ASSOCIATION OF *ISG20 rs4566136* POLYMORPHISM WITH HEPATITIS B VIRUS-RELATED HEPATOCELLULAR CARCINOMA

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Summary

Objectives: Interferon-stimulated gene 20 (*ISG20*) plays an important role in viral infection and cancers. This study investigated the association between *ISG20 rs4566136* polymorphism with HBV-related hepatocellular carcinoma (HCC). ***Subjects and methods:*** The SNP *ISG20 rs4566136* was genotyped by Sanger sequencing in 115 patients with HCC, 100 liver cirrhosis patients (LC) and 120 healthy controls (HC). ***Results:*** We observed that the frequencies of genotype *ISG20 rs4566136CC* and allele *rs4566136C* were significantly lower in HCC patients compared to LC patients and HC individuals. These results indicated that the SNP *ISG20 rs4566136* was associated with HCC (HCC vs. LC: OR (95%CI) = 0.67 (0.46-0.99), p = 0.04; HCC vs. HC: OR (95%CI) = 0.2 (0.12 - 0.32), p < 0.001. Patients with genotype *rs4566136TT* had higher AST and ALT levels, followed by patients with genotype *rs4566136TC* and *rs4566136CC*. However, the difference was not significant. ***Conclusion:*** *ISG20 rs4566136* polymorphism is associated with HBV-related HCC in the Vietnamese population. Allele *rs4566136C* contributes to a decreased risk of HBV-related HCC.

** Keywords: ISG20; SNPs; rs4566136; HBV; HCC.*

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INTRODUCTION

Hepatocellular carcinoma is a primary tumor of the liver and accounts for over 90% of the primary liver cancers. HCC occurs in 80% to 90% of patients with cirrhosis [6] and is the fifth most common cancer worldwide and the second leading cause of cancer-related death after lung cancer [3]. significant risk factors for HCC include hepatitis B virus (HBV). Chronic HBV and HCV (hepatitis C virus) infections account for more than 70% of HCC cases. HBV affects more than 250 million individuals in the world and is the most common cause of chronic hepatitis globally, and Vietnam is one of the countries with a high prevalence of HBV infection [5].

The replication of viruses is inhibited by type I interferon-stimulated genes (ISGs). Despite the understanding of the molecular basis of ISG restriction, the antiviral mechanisms remain unclear. One of the proteins in the ISG family is the 20-kDa exonuclease interferon-stimulated gene 20 (*ISG20*), which has antiviral activity against viruses. *ISG20* can inhibit the infection of a broad range of viruses including HBV infection. *ISG20* has been shown as an innate anti-HBV effector that can inhibit HBV infection through degrading HBV-RNA [4].

HBV-related and induced HCC development is a significant research area. Single nucleotide polymorphisms (SNPs) were a crucial factor in the development of the tumor. *ISG20* might be a potential indicator for liver injury and the clinical outcome of HBV-related HCC disease [1]. However, so far, no studies have reported the association between *ISG20* polymorphisms and HCC, and whether *ISG20* polymorphisms could affect the development of HBV-related HCC in the Vietnamese population.

We conducted this study: *To investigate the relationship between ISG20 rs4566136 polymorphisms and the risk of HBV-related HCC. The study of hereditary factors may shed more light on a better understanding of the molecular mechanisms and the pathogenicity of HBV-related HCC.*

SUBJECTS AND METHODS

1. Subjects

A total of 115 patients with HBV-related HCC and 100 patients with HBV-related liver cirrhosis (LC) were enrolled in this study. HCC patients were diagnosed according to the AASLD 2010 recommendation. This includes liver tumor size > 1 cm and rapidly absorbing drug in the fast-release artery in the venous tenses, and later on the CT scan film with contrast injection and/or histopathology

confirmed HCC. The patients were positive for HBsAg and negative for anti-HCV and anti-HIV. HCC stages were classified based on Barcelona criteria. Liver cirrhosis (LC) patients were diagnosed with two symptoms, including impaired liver function syndrome and portal hypertension syndrome. Demographic and clinical parameters including age, sex, AST and ALT, GGT levels, bilirubin total and direct levels, protein, albumin, RBC, WBC, PLT, and prothrombin levels were collected. The liver cirrhosis stages were classified based on Child-Pugh criteria. We also included 120 healthy individuals which were negative for HBV, HCV, and HIV infections as the control group. A sample of 5 mL of venous blood was collected from each patient or healthy individual, and sera were separated and stored at -80ºC until use.

Written informed consent from patients and healthy controls were obtained. The study was approved by the institutional review board of the Vietnam Military Medical University (VMMU). All samples were anonymized after the completion of the collection.

2. Methods

** Genotyping of ISG20 rs4566136 polymorphism:*

Total DNA was extracted from venous blood samples by a Gene JET Whole Blood Genomic DNA Purification Mini Kit (Thermo, USA). Primers were designed to amplify a fragment covering exon 2 and intron 2-3 using the Primer3 and NCBI primer BLAST software. PCR primer sequences were ISG20F:5'-GAG GGG CTT ACC TTT GTA GC-3' and ISG20R:5'TCA GAA CAC ATC CCA CTC CT-3'. The temperature cycling was as follows: 95°C for 2 min, followed by 40 cycles of denaturalization at 95°C for 30 sec, annealing at 60°C for 25 sec and extension at 72°C for 30 sec, and final extension for 5 min at 72°C. The total reaction mixture (25 µl) contained 2 µl DNA template, 12.5 µl Master Mix (2X), (2X) Dream Taq, and 0.625 µl of each primer. PCR products were purified using the GeneJET Genomic DNA Purification Kit (Thermo, USA). Sanger sequencing was carried out on all the purified PCR products on the ABI 3130 automated genetic analyzer (Applied Biosystems, USA). Sequencing data were analyzed using the Bioedit software version 7.0 to determine *ISG20 rs4566136* SNP.

** Statistical analysis:*

All statistical analysis was conducted by using SPSS 22.0. Continuous variables were presented as means or median where appropriate. Categorical variables are expressed as frequencies and percentages. Comparisons of continuous data between groups were

performed with the Mann-Whitney U test or Kruskal-Wallis test. The comparison of the differences in categorical variables between groups was performed by Pearson's Chi-square or Fisher's exact test. The logistic regression model was used to analyse the association of *ISG20 rs4566136* SNP with HBV-related HCC adjusted for confounding factors such as age and gender. Odds ratios (OR) and 95%CI were also calculated, and a p-value of < 0.05 was considered statistical significance.

RESULTS

1. Baseline characteristics of the study groups

Table 1: Baseline characteristics of patient group.

Characteristics	Median	Min	Max
Ages (years)	60.5 ± 10.9		
Gender (male; %)	107 (93)		
AST (U/L)	60	17	448
ALT (U/L)	45	14	469
GGT (U/L)	165.8	20.7	771.9
Bilirubin total (µmol/L)	17.1	7.5	470
Bilirubin direct (µmol/L)	4.8	0.6	249.7
Protein total (g/L)	78.9	55.7	91.4
Albumin (g/L)	39.6	18.9	47.3
RBC (T/L)	4.7	2.6	6.5
WBC (G/L)	6.6	2.7	15.3
PLT (G/L)	169	44	492
Prothrombin (%)	86	33	119

The demographic and clinical parameters such as age, sex, AST, ALT and GGT levels, RBC, WBC, PLT, and Prothrombin levels of the patients were presented in table 1. AST, ALT, and GGT levels increased slightly compared to normal levels. Bilirubin total and direct protein, albumin, and prothrombin levels were not significantly different compared to normal levels.

Table 2: Characteristic of liver function and stage of disease.

Stage	BCLC (n = 115)	Child Pugh (n = 115)
A	14 (12.3)	95 (83.6)
B	62 (53.9)	15(13)
C	33 (28.7)	5 (4.4)
D	6 (5.2)	

HCC patients in Child A stage were 83.6% (95 patients) and HCC patients in the intermediate stage were 53.9% (62 patients).

2. *ISG20 rs4566136* polymorphism analysis

The polymorphism *rs4566136* is located in the Intron 2 region of the *ISG20* gene. A DNA fragment covering Exon 2 and Intron 2-3 was amplified by the specific primers. PCR products were observed by Agarose 1.5% gel electrophoresis with standard maker 100bp (*Figure 1*). PCR products were purified and subjected to Sanger sequencing and the DNA sequences and genotype of the *ISG20 rs4566136* SNP were analysed by Bioedit software aligning with the reference sequence (*Figure 2*).

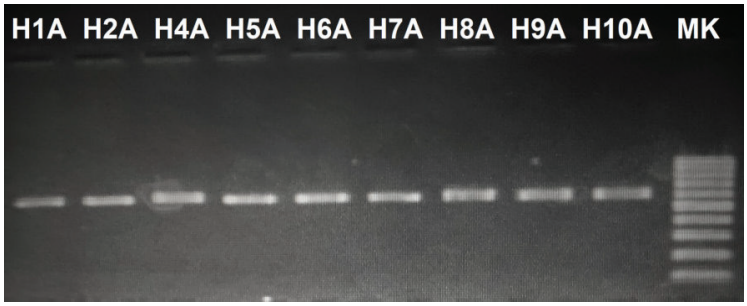

Figure 1: PCR products amplified DNA fragments of *ISG20* gene.

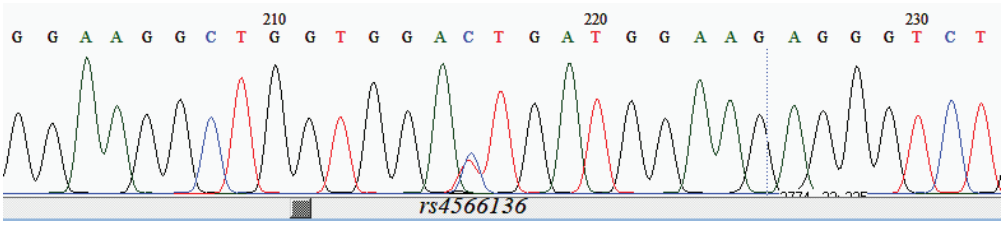

Figure 2: Sequence by Sanger method showing *rs4566136* genotype

Association of *ISG20 rs4566136* polymorphism with hepatocellular carcinoma.

The frequencies of genotype and allele of *ISG20 rs4566136* polymorphism in patients and healthy controls were presented in table 3. We analysed the association of *ISG20 rs4566136* polymorphism with HBV-related HCC in different genetic models, including genotypic, allelic, dominant, and recessive models.

Table 3: Genotype and allele distribution of *rs4566136* SNP in study groups.

***ISG20* SNP**	**HCC n = 115 (%)**	**LC n = 100 (%)**	**HC n = 120 (%)**	**OR (95%CI) HCC vs. LC**	**P**	**OR (95%CI) HCC vs. HC**	**p**
Genotype (n)							
TT	38 (33)	29 (29)	42 (35)	Reference		Reference	
CT	61 (53)	48 (48)	52 (43.3)	0.8 (0.43 - 1.5)	0.49	0.14 (0.06 - 0.31)	< 0.001
CC	16 (14)	23 (23)	26 (21.7)	0.45 (0.2 - 1.04)	0.06	0.21 (0.09 - 0.53)	0.001
Allelic (2n)							
T	137 (59.6)	106 (53)	136 (56.7)	Reference		Reference	
C	93 (40.4)	94 (47)	104 (43.3)	0.67 (0.46 - 0.99)	0.04	0.2 (0.12 - 0.32)	< 0.001
Dominant (n)							
TT	38 (33)	29 (29)	42 (35)	Reference		Reference	
TC + CC	77 (67)	71 (71)	78 (65)	0.67 (0.37 - 1.2)	0.18	0.09 (0.04 - 0.18)	< 0.001
Recessive (n)							
TT + TC	99 (86)	77 (77)	94 (78.3)	Reference		Reference	
CC	16 (14)	23 (23)	26 (21.7)	0.48 (0.24 - 0.98)	0.04	0.26 (0.12 - 0.57)	0.001

OR and p values were calculated by a logistic regression model adjusted for age and gender.

We observed that the frequencies of genotype *ISG20 rs4566136CC* and allele *rs4566136C* were significantly lower in HCC patients (14% and 40.4%, respectively) compared to LC patients (23% and 47%, respectively) and HC individuals (21.7% and 43.3%, respectively). In the allelic model, the results indicated that the SNP ISG20 rs4566136 was associated with HCC (HCC vs. LC: OR (95%CI) = 0.67 (0.46 - 0.99), p = 0.04; HCC vs. HC: OR (95%CI) = 0.2 (0.12 - 0.32), p < 0.001. Similarly, in the recessive genetic model, the results showed that the SNP *ISG20 rs4566136* was associated with HCC (HCC vs. LC: OR (95%CI) = 0.48 (0.24 - 0.98), p = 0.04; HCC vs. HC: OR (95%CI) = 0.26 (0.12 - 0.57), p = 0.001.

3. Association of *rs4566136* SNP with clinical parameters of HCC patients

Table 4: Association of *rs4566136* SNP with clinical characteristics of HCC patients.

Characteristic		**TT (n = 38)**	**TC (n = 61)**	**CC (n = 16)**	**p**
AST (U/L)	Median	64.3	61	49	0.3
	Min-max	17.3 - 448	24.6 - 427.4	26 - 356	
ALT (U/L)	Median	48.7	47	39	0.15
	Min-max	14 - 154	16.9 - 412.8	14 - 469	
Bilirubin total (µmol/L)	Median	16.4	19.4	15.74	0.43
	Min-max	7.9 - 404.5	7.5 - 470	10.7 - 27.3	
Albumin (g/L)	Median	39.18	38.9	40.45	0.31
	Min-max	23.5 - 47.3	18.9 - 46.4	30 - 46.1	
Prothrombin (%)	Median	86.5	87	79.5	0.56
	Min-max	33 - 110	34 - 119	51 - 118	
PLT (G/L)	Median	177	150	187	0.4
	Min-max	44 - 362	54 - 492	77 - 443	

We analysed the association of *rs4566136* polymorphism genotype with clinical parameters of HCC patients by comparing the clinical parameters such as AST, ALT, bilirubin, protein, albumin, prothrombin levels, RBC and PLT among

patients with different genotypes. The results showed that patients with genotype TT had higher AST, and ALT levels, followed by patients with genotype TC and CC. However, the difference was not significant. In contrast, patients with genotype TT had significantly lower albumin levels, followed by patients with genotype TC and CC. However, the difference was not also significant.

Table 5: Association of *rs4566136* SNP with development of HCC.

Characteristic		***rs4566136* genotypes**		
		TT	**TC**	**CC**
Child-Pugh	A	30 (78.9)	52 (85.2)	13 (81.3)
	B + C	8 (21.1)	9 (14.8)	3 (18.7)
	p	0.47	0.43	0.88
Barcelona	A +B	23 (60.5)	42 (68.9)	11 (68.8)
	C+ D	15 (39.5)	19 (31.1)	5 (31.2)
	p	0.38	0.51	0.81

We also classified HCC patients into subgroups with different development stages according to Child-Pugh and Barcelona criteria and compared genotypes of *ISG rs4566136* SNP among classified groups. The results showed that *ISG rs4566136* SNP was not significantly related to Child-Pugh and BCLC with $p > 0.05$.

DISCUSSION

Human genetic polymorphism regulates the susceptibility to a certain disease of individuals and several studies have shown the association of SNPs with cancers. *ISG20 rs4932196* SNP had been shown to play an important role in hearing loss syndrome in elderly individuals. However, to the best of our knowledge, no study has determined the role of *rs4566136* polymorphism in HCC disease. This is the first study showing an association of *rs4566136* polymorphism with the risk of HCC and liver cirrhosis in the Vietnamese population. *ISG20* has been shown to promote metastasis and angiogenesis via the IL-8/p-JAK2/p-STAT3 signaling pathway suggesting a pro-tumour role of *ISG20* [2, 7]. Our results additionally contribute to verifying the functional role of *ISG20* in the pathogenesis of HCC.

The risk of HCC of *rs4566136* polymorphism has been assessed by comparing the frequency of genotypes and alleles. Additionally, different genetic models such as dominant and recessive models and binary logistic regression adjusted for confounding factors (age and gender) have been used to determine the association of *ISG20 rs4566136* polymorphism with HCC and LC. When the LC group was used as a control group, the frequency of genotypes and dominant models were not significantly different. However, the frequency of alleles and recessive models were significantly different, with $p < 0.05$. Our result indicated that the *ISG20 rs4566136*C allele was associated with a decreased risk of HCC in liver cirrhosis patients. When HC group was used as a control group, the frequency of genotypes, alleles, and dominant recessive models were significantly different. Our result showed that *ISG20 rs4566136TC* and CC genotypes, *rs4566136C* allele was associated with a decreased risk of HCC in healthy individuals.

In the HCC group, the TT genotype had higher liver enzyme levels. However, the differences were not statistically significant. Albumin plays an important role in liver function when the liver function is decreased, the non-branding amino acids are not synthesized therefore leading to the fact that albumin levels in the blood are decreased. This could lead to a decrease in intravascular colloid pressure, and that was the mechanism for interpreting the clinical symptoms of liver cirrhosis in HCC patients. This study has shown that individuals with TT genotypes had lower albumin concentrations compared to those with TC and CC genotypes. However, the difference was not statistically significant. In this study, we did not observe the association between the genotype of *rs4566136* polymorphism with liver cirrhosis according to Child-Pugh scores and disease stages according to the Barcelona classification.

In conclusion, allele C, genotype TC, and CC are associated with a decreased risk of HBV-related HCC. *ISG20 rs4566136* SNP could be considered as a marker and need further studies to clarify the functional role in the pathogenesis and prognosis of HBV-related HCC.

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