STUDY OF rs1059513 *STAT6* POLYMORPHISM IN HBsAg(+) HEPATOCELLULAR CARCINOMA PATIENTS

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Abstract

Objectives: To determine the genotype frequency of rs1059513 *STAT6* polymorphism and its correlation with clinical, paraclinical characteristics, and cancer risk in Hepatitis B Surface Antigen positive (HBsAg (+)) hepatocellular carcinoma (HCC) patients. ***Methods:*** A cross-sectional study was conducted on 118 HBsAg-positive HCC patients, compared with 86 HBsAg-positive cirrhosis patients and 195 healthy individuals at Military Central Hospital 108, Military Hospital 103, and Can Tho City General Hospital from July 2017 to August 2020. The rs1059513 polymorphism in *STAT6* was analyzed from peripheral blood samples of the study subjects using Sanger sequencing. ***Results:*** The genotype frequency of GG in the rs1059513 *STAT6* polymorphism was lowest in cirrhosis patients (10.2%), but significantly higher compared to the corresponding values in HCC patients and healthy individuals (1.2% and 0%, respectively), $p < 0.05$. Individuals with the GG genotype had a higher risk of HBsAg-positive HCC compared to those with the AA genotype in both the cirrhosis group (OR = 11.54, 95%CI: 1.47 - 90.96, $p < 0.01$) and the healthy group (OR = 50.47, 95%CI: 2.95 - 863.43, $p < 0.01$). There was no correlation between *Rs1059513 STAT6* polymorphism and age, serum AFP (Alpha fetoprotein) concentration, and some tumor characteristics. ***Conclusion:*** The GG genotype of rs1059513 *STAT6* polymorphism increases the risk of HBsAg-positive HCC but is not associated with serum AFP concentration and some tumor characteristics.

Keywords: rs1059513 *STAT6* polymorphism; Hepatocellular carcinoma; Cirrhosis.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide and ranks first in both incidence and mortality rates in Vietnam [1]. The disease has many risk factors, among which hepatitis B virus (HBV) infection is the most important risk factor, presenting in around 80 - 90% of HCC cases [1, 2]. The pathogenesis of HBV-induced HCC is very complex, involving the integration of viral genetic material into the hepatocyte nucleus and the induction of hepatocyte damage through various signalling pathways, such as Wnt/β-catenin, PI3K/Akt/mTOR, Ras/Raf/MAPK, JAK/STAT, and other genes [3]. Among these signalling pathways, JAK/STAT (Janus kinase/Signal transducer and activator of transcription) plays a crucial role in transmitting signals from many cytokines and growth factors, responsible for cellular functions such as cell growth, maintenance of stem cells, cell differentiation, as well as regulation of immune response, inflammation, and has been shown to play an important role in the formation, progression, and development of HCC [4]. Among the 7 members of the *STAT* family, several studies have shown that *STAT1*, *STAT2*, and *STAT4* exhibit inhibitory effects on cancer formation (including HCC) both in vitro and in vivo, while *STAT3* has been demonstrated as an oncogenic factor, promoting angiogenesis, maintaining cancer stem cell population, thereby promoting the development, invasion, and metastasis of HCC [4]. Recently, a study has reported that *STAT6* expression is higher in HCC tissue than in non-tumor liver tissue and has prognostic significance [5]. However, research on rs1059513 *STAT6* polymorphisms in HCC patients has not been widely reported worldwide, although more than 10 polymorphic sites have been identified, including rs1059513 *STAT6* polymorphism, which has been associated with the treatment efficacy of the hepatitis C virus [6] and demyelinating neuropathy [7].

Currently, research on the association between rs1059513 *STAT6* polymorphism and HBV-induced HCC has not been reported worldwide or in Vietnam. Therefore, our study was conducted: *To determine the frequency of rs1059513 STAT6 polymorphism and its association with some clinical, paraclinical, and cancer risk factors in patients with HBsAg-positive HCC.*

MATERIALS AND METHODS

1. Subjects

The study was conducted on 399 participants, divided into 3 groups: Hepatocellular carcinoma patients' group, cirrhosis patients' group, and healthy group

* *Inclusion criteria:* Hepatocellular carcinoma patients' group (study group): Including 118 patients diagnosed with hepatocellular carcinoma according to the standard of the Vietnamese Ministry of Health in 2012 and tested positive for HBsAg (+) [8]; Cirrhosis patients' group: 86 patients diagnosed with cirrhosis based on clinical symptoms and tests with portal hypertension syndrome, liver dysfunction syndrome, changes in liver morphology, or cirrhosis degree F4, while testing positive for HbsAg; Healthy group: 195 volunteer blood donors without clinical symptoms or history of hepatitis, cirrhosis or liver cancer, with HBsAg, Anti-HCV, and Anti-HIV negative.

* *Exclusion criteria:* Patients with anti-HCV (+), anti-HIV (+), concomitant cancer, or without testing for the rs1059513 *STAT6* polymorphic gene were not included in the study; Exclusion criteria for cirrhosis patients included HCV infection, alcohol abuse, and use of hepatotoxic drugs, etc.

* *Study location:* The hepatocellular carcinoma and cirrhosis groups were collected at Military Hospital 103, 108 Military Central Hospital, and Can Tho City General Hospital. The healthy group was collected at the Hematology-Transfusion Center, Military Hospital 103.

* *Study period:* from July 2017 to August 2020.

2. Methods

* *Study design:* The study was conducted using a cross-sectional descriptive method.

* *Research process:*

All eligible patients were carefully examined for medical history, clinical symptoms, and laboratory tests for disease diagnosis (including serum AFP levels (ng/mL), tumor characteristics such as quantity, size, and vascular proliferation), disease stage (portal vein invasion (PVI) and extrahepatic metastasis).

The analysis of the polymorphism rs1059513 on *STAT6* was performed on peripheral blood samples of the study subjects following these steps:

- Step 1: Total DNA was extracted from peripheral blood using the GenJET Whole Blood Genomic DNA Purification Mini Kit (Thermo, USA) following the manufacturer's instructions.

The total DNA product was quantified, and purity was checked by measuring absorbance at 260 nm and 280 nm using a Nanodrop spectrophotometer.

- Step 2: The target gene segment was amplified by the Polymerase chain reaction (PCR) technique using the following primer pair:

GCACACTTGCTGCTGTCTTC (forward)

CTGCTCTGGACACTTGCTCA (reverse)

The PCR product was electrophoresed to check the specificity of the primers.

- Step 3: The PCR product was purified using the Gene JET PCR Purification Kit (Thermo, USA) according to the manufacturer's instructions.

- Step 4: 20 µL of the purified PCR product was sent to Apical Scientific Sequencing Company, Malaysia, for Sanger sequencing. The sequencing result was analyzed using Geneious software to compare with the standard Genbank in humans, thereby determining the polymorphism at rs1059513 on STAT6.

* *Data processing and analysis*: Using SPSS 20.0 software. Statistical analysis was carried out using frequency, percentage, mean, and comparison of categorical variables using the χ^2 test or Fisher's exact test. The percentage was rounded to one decimal place. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Table 1. Characteristics of age and gender of the study participants.

Participants	n	Male	Female	Male-to-female ratio	$\bar{X}$ ± SD
Hepatocellular carcinoma	118	104	14	7.4	65.5 ± 11.1
Cirrhosis	86	62	24	2.6	59.5 ± 10.5
Healthy	195	118	77	1.5	19.5 ± 1.2
Total	399	284	115	2.5	41.7 ± 23.2

The hepatocellular carcinoma patients had a mean age of 65.5, higher than the mean age of the cirrhosis group (59.5) and the healthy group (19.5). 88.1% of hepatocellular carcinoma patients were male, with a male-to-female ratio of 7.4, compared to a ratio of 2.6 in the cirrhosis group and 1.5 in the healthy group.

Table 2. Genotype and allele frequencies of rs1059513 *STAT6* polymorphism in hepatocellular carcinoma patients.

Genotypes and alleles		Frequency	Percent (%)
rs1059513 *STAT6* polymorphism	*Genotypes* (n = 118)		
	AA	79	66.9
	AG	27	22.9
	GG	12	10.2
	Alleles (2n = 236)		
	A	185	78.4
	G	51	21.6

In patients with hepatocellular carcinoma, the homozygous AA genotype of the rs1059513 *STAT6* polymorphism had the highest frequency (66.9%), while the homozygous GG genotype had the lowest frequency (10.2%). The A allele frequency was 78.4%, while the G allele frequency was only 21.6%.

Table 3. Comparing the genotype and allele distribution of the rs1059513 *STAT6* polymorphism between the hepatocellular carcinoma group and the healthy control group.

Genotypes and alleles		Hepatocellular carcinoma		Healthy control group		p
		n = 118	%	n = 195	%	
rs1059513 *STAT6* polymorphism	*Genotypes* (n)					
	AA	79	66.9	160	82.1	< 0.01
	AG	27	22.9	35	17.9	0.36
	GG	12	10.2	0	0	< 0.01
	Alleles (2n)					
	A	185	78.4	355	910	
	G	51	21.6	35	9.0	< 0.01

The homozygous GG genotype and G allele of the rs1059513 *STAT6* polymorphism were found to have a higher frequency in the hepatocellular

carcinoma group than in the healthy group (10.2% vs. 0% and 21.6% vs. 9.0%, respectively), with statistical significance at $p < 0.01$.

Table 4. Comparing the distribution of genotypes and alleles of the rs1059513 *STAT6* polymorphism in the hepatocellular carcinoma group and the cirrhosis group.

Genotypes and alleles		Hepatocellular carcinoma		Cirrhosis		p
		n = 118	%	n = 86	%	
		Genotypes (n)				
rs1059513 *STAT6* polymorphism	AA	79	66.9	76	88.4	< 0.01
	AG	27	22.9	9	10.5	< 0.05
	GG	12	10.2	1	1.2	< 0.01
		Alleles (2n)				
	A	185	78.4	161	93.6	< 0.01
	G	51	21.6	11	6.4	

The homozygous GG genotype and G allele of rs1059513 *STAT6* were found to have a higher frequency in the hepatocellular carcinoma group than in the cirrhosis group (10.2% vs. 1.2% and 21.6% vs. 6.4%, respectively), with statistical significance at $p < 0.01$. Meanwhile, the AA genotype was found to be significantly lower in the carcinoma group than in the cirrhosis group (66.9% vs. 88.4%, $p < 0.01$).

Table 5. The relationship between the rs1059513 *STAT6* polymorphism and risk factors of hepatocellular carcinoma (with a healthy control group).

Genotypes and alleles		Hepatocellular carcinoma (n = 118)	Healthy control group (n = 195)	OR (95%CI)	p
rs1059513 *STAT6* polymorphism	*Genotypes (n)*				
	AA	79	160	Ref	
	AG	27	35	1.56 (0.88 - 2.76)	0.12
	GG	12	0	50.47 (2.95 - 863.43)	< 0.01
	AG + GG (G-)	39	35	2.26 (1.33 - 3.83)	< 0.01
	Alleles (2n)				
	Allele A	185	355	2.80 (1.76 - 4.45)	< 0.01
	Allele G	51	35		

Individuals carrying the GG genotype and G allele of rs1059513 *STAT6* have a higher risk of hepatocellular carcinoma than those carrying the AA genotype and A allele, with corresponding odds ratios of 50.47 (95%CI: 2.95 - 863.43, $p < 0.01$) and 2.80 (95%CI: 1.76 - 3.83, $p < 0.01$), respectively. Additionally, the AG + GG genotypes were found to increase the risk of hepatocellular carcinoma compared to the AA genotype, with an odds ratio of 2.26 (95%CI: 1.33 - 3.83, $p < 0.01$).

Table 6. The relationship between rs1059513 *STAT6* polymorphism and risk factors of hepatocellular carcinoma (with a cirrhosis control group).

Genotypes and alleles		Hepatocellular carcinoma (n = 118)	Cirrhosis (n = 86)	OR (95%CI)	p
		Genotypes (n)			
rs1059513 *STAT6* polymorphism	AA	79	76	Ref	
	AG	27	9	2.89 (1.27 - 6.54)	< 0.05
	GG	12	1	11.54 (1.47 - 90.96)	< 0.05
	AG + GG (G-)	39	10	3.75 (1.75 - 8.0)	< 0.01
		Alleles (2n)			
	Allele A	185	161	4.03 (2.03 - 8.0)	< 0.01
	Allele G	51	11		

Individuals carrying the AG, GG, and G allele of rs1059513 *STAT6* were found to have a higher risk of hepatocellular carcinoma than those carrying the AA genotype and A allele, with corresponding odds ratios of 2.89 (95%CI: 1.27 - 6.54, $p < 0.05$), 11.54 (95%CI: 1.47 - 90.96, $p < 0.01$), and 4.03 (95%CI: 2.03 - 8.0, $p < 0.01$), respectively.

Table 7. The relationship between the rs1059513 *STAT6* polymorphism and some clinical, subclinical symptoms in hepatocellular carcinoma patients.

Clinical and subclinical symptoms		AA	AG	GG	p
Age		65.2 ± 10.6	64.4 ± 12.2	69.8 ± 12.1	0.35
Alpha-fetoprotein (AFP)	< 400 ng/mL	40 (50.6)	14 (51.9)	7 (58.3)	0.88
	≥ 400 ng/mL	39 (49.4)	13 (48.1)	5 (41.7)	
Number of tumors	1	29 (36.7)	9 (33.3)	6 (50.0)	0.60
	≥ 2	50 (63.3)	18 (66.7)	6 (50.0)	
Tumor size	< 5 cm	37 (46.8)	11 (40.7)	5 (41.7)	0.84
	≥ 5cm	42 (53.2)	16 (59.3)	7 (58.3)	
Portal vein thrombosis	No	65 (82.3)	24 (88.9)	9 (75.0)	0.54
	Yes	14 (17.7)	3 (11.1)	3 (25.0)	
Metastasis	No	72 (91.1)	22 (81.5)	9 (75.0)	0.17
	Yes	7 (8.9)	5 (18.5)	3 (25.0)	

There was no significant difference in age, serum AFP concentration and some characteristics of the tumor (number, size, presence of portal vein thrombosis and metastasis) among different genotypes of the rs1059513 *STAT6* polymorphism with $p > 0.05$.

DISCUSSION

The study was conducted on 118 inpatient hepatocellular carcinoma patients at three hospitals, including Military Central Hospital 108, Military Hospital 103, and Can Tho Central General Hospital, with an average age of 65.5 ± 11.1. Our study results are relatively consistent with those of many studies in Vietnam, such as the study by Phan Thi Hien Luong (2020) on 102 HBV-infected hepatocellular carcinoma patients at Bach Mai Hospital to determine the TNF-α-308 and TGF-β1-509 gene polymorphisms, with an average age of 57.4 ± 9.7 [9]. Therefore, hepatocellular carcinoma in our country is usually detected in middle-aged people because our country is in an area of hepatitis B epidemiology with a high transmission rate from mother to child during birth, while in European countries, the main causes are alcohol and hepatitis C virus infection. Most authors worldwide acknowledge that the age of hepatocellular carcinoma disease depends on many factors such as gender and hepatitis virus infection status, and varies by region [1, 2].

88.1% of hepatocellular carcinoma patients in our study were male, while only 11.9% were female, with a male-to-female ratio of 7.4/1.0. Thus, this male-to-female ratio is higher than the results of many previous studies (ranging from 2 - 8/1, with an average of 4/1) but is consistent with recent studies such as Phan Thi Hien Luong's study (2020), which had a male-to-female ratio of 11.8/1.0 [9]. The higher male-to-female ratio in hepatocellular carcinoma may be due to men having more exposure to risk factors such as alcohol abuse, smoking, body mass index, iron reserves, and especially a higher rate of hepatitis B and C virus infection than women. In addition, the relationship between sex hormones and the development of hepatocellular carcinoma has been confirmed in many studies, in which testosterone stimulates the development of hepatocellular and accelerates the formation of hepatocellular carcinoma. In contrast, estrogen inhibits cell cycle regulation and inhibits inflammation through Interleukin 6, thereby reducing liver damage and limiting the development of liver cancer [2, 3].

Hepatocellular carcinoma (HCC) remains a highly malignant disease with a complex pathogenesis, in which the JAK/STAT signalling pathway has been widely reported to play an important role in the formation, progression, and development of HCC.

Among the members of the STAT family, we observed a lack of research

on the rs1059513 *STAT6* polymorphism in patients with HCC. Most published studies on this polymorphism are related to patients with an allergic predisposition (such as asthma, eczema, etc.) [10], with a few studies reporting its association with the efficacy of treatment for hepatitis C virus [6] and multiple sclerosis [7].

Among 195 healthy individuals without liver disease, the genotype distribution of the rs1059513 *STAT6* polymorphism was as follows: The homozygous AA genotype had the highest frequency at 66.9%, while the homozygous GG genotype had the lowest frequency at 10.2%. The A allele frequency was 78.4%, while the G allele frequency was only 21.6%.

Our study results were similar to those of Ruan Z. et al. (2011) on 693 healthy Chinese individuals, which also reported the AA genotype of the rs1059513 *STAT6* polymorphism to have the highest frequency at 86.9% and the GG genotype to have the lowest frequency at 0.1% [7]. In a study by Duetsch G. et al. (2002) on 474 white individuals, the A allele frequency of the rs1059513 *STAT6* polymorphism was found to be 92.08%, while the G allele frequency was relatively low at 7.92% [10].

Comparing the frequency of genotypes and alleles of the rs1059513 polymorphism of the *STAT6* between the group of patients with hepatocellular carcinoma (HCC) and healthy individuals, we found that the GG homozygous genotype and G allele of rs1059513 *STAT6* polymorphism were more prevalent in the HCC group than in the healthy group (10.2% vs. 0% and 21.6% vs. 9.0%, respectively, $p < 0.01$). Individuals carrying the GG genotype and G allele had a higher risk of HCC than those carrying the AA genotype and A allele, with respective odds ratios (OR) of 50.47 (95%CI: 2.95 - 863.43, $p < 0.01$) and 2.80 (95%CI: 1.76 - 3.83, $p < 0.01$). Moreover, individuals carrying the G allele had a higher risk of HCC than those carrying the A allele, with an OR of 2.80 (95%CI: 1.76 - 4.45, $p < 0.01$) *(Table 5)*. Similarly, the GG homozygous genotype and G allele of rs1059513 *STAT6* polymorphism were more prevalent in the HCC group than in the cirrhosis group (10.2% vs. 1.2% and 21.6% vs. 6.4%, respectively, $p < 0.01$), and individuals carrying the AG and GG genotypes and G allele had a higher risk of HCC than those carrying the AA genotype and A allele, with respective OR of 2.89 (95%CI: 1.27 - 6.54, $p < 0.05$), 11.54 (95%CI: 1.47 - 90.96, $p < 0.01$), and 4.03 (95%CI: 2.03 - 8.0, $p < 0.01$) *(Table 6)*. However, we did not find any

association between the rs1059513 *STAT6* polymorphism and clinical or laboratory symptoms in HCC patients.

Currently, research on *STAT6* polymorphism in HCC patients is limited worldwide and has not been conducted in Vietnam. Some experimental studies have shown that *STAT6* inactivation significantly inhibits the survival and migration of two cell lines derived from HCC (HepG2 and Hep3B) and induces programmed cell death [11]. More recently, Kamerkar et al. (2021) used exoASO-*STAT6* (a targeted oligonucleotide-loaded extracellular vesicle) on a HCC cell model and found a significant reduction in tumor growth and complete regression in 50% of cases [12]. In humans, Dong et al. (2019) evaluated the mRNA expression of *STAT6* in HCC tissue and found that tumors with high *STAT6* mRNA expression had a better prognosis and a longer median overall survival time than those with low *STAT6* expression [5].

The relationship between the rs1059513 *STAT6* polymorphism and cancer pathology in general and hepatocellular carcinoma (HCC) in particular needs to be further investigated in order to reach a final conclusion and apply this marker in clinical practice.

CONCLUSION

Based on the study of the rs1059513 *STAT6* polymorphism in 118 HBsAg (+) HCC patients compared to the corresponding polymorphism in 86 cirrhosis patients and 195 healthy individuals, we draw the following conclusions:

- The frequency of the GG genotype at rs1059513 on *STAT6* is lowest in hepatocellular carcinoma patients (10.2%), but higher and statistically significant compared to the corresponding index in cirrhosis patients and healthy individuals (1.2% and 0%, respectively; $p < 0.05$). Individuals with the GG genotype have a higher risk of HCC than those with the AA genotype in the cirrhosis group (OR = 11.54, 95% CI: 1.47 - 90.96, $p < 0.01$) and the healthy group (OR = 50.47, 95%CI: 2.95 - 863.43, $p < 0.01$).

- There is no association between the rs1059513 *STAT6* polymorphism and age, serum AFP levels, and some tumor characteristics in HCC patients.

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