA levels were significantly correlated with its genotypes (p = 0.0498 by Kruskal-Wallis test, Fig. 3). These results suggest that MICA protein expression likely correlate with the *MICA* genotype.

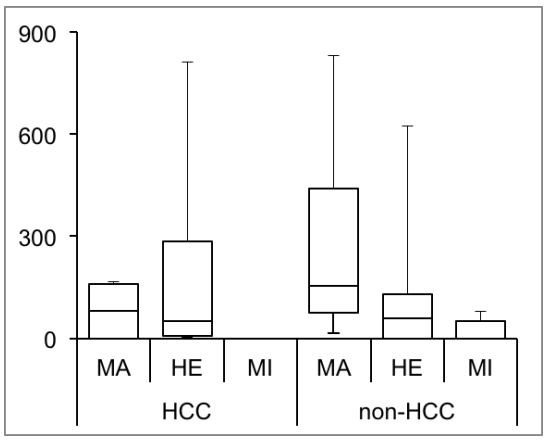


Figure 3: *MICA* SNP genotypes and soluble protein levels. Median protein levels in HCC or non-HCC samples with MA, HE, and MI genotypes are shown as horizontal lines inside the box of interquartile range. The whiskers are the maximum and minimum values. We assessed the difference in the median values among genotypes by using Kruskal-Wallis tests (p = 0.1284 in HCC group; p = 0.0498 in non-HCC group).

(5) *The* MICA *MI genotype is related to HCC development in patients older than 70 years*

We determined whether the risk allele rs2596542 was related to HCC development when patients were stratified by age. By comparing patients with and without HCC across 3 groups of age (younger than 65 years old, 65-70 years old, and above 70 years old), we found that the MI genotype was significantly associated with HCC development in the subset of patients with CHC above 70 years old (p = 0.004, Fig. 4).

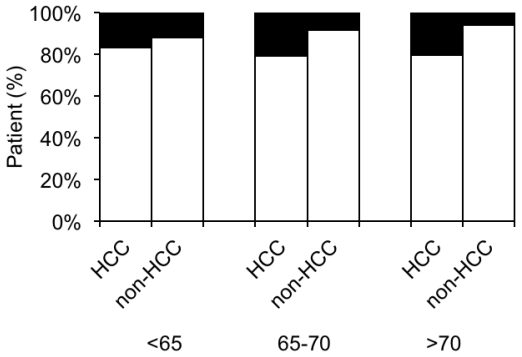


Figure 4: *MICA* rs2596542 MI genotype and the age of patients. Patients with or without HCC were divided into 3 age groups: < 65, 65-70, and > 70. The vertical axis shows the percentage of each genotype.

Moreover, we analysed the ratio of patients with HCC to those without HCC with respect to the *MICA* genotypes in 5-year age ranges: younger than 55 years old, 55-59 years old, 60-64 years old, 65-69 years old, 70-74 years old, and above 74 years old. This ratio varied among MI allele carriers from 0.06 to 2.0, which was greater than the range of 0.06 to 0.46 observed among patients with the non-MI allele (Fig. 5).

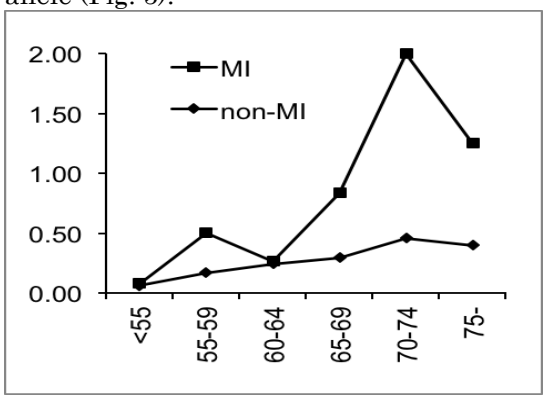


Figure 5: HCC/non-HCC ratio of each 5-year age group with respect to the *MICA* rs2596542. The vertical axis shows the HCC/non-HCC ratio in the MI genotype (□) or non-MI genotype (□), and the horizontal axis shows age grouped by a 5-year period.

(6) *The* MICA *MI genotype is associated with the development of HCC in patients with platelet counts in the range of 10-15 × 10^4/μL*

A low platelet count is a risk factor for HCC. In this study, we determined the platelet count range at which MI rs2596542 was related to the development of HCC. We found that the *MICA* MI genotype was associated with HCC development when the platelet count was 10-15 × 10^4/μL but was not lower than 10 or higher than 15 × 10^4/μL (Fig. 6).

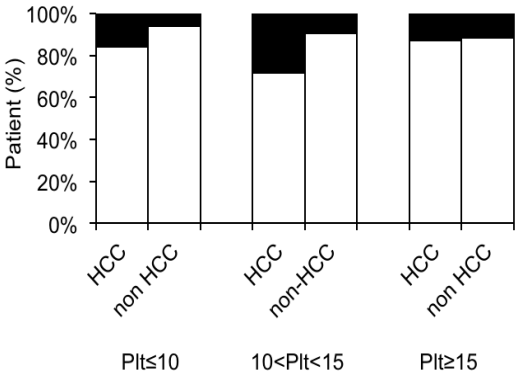


Figure 6: *MICA* SNP MI genotype and platelet counts. The vertical axis shows the percentage of each genotype in HCC or non-HCC patients. Plt ≤10, platelet counts equal or less than 10 × 10^4/μL blood; 10<Plt<15, platelet counts in the range of 10 – 15 × 10^4/μL blood; Plt ≥15, platelet counts equal or more than 15 × 10^4/μL blood.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　5　件）

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〔学会発表〕（計　4　件）

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2. NS5A RAVs in DAA-naïve HCV genotype 1b Japanese patients were associated with patients background. 25th Asian Pacific Association for the Study of the Liver (APASL), February 20-24, 2016, Tokyo, Japan.
3. *MICA* SNP, but not *DEPDC5, PNPLA3* and *HCP5* SNPs, is associated with chronic hepatitis C-related hepatocellular carcinoma. AASLD The Liver Meeting Nov 13-17, 2015 San Francisco, CA, USA.
4. The *MICA* but not the *DEPDC5, PNPLA3* and *HCP5* polymorphism is associated with chronic hepatitis C-related hepatocellular carcinoma. 51st Japan Society of Hepatology Annual Meeting, May 20-21, 2015, Kumamoto, Japan.

〔図書〕（計　0　件）

〔産業財産権〕

○出願状況（計　0　件）

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発明者：
権利者：
種類：
番号：
出願年月日：
国内外の別：

○取得状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
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