Pathology & Oncology Research (2020) 26:1519–1525
https://doi.org/10.1007/s12253-019-00738-6

ORIGINAL ARTICLE

Association between *MICA* rs2596542 Polymorphism with the Risk of Hepatocellular Carcinoma in Chronic Hepatitis C Patients

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Received: 20 February 2019 / Accepted: 27 August 2019 / Published online: 31 August 2019

Abstract

In this study we investigated the impact of rs2596542A/G single nucleotide polymorphism (SNP) in the major histocompatibility complex class I chain-related sequence A (*MICA*) gene on HCV-induced hepatocellular carcinoma (HCC) susceptibility in a Brazilian population. In total, 252 HCV-infected patients (98 with HCV-induced HCC and 154 non-malignant HCV-induced liver cirrhosis) were enrolled and 98 healthy control subjects (negative anti-HCV). The *MICA* rs2596542 SNP genotypes were determined by real-time PCR assay. No differences in *MICA* genotype frequencies between HCV-induced cirrhosis patients and controls were observed. However, genotype frequencies of rs2596542A/G SNP were statistically different between HCV-induced HCC patients and controls ($p = 0.048$), and also between HCC and HCV-induced cirrhosis patients ($p = 0.039$). The highest frequency of the rs2596542AA genotype was observed in HCC patients (31.6%) when compared with HCV-induced cirrhosis patients (18.8%) and healthy controls (19.4%). Also, rs2596542AA genotype carriers have an increased risk for HCC when compared to HCV-induced cirrhosis status [odds ratio (OR) = 1.99; 95% confidence interval (CI) = 1.06–3.74, $p = 0.020$)] and healthy individuals (OR = 1.92, 95% CI = 1.00–3.70, $p = 0.049$). Taken together our study suggest that *MICA* rs2596542 SNP impacts HCV-induced HCC susceptibility suggesting that genetic variants in *MICA* are of clinical relevance to hepatocarcinogenesis by impacting host immune response in chronic HCV infection.

Keywords HCV · Hepatocellular carcinoma · Liver cirrhosis · *MICA* gene · rs2596542, HCC

Introduction

Hepatitis C virus (HCV) is a single-stranded positive RNA virus of the *Flaviviridae* family [1]. HCV causes acute and chronic liver infection and is a serious human health issue [2, 3]. Acute HCV infection is generally asymptomatic, and 15 to 25% of the infected individuals eliminate the virus spontaneously [4]. In the remaining patients, HCV infection becomes chronic [4], causing liver inflammation [5]. Chronic HCV infection may result in severe liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC) [4]. Of note, 20 to 30% of chronically HCV infected patients progress to cirrhosis within 25 to 30 years after HCV infection. Once liver cirrhosis is established, the annual risk for HCC is about 1–4% [4]. HCC is an aggressive tumor and is one of the most frequent causes of cancer-related death in the world [6]. Therefore, the progression of HCV-related chronic liver diseases increases the risk of morbimortality, affecting the quality of life and represent a severe economic burden to the public healthcare systems [2].

Genome-wide association studies (GWAS) have reported various single nucleotide polymorphisms (SNPs) associated with liver disease progression in HCV-infected subjects [7–9]. The SNP rs2596542A/G, in the 5′ flanking region of the major histocompatibility complex class I polypeptide-related sequence A (*MICA*) gene, was associated to HCV-

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12253-019-00738-6) contains supplementary material, which is available to authorized users.

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related HCC in Japanese individuals [10]. The rs2596542A allele increases the risk for HCC as well as it is associated with low levels of soluble MICA (sMICA) protein in the serum of the individuals with HCV-induced HCC [10].

MICA is located on chromosome 6, and it encodes a transmembrane protein which is up-regulated by cellular stress, such as viral infection and oncogenic transformation [11, 12]. MICA is mainly expressed intracellularly in epithelial cells of various normal tissues and also in several tumors [13], but it is virtually expressed in all body tissues except the central nervous system [14]. The membrane-bound MICA interacts with the natural killer group 2 member D (NKG2D) activatory receptor expressed on the surface of natural killer (NK) cells and CD8+ T-cells [12]. Interestingly, membrane-bound and soluble MICA (sMICA) molecules exhibit distinct functional properties. Recognition of MICA molecules is necessary for the immune system to detect and eliminate tumoral or viral infected cells, thus displaying a critical role in immune surveillance [15]. However, shedding of sMICA molecules represents a mechanism of tumor immune escape [16] and suppression of allograft immune response in transplanted patients (reviewed in [17]). Also, high sMICA levels have been detected in the serum of patients with malignancies, including HCC [18–20].

It has been suggested that the expression of MICA is decreased in response to HCV infection in Japanese individuals carrying the rs2596542A allele, which may affect the immunologic response and elimination of virus-infected cells [10]. Noteworthy, viral infection may progress and increases the risk of HCC development [10]. However, studies in other populations reported conflicting results [21–25]. Currently, the role of the *MICA* polymorphism as a prognostic genetic marker for liver disease is still not well established.

Therefore, in the present study, we evaluated the impact of *MICA* rs2596542 A/G SNP on the susceptibility to liver cirrhosis and HCC in a population of chronic HCV-infected patients from Brazil.

Material and Methods

Study Population

The study recruited chronic hepatitis C patients (aged ≥18 years) attending in an outpatient care setting at *Hospital de Clínicas de Porto Alegre* (Porto Alegre, Rio Grande do Sul, Brazil) from 2015 to 2016. HCV infected patients were grouped into non-malignant HCV-induced liver cirrhosis ($n = 154$) and HCV-induced HCC ($n = 98$). Cirrhosis was defined according to the METAVIR score of liver biopsy [26], image analysis or combining clinical and laboratory parameters [27]. HCC was diagnosed according to the American Association for the Study of Liver Diseases (AASLD) guideline [28]. A total of 98 healthy blood donors (negative for anti-HCV) comprised the control group. Subjects with hepatitis B virus (HBV) or human immunodeficiency virus (HIV) co-infection were excluded, as well as patients with other liver related-diseases such as hemochromatosis, autoimmune hepatitis and Wilson's disease. Sociodemographic data were obtained through a structured questionnaire. Ethnicity was investigated as self-reported skin color. Clinical and laboratory data were collected from the patients' clinical charts. The local Institutional Review Board approved the study protocol (*Hospital de Clínicas de Porto Alegre*, protocol #15–0126), and informed consent was obtained from all subjects.

DNA Extraction and SNP Genotyping

DNA samples were extracted through the salting-out method from blood samples as described previously [29]. The genotypes of the *MICA* rs2596542A/G polymorphism were determined by real-time PCR (TaqMan®: C__27301153_10, Applied Biosystems, Thermo Fisher Brand, Foster City, USA) on a StepOnePlus™ Real-Time PCR Systems (Applied Biosystems Inc., Foster City, USA) according to the manufacturer's instructions.

Statistical Analysis

Allele frequencies were determined by direct counting. Deviation from Hardy-Weinberg equilibrium was assessed using a Web program available at http://www.oege.org/software/hwe-mr-calc.shtml [30]. The Kolmogorov-Smirnov test was used to evaluate if a variable is normally distributed. Continuous variables with normal distribution were compared between groups using the Student *t*-test and variables with nonparametric distribution were compared using the Mann-Whitney *U* test. Categorical variables were compared using the chi-square or *Fisher's exact test* when indicated. The strength of the association between *MICA* polymorphism and risk to cirrhosis and HCC was assessed by odds ratio (OR) and corresponding 95% confidence interval (CI). Potential confounding factors were entered in the logistic regression models based on statistical criteria (only if the variable was associated with the study factor and with the outcome at $p < 0.20$). All statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, USA). p values <0.05 were considered statistically significant.

Results

Sociodemographic and clinical characteristics of the chronic HCV infected patients are shown in Table 1. There was a significant difference in male frequency between HCV-induced HCC (58.2%) and non-malignant HCV-induced cirrhosis (43.5%) groups ($p = 0.023$). The HCC group was older than cirrhotic

Table 1 Sociodemographic and clinical characteristics of patients with chronic HCV infection

Variable	All ($n = 252$)	Non-malignant HCV-induced cirrhosis ($n = 154$)	HCV-induced HCC ($n = 98$)	p*
Gender (male)	124 (49.2)	67 (43.5)	57 (58.2)	**0.023**
Age (years)	60.4 ± 8.4	59.5 ± 8.4	61.8 ± 8.2	0.056
Caucasians (ethnicity)	183 (72.6)	110 (71.4)	73 (74.5)	0.595
BMI (kg/m^2)	27.2 ± 5.0	27.8 ± 5.4	26.3 ± 4.2	0.693
Age at infection of HCV (years)	27.2 ± 9.9	27.5 ± 9.8	26.6 ± 10.3	0.688
Age at diagnosis of HCV (years)	49.6 ± 10.6	49.2 ± 11.0	50.2 ± 10.1	0.765
Route of infection				0.811
Blood transfusion	101 (40.1)	64 (41.6)	37 (37.8)	–
Intravenous drug	10 (4.0)	6 (3.9)	4 (4.1)	–
Tattoo	3 (1.2)	3 (1.9)	–	–
Surgery	4 (1.6)	2 (1.3)	2 (2.0)	–
Sexual contact	3 (1.2)	2 (1.3)	1 (1.0)	–
Labor	4 (1.6)	2 (1.3)	2 (2.0)	–
Other	8 (3.2)	6 (3.9)	2 (2.0)	–
Unknown	119 (47.2)	69 (44.8)	50 (51.0)	–
HCV RNA ($\log_{10}$ UI/mL)	6.7 ± 6.9	6.7 ± 6.9	6.5 ± 6.8	0.389
HCV genotypes				0.060
1	124 (51.0)	86 (57.0)	38 (41.3)	–
2	7 (2.9)	4 (2.6)	3 (3.3)	–
3	112 (46.1)	61(40.4)	51 (55.4)	–
Diabetes	85 (33.7)	50 (32.5)	35 (35.7)	0.595
Steatosis	24 (9.6)	13 (8.4)	11 (11.5)	0.431
Ascites	66 (26.3)	31 (20.1)	35 (36.1)	**0.005**
Portal hypertension	146 (58.2)	72 (46.8)	74 (76.3)	**<0.001**
Esophageal varices	156 (62.4)	91 (59.5)	65 (67.0)	0.231
Upper gastrointestinal bleeding	49 (19.5)	26 (16.9)	23 (23.7)	0.184
Spontaneous bacterial peritonitis	13 (5.2)	7 (4.5)	6 (6.2)	0.568
Hepatic encephalopathy	22 (8.8)	14 (9.1)	8 (8.2)	0.818

In bold statistically significant results. Variables expressed as number (percentage) or mean ± standard deviation. HCC, hepatocellular carcinoma; BMI, body mass index. *p values corresponding to comparisons between non-malignant HCV-induced cirrhosis vs. HCV-induced HCC patients

group, and most of them had Caucasian ethnicity (72.6%). Among the possible routes involved in HCV infection reported by patients, blood transfusion and unknown routes were the most frequent. Further, significant differences between HCC and cirrhotic patients were observed in the frequency of ascites ($p = 0.005$) and portal hypertension ($p < 0.001$). Among HCC patients the mean size of tumors was 3.3 cm ± 1.8 and 62 (63.9%) patients had one tumor (data not shown).

The rs2596542A/G genotype frequencies in the control group adhered to Hardy-Weinberg equilibrium. The allele and genotype frequencies of the *MICA* polymorphism in chronic HCV-infected patients groups and healthy controls are shown in Table 2. In all groups evaluated the rs2596542A/G allele frequencies were similar among studied groups ($p > 0.05$). Because sMICA levels and progression from HCV chronic infection to HCC are strongly associated with MICA rs2596542A allele (Kumar et al., 2011), we performed analyses based on the effect of the rs2596542A risk allele in homozygosis. Differences were observed in *MICA* rs2596542AA genotype frequency among HCV-induced HCC patients vs. controls ($p = 0.048$) and HCV-induced HCC vs. HCV-induced cirrhosis status ($p = 0.039$). While individual genotypes have no influence on cirrhosis and HCC susceptibility, we observed that rs2596542AA homozygous status (AA vs. AG + GG) was associated with the risk for HCV-induced HCC in our population (HCC vs. controls, HCC vs. cirrhosis, $p < 0.05$, Table 3). Stratified analyses of genotypes according to clinical

Table 2 Allele and genotype frequencies of SNP rs2596542 in patients with chronic HCV infection and healthy controls subjects

rs2596542	Control ($n = 98$)	Non-malignant HCV-induced cirrhosis ($n = 154$)	HCV-induced HCC ($n = 98$)	Non-malignant HCV-induced cirrhosis vs. Control p-value	HCV-induced HCC vs. Control	Non-malignant HCV-induced cirrhosis vs. HCV-induced HCC	Control vs. HCV-infected patients*
Allele							
G	106 (54.1)	173 (56.2)	98 (50.0)	0.646	0.419	0.176	0.941
A	90 (45.9)	135 (43.8)	98 (50.0)				
Genotype							
GG	27 (27.5)	48 (31.2)	31 (31.6)	0.826	**0.048**	**0.039**	0.376
AG	52 (53.1)	77 (50.0)	36 (36.8)				
AA	19 (19.4)	29 (18.8)	31 (31.6)				

In bold statistically significant results. Variables expressed as number (percentage). HCC, hepatocellular carcinoma. *All HCV-infected patients: non-malignant HCV-induced cirrhosis plus HCV-induced HCC

Table 3 The impact of rs2596542A/G SNP on HCC susceptibility in HCV-chronic infected patients

Variable	Control ($n = 98$)	Non-malignant HCV-induced cirrhosis ($n = 154$)	HCV-induced HCC ($n = 98$)	Non-malignant HCV-induced cirrhosis vs. Control		HCV-induced HCC vs. Control		HCV-induced HCC vs. Non-malignant HCV-induced cirrhosis		Control vs. HCV-infected patients*	
				OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
rs2596542A/G											
GG	27 (27.5)	48 (31.2)	31 (31.6)	1.00 (Ref.)	–	1.00 (Ref.)	–	1.00 (Ref.)	–	1.00 (Ref.)	–
AG	52 (53.1)	77 (50.0)	36 (36.8)	0.83 (0.44–1.56)	0.542	0.60 (0.29–1.24)	0.137	0.72 (0.38–1.38)	0.291	0.74 (0.41–1.32)	0.285
AA	19 (19.4)	29 (18.8)	31 (31.6)	1.16 (0.38–1.95)	0.826	1.42 (0.62–3.30)	0.370	1.66 (0.79–3.45)	0.144	1.08 (0.52–2.26)	0.825
Dominant model											
GG + AG	79 (80.6)	125 (81.2)	67 (68.4)	1.00 (Ref.)	–	1.00 (Ref.)	–	1.00 (Ref.)	–	1.00 (Ref.)	–
AA	19 (19.4)	29 (18.8)	31 (31.6)	0.96 (0.49–1.95)	0.913	1.92 (1.00–3.70)	**0.049**	1.99 (1.06–3.74)	**0.020**	1.30 (0.71–2.46)	0.374

In bold statistically significant results. Variables expressed as number (percentage). HCC, hepatocellular carcinoma; OR, odds ratio; CI, confidence interval. Ref., reference. *All HCV-infected patients: non-malignant HCV-induced cirrhosis plus HCV-induced HCC

and sociodemographic variables suggested that groups were equally distributed in our analysis (Table S1). Noteworthy, our results suggested that rs2596542A/G SNP is exclusively associated with HCC risk since no association was observed in comparisons between joint HCV-induced clinical outcomes (HCC plus cirrhosis only) vs. healthy controls ($p > 0.05$). Such analysis also indicated that rs2596542A/G SNP is not associated with HCV infection risk.

Discussion

In this study, we investigated the association between *MICA* SNP rs2596542A/G with the risk of HCC development in chronically HCV infected patients. We observed that rs2596542AA genotype frequency was higher in HCV-induced HCC patients than in controls and non-malignant HCV-induced cirrhosis patients. Furthermore, our analyses showed that HCV infected individuals carrying the AA genotype are approximately twice as likely to progress to HCC as those carrying AG or GG genotypes.

Similar findings were observed in previous studies evaluating other populations, such as Japanese [10, 21, 25]. However, conflicting results regarding rs259654A/G SNP on HCC susceptibility in the literature has been reported [22–24]. The variable results observed in previous studies may be due to differences in sample size and ethnicity. In addition, HCC pathogenesis involves a complex interaction among viral, host and environmental factors [31]. Therefore, population-specific features are important factors for taking into account the differences observed.

Notably, the functional genetic variant *MICA* rs2596538 (G/A) was shown to influence sMICA levels in HCV-induced HCC patients [32]. Interestingly, this SNP is located in the promoter region and acts directly on the *MICA* transcription [32]. According to authors, HCV proteins are able to induce phosphorylation of transcription factor Specificity Protein 1 (SP1), which has a high affinity for the G allele of SNP rs2596538 and activates the expression of *MICA* in the hepatocytes [32]. Therefore, patients carrying the rs2596538G allele have high membrane-bound MICA levels and generate a robust immune response to the viral infection, reducing the risk of disease progression [32]. In addition, the rs2596538G carriers are associated with increased sMICA levels in healthy individuals [33].

Interestingly, the *MICA* rs2596538G allele is found in nearly perfect linkage disequilibrium with the rs2596542G allele (D' = 1.0, $r^2 = 0.99$) when considering individual from 1 k genomes project (phase 3, version 5) [34]. Hence, this observation could partially explain why cirrhotic HCV patients carrying the rs2596542A allele (GA + AA) and those not carrying but with high sMICA levels and are at increased risk for HCC [35]. Further, sMICA levels associates with liver fibrosis in chronically HCV-infected individuals carrying the rs2596542A allele [36]. It should be noted that membrane-bound MICA and sMICA have opposing effects on effector cytotoxic functions of CD8+ T and NK cells being relevant in host-viral response [17]. Also, a functional variant in *MICA* (i.e., rs1051792, also known as Val129Met) has been shown to influence NKG2D affinity [37, 38].

Despite *MICA* high polymorphism several genetic variants have been identified in their nucleotide sequence affecting cancer susceptibility, such as cervical and breast cancers [15, 39, 40]. Moreover, it has been suggested that *MICA* SNPs may be associated with HBV-induced HCC [18, 41] and also with the risk for CMV and HIV infections suggesting that such viruses may usurp MICA expression favoring their pathogenesis [42–44].

Finally, studies on *MICA* have improved our understanding of the biological role of the MICA–NKG2D immunological axis in HCV chronic infection and in the hepatocarcinogenesis [45]. Since MICA significantly impact host viral response, it represents a promising therapeutic strategy based on chemoimmunotherapeutic for HCC, especially in individuals with an increased genetic risk of insufficient MICA induction [46]. It has been suggested that drugs (e.g., anti-cancer agent vorinostat) could restore MICA expression in HCC cells, and thereby boosting the anti-HCC effects of NK cells [46]. Similar approaches have been suggested in other malignant disorders [47]. It should be noted that our study has some limitations. Despite the fact that sample size of our study is relatively small, this is the first study suggesting an association of the rs2596542AA genotype with HCV-induced HCC in a Brazilian population. With respect to established risk factors for HCC development, smoking and alcohol consumption are associated with HCC in a dose-dependent manner, information that we were not able to measure [48]. Also worthy to mention, the genetic diversity of *MICA* alleles was not evaluated. Further studies are necessary to evaluate the clinical relevance since it has been shown that soluble NKG2D affinity to MICA molecules is greatly impacted by different MICA molecules [49]. Also, *NKG2D* haplotypes have been associated with low or high cytotoxic activity of immune cells [50]. Thus, larger and well-designed collaborative studies are necessary to conclude the impact of rs2596542 SNP and *MICA* gene on HCV-induced HCC susceptibility.

In conclusion, the *MICA* rs2596542 SNP was associated with HCC in a population of HCV-infected patients from Southern Brazil. Our results suggest that *MICA* rs2596542 is potentially involved in viral immune response and that rs2596542AA homozygous are at increased risk for developing HCC. In this context, genetic variants are promising predictors of disease progression and can guide health professionals in the management of chronically HCV-infected patients.

Acknowledgments The authors thank the patients of the *Hospital de Clínicas de Porto Alegre* for their collaboration on this study.

Author Contributions Conceived and designed the experiments: CGM MRAS DS. Performed the experiments: CGM JTB DCS. Analyzed the

data: CGM MRAS RTM DS. Contributed reagents/analysis tools: VRL. Wrote the paper: CGM RTM DS.

Funding This work was supported by the *Universidade Luterana do Brasil*, *Hospital de Clínicas de Porto Alegre,* and by the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES* - Finance Code 001).

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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