STUDY ON *STAT6* rs324015 POLYMORPHISM IN HEPATITIS B VIRUS-RELATED HEPATOCELLULAR CARCINOMA PATIENTS

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Abstract

Objectives: To determine the genotype frequency of the *STAT6* rs324015 polymorphism and its association with cancer risk in hepatitis B virus (HBV) - related hepatocellular carcinoma (HCC) patients. ***Methods:*** A cross-sectional descriptive study on 118 HBV-related HCC patients, compared to 86 HBV-related cirrhosis patients and 195 healthy people at 108 Military Central Hospital, Military Hospital 103, and Can Tho General Hospital from July 2017 to August 2020. Polymorphism analysis of the *STAT6* rs324015 gene was conducted from peripheral blood samples of subjects using the Sanger sequencing method at Apical Scientific Sequencing Company (Malaysia). ***Results:*** The AG/GA heterozygous genotype of the *STAT6* rs324015 polymorphism accounted for the highest rate in HCC patients at 51.7%, higher than the corresponding index in the cirrhosis group at 39.5%, and healthy people (48.2%); the GG genotype was the highest in cirrhosis patients (40.7%), the difference was not statistically significant, $p > 0.05$. ***Conclusion:*** *STAT6* rs324015 polymorphism is not associated with cancer risk in patients with HBV-related HCC.

Keywords: *STAT6* gene polymorphism; Hepatocellular carcinoma (HCC).

INTRODUCTION

Hepatocellular carcinoma is the most common type of cancer, with the 6th highest prevalence and the 3rd highest mortality rate, following lung cancer and colorectal cancer [1]. There are several causes of HCC, of which HBV is the main one, accounting for over 50% of HCC cases. However, HBV infection can be asymptomatic or

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cause acute hepatitis, chronic hepatitis, cirrhosis, etc, and partly be able to form HCC [2]. The pathophysiological mechanism of these clinical disease manifestations is still controversial, depending closely on HBV characteristics (genotype, gene mutations, replication level, etc.) as well as the interaction between HBV and other pathogens as related factors of the host body (genes and signaling pathways).

Notably, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is a signal transduction pathway of many cytokines and growth factors, responsible for cellular functions such as cell proliferation, stem cell maintenance, cell differentiation, as well as regulation of immune and inflammatory responses, and therefore this signaling pathway is closely related to many infectious diseases and cancers including HCC [3, 4]. Among the seven members of the STAT family, *STAT1, STAT2*, and *STAT4* exhibit inhibitory effects on HCC formation both in vitro and in vivo; on the contrary, *STAT3* is a carcinogenic agent, stimulating angiogenesis, maintaining cancer stem cell populations, thereby promoting the development, invasion and metastasis of HCC, while *STAT5* (including *STAT5a* and *STAT5b*) has a higher expression level in HCC tissue than in non-tumor liver tissue and has significance in disease prognosis [4, 5].

The *STAT6* gene is located on the long arm of chromosome 12, includes 23 exons and more than 10 gene polymorphisms have been identified, of which the polymorphisms at exon 23, such as rs324015, rs703817, etc, are the most studied because exon 23 is the transcriptional activation region and contains important information that affects the structure and function of STAT6 protein [6, 7]. However, new studies only focus on their role in some allergy-related diseases because the *STAT6* gene plays a role in the IL4/IL13/*STAT6* signaling pathway, activating mastocytes and increasing IgE production [7]; study on cancer patients is still limited, especially there has been no study on the *STAT6* rs324015 polymorphism in relation to HBV-infected HCC. Therefore, we conducted the study: *To determine the genotype rate of STAT6 rs324015 polymorphism and its relationship with cancer risk in patients with HBV-related HCC.*

MATERIALS AND METHODS

1. Subjects

The study was conducted on 3 groups of patients:

- Group of patients with HCC: 118 patients were diagnosed with HCC

according to the standards of the Vietnam Ministry of Health in 2012 and HBsAg (+) tested [8].

Excluded criteria: Patients with HCC who had anti-HCV (+), anti-HIV (+) tests; had combined cancer; did not have a test to determine the *STAT6* rs324015 polymorphism; cirrhotic patients with alcohol abuse; use of hepatotoxic drugs, etc.

- Cirrhosis group: 86 patients diagnosed with cirrhosis based on clinical and laboratory tests with portal hypertension syndrome, liver failure and changes in liver morphology or F4 fibrosis, and HBsAg (+).

The group of HCC patients and cirrhosis was selected at Military Hospital 103, 108 Military Central Hospital, and Can Tho City General Hospital.

- Healthy group: 195 blood volunteers at the Center for Hematology - Blood Transfusion, Military Hospital 103, with no clinical symptoms or history of hepatitis, cirrhosis, or liver cancer. HBsAg, Ani-HCV and Anti-HIV tests were negative

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

2. Methods

* *Research design:* A cross-sectional descriptive study.

All patients eligible for the study were carefully examined for medical history, clinical symptoms, and tests to confirm the diagnosis and stage of the disease.

Analyze the rs324015 polymorphism on *STAT6* from peripheral blood samples of subjects at the Biosafety department of the Institute of Biomedicine and Pharmacy, Vietnam Military Medical University according to the following steps:

- Step 1: Separate the whole DNA from peripheral blood using GenJET Whole Blood Genomic DNA Purification Mini Kit (Thermo; USA), following the manufacturer's instructions. The whole DNA product was measured for concentration and checked for purity by measuring optical absorbance at wavelengths of 260nm and 280nm on a Nanodrop machine.

- Step 2: Amplify the target gene segment. Use PCR (Polymerase chain reaction) technique to amplify the *STAT6* gene segment with the following primer pair:

GCACACTTGCTGCTGCTTC (forward)

CTGCTCTGGACACTTGCTCA (reverse)

Electrophoresis of PCR products to check primer specificity.

- Step 3: PCR products will be purified using GeneJET PCR Purification Kit (Thermo; USA), following the manufacturer's instructions.

- Step 4: Take 20µL of purified PCR product and sequence it using the Sanger method at Apical Scientific Sequencing Company, Malaysia. Sequencing results were then entered into Geneious software to compare with the human standard Genbank, thereby identifying the rs324015 polymorphism on *STAT6*.

* *Data processing and analysing*: Using SPSS 20.0 medical statistical software. Statistical analysis by calculating percentages and average values, comparing proportions using the $\chi 2$ test or Fisher exact test. Calculated percentage values are taken 1 digit after the decimal number. The difference is considered statistically significant when $p < 0.05$.

3. Ethics

The study was reviewed and approved by the Ethical Committee, Vietnam Military Medical University. The study complied with the Declaration of Helsinki principles. All study participants or their legal guardians provided informed written consent before study enrollment. The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy and ethics.

RESULTS

1. Age and gender characteristics of the study group

Table 1. Age and gender characteristics of the study group.

Subjects	n	Male	Female	Male/female ratio	Mean ± SD
HCC	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

HCC patients had an average age of 65.5 years, higher than the average age of the cirrhosis group of 59.5 and the healthy group of 19.5.

88.1% of HCC patients were male, and the male/female ratio was 7.4. This ratio in the cirrhosis group was 2.6, and in the healthy group was 1.5.

2. Gene and allele distribution of *STAT6* rs324015 polymorphism

Table 2. Genotype and allele ratio of *STAT6* rs324015 polymorphism in patients with HCC.

Genotype and allele	Number	Percentage (%)
Genotype (n = 118)		
AA	25	21.2
AG/GA	61	51.7
GG	32	27.1
Allele (2n = 236)		
A	111	47.0
G	125	53.0

The majority of HCC patients had the heterozygous AG/GA genotype (51.7%), and the homozygous AA and GG genotypes had a low frequency (21.1% and 27.1%, respectively).

The rate of G allele of *STAT6* rs324015 polymorphism in the HCC group was higher than the rate of A allele (53.0% vs. 47.0%).

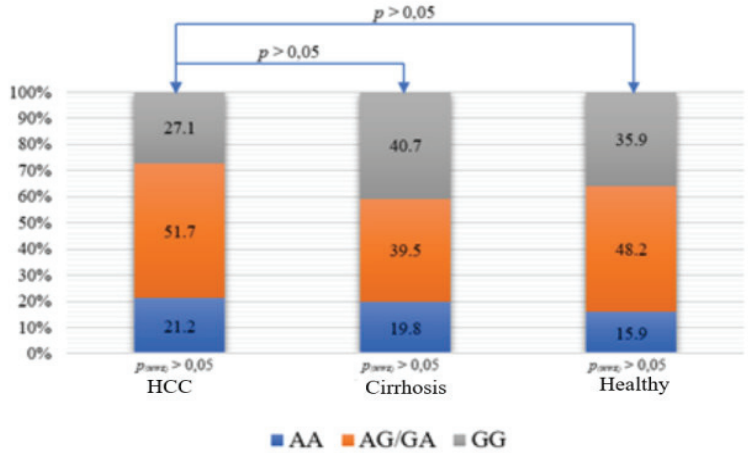

Chart 1. Comparison of genotype distribution of *STAT6* rs324015 polymorphism in HCC, cirrhosis, and healthy people.

The genotype distribution of *STAT6* rs324015 polymorphism in HCC, cirrhosis, and healthy groups all comply with Hardy Weinberg's *equilibrium*, p > 0.05.

The genotype ratio of the *STAT6* rs324015 polymorphism between the HCC group, cirrhosis, and the healthy group was not statistically different, p > 0.05.

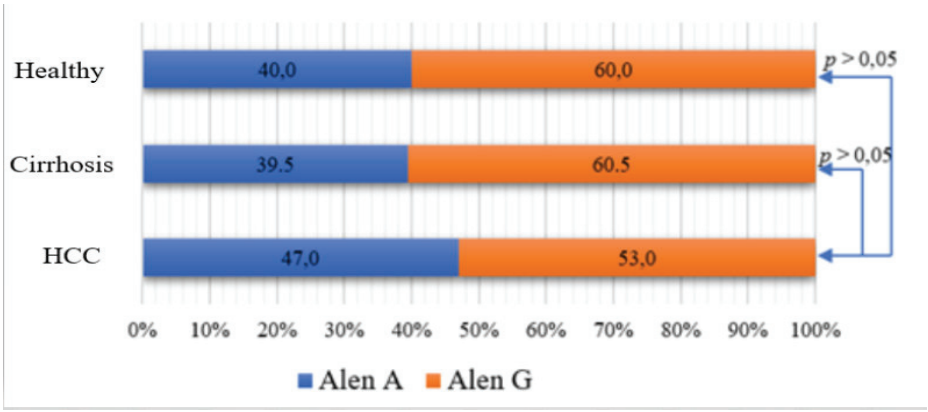

Chart 2. Comparison of allele distribution of *STAT6* rs324015 polymorphism in HCC, cirrhosis, and healthy people.

The allele ratio of *STAT6* rs324015 polymorphism between HCC, cirrhosis, and healthy groups was not statistically different, p > 0.05.

DISCUSSION

1. Patient characteristics by age and gender

* *Age:* The average age of the 118 HCC patients in our study was 65.5 ± 11.1, consistent with the results of domestic study by Phan Thi Hien Luong (2020) on 102 HCC patients with HBV infection at Bach Mai Hospital with the average age of 57.4 ± 9.7 [9]. Thus, in Vietnam, HCC is often detected in middle-aged people because the country is located in an HBV endemic area, with a high rate of mother-to-child infection, while in European countries, the main cause of HCC is alcohol and hepatitis C virus infection. Most authors around the world have noted that the age of HCC depends on many factors such as gender, hepatitis virus infection status, and the difference from region to region [1, 2].

* *Gender:* 88.1% of HCC patients in our study were male, 11.9% were female; the male/female ratio was 7.4/1. This ratio is higher than the results of many previous studies (only ranging from 2 - 8/1, with an average of 4/1), but consistent with the results of Phan Thi Hien Luong (2020), which has a male/female ratio of 11.8/1 [9]. The reason why HCC is more common in men than in women may be because

men are more exposed to risk factors such as alcohol abuse, smoking, and high body mass index, and especially the rate of HBV and HCV infection is higher than in women. In addition, the relationship between sex hormones and the occurrence and progression of HCC has also been confirmed. Testosterone is a factor that plays a role in regulating the liver cell cycle, promoting the signal transduction pathway through the androgen receptor, stimulating the growth of liver cells, and thereby accelerating the pathogenesis of HCC. In contrast, the female sex hormone (oestrogen) inhibits cell cycle regulation and inflammation through Interleukin 6, thereby reducing liver damage and limiting the development of liver cancer [2, 4].

2. Gene and allele distribution of *STAT6* rs324015 polymorphism

HCC is still a malignant disease with a very complex pathogenesis with the impact of many risk factors and the participation of many signaling pathways such as Wnt/β-catenin, PI3K/Akt/ mTOR, Ras/Raf/MAPK, JAK/STAT, and many other genes involved. The JAK/STAT (Janus kinase/Signal transducer and activator of transcription) signaling pathway has been recognized by many studies to play an important role in the formation, emergence, and progression of HCC.

Among members of the STAT family, we found that very few studies have been conducted on *STAT6* polymorphisms in HCC patients (especially polymorphisms at position rs324015).

Our study found that on 195 healthy people, the rate of AG/GA heterozygous genotype of the *STAT6* rs324015 polymorphism (4219 G>A) was the highest at 48.2%, followed by the AG/GA genotype. Homozygous gene GG accounted for 35.9%, and the lowest was genotype AA accounting for 15.9%. The ratio of the G allele was more dominant than the A allele (60.0% vs. 40.0%, respectively) (*Chart 1, 2*).

Results by Ruan Z et al. (2011) on 693 healthy Chinese people with an average age of 39.6 ± 18.3 (varying from 1 - 86 years old) showed similar results to ours. In our study, in the *STAT6* rs324015 polymorphism, the highest rate of genotype was AG (48.9%), while the rate of homozygous genotypes GG and AA was nearly equal at 24.3% and 26.9%, respectively [10]. Research by Duetsch G et al. (2002) analyzing gene polymorphisms on 449 subjects living in Germany and Sweden also showed that the rate of G allele of *STAT6* rs324015 gene

polymorphism was 76.57%, while the rate of allele A was lower at only 23.43% [6]. Thus, according to the above studies, the *STAT6* rs324015 polymorphism has a dominant ratio of the G allele and heterozygous AG genotype compared to the A allele and the remaining genotypes. However, a recently published study by Dai L et al. (2021), when directly analyzing the *STAT6* rs324015 polymorphism by qRT-PCR method on 355 healthy Chinese people, recorded that the AA genotype accounted for the highest proportion. The rate was 56.3%, higher than the remaining two genotypes AG (37.5%) and GG (6.2%); the A allele rate was up to 75.1% compared to the G allele rate, which only accounted for 24.9% [11]. Obviously, the allele and genotype rates of the *STAT6* rs324015 gene polymorphism still seem to largely depend on the human race, so further research is needed.

In the group of HCC patients with HBsAg (+), the results of *STAT6* rs324015 polymorphism analysis were similar to the healthy group: The heterozygous AG genotype had the highest rate of 51.7%, followed by the GG genotype at 27.1% and AA genotype at 21.2%. The G allele was still dominant at 53.0% compared to the A allele at 47.0%. In the group of cirrhotic patients, we noted a slight difference: The proportion of homozygous GG and heterozygous AG genotypes was almost equal (40.7% and 39.5%, respectively). However, we did not observe any difference in the distribution of genotypes and alleles of the *STAT6* rs324015 polymorphism between the 3 study groups. Thus, *STAT6* rs324015 polymorphism does not change the risk of HCC in HBV-infected patients.

Currently, we have not recorded any study published around the world on the relationship of the *STAT6* rs324015 polymorphism with HCC. Most of the studies on *STAT6* rs324015 polymorphism are on subjects with allergies, bronchial asthma, etc, because it is related to the IL4/IL13/*STAT6* signaling pathway that activates mastocytes and increases blood IgE production. A systematic review conducted by Qian X et al. (2014), when subgroup analysis by race (Caucasians and Asians) and age (children and adults), also failed to note an association with asthma. However, this review showed that the *STAT6* rs324015 polymorphism appeared to reduce the risk of allergic asthma with OR = 0.83, 0.68, and 0.79 for the A allele, AA genotype, and AA + AG genotype combination, respectively [12]. Recently, research by Dai L et al. (2021) noted that the *STAT6* rs324015 polymorphism increased the risk of

ulcerative colitis in both homozygous models, dominant models, and allele models. Subgroup analysis also showed a clear association between this polymorphism and alcohol consumption, cigarette smoking, young age < 40, as well as the severity and extent of disease [11].

In a group of cancer patients, we noted the study of Ruan Z et al. (2011) determining the relationship of *STAT6* polymorphisms with brain gliomas. The results show that the *STAT6* rs324015 polymorphism was not associated with the risk of glioma formation when analyzed in both homozygous models, dominant models, and recessive models, as well as when analyzed in subgroups according to smoking status, histopathological results, and disease stage according to WHO [9].

Thus, the relationship between the *STAT6* rs324015 polymorphism and cancer in general and HCC in particular needs to continue to be studied to reach a final conclusion to apply this marker in clinical practice.

CONCLUSION

Studying *STAT6* rs324015 polymorphism in 118 patients with HBV-related HCC, compared with the corresponding polymorphism expression in 86 HBV-related cirrhosis patients and 195 healthy people, we draw the conclusion as follows:

- The AG/GA genotype of the *STAT6* rs324015 polymorphism accounted for the highest proportion in HCC patients at 51.7%, higher than the corresponding index in the cirrhosis group of 39.5% and healthy people at 48.2%. While the GG genotype was the highest in cirrhosis patients (40.7%), the difference was not statistically significant, $p > 0.05$.

- *STAT6* rs324015 gene polymorphism was not associated with HCC risk when compared to the cirrhotic and healthy people groups.

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