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Original Research

Evaluation of STAT3 rs1053004 single nucleotide polymorphism in patients with chronic hepatitis B and hepatocellular carcinoma

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Abstract: Chronic infection with hepatitis B (CHB) virus is one of the most important risk factors for Hepatocellular carcinoma (HCC). Hepatitis B virus (HBV) causes liver cancer in various ways. One of these ways is increasing the expression of the signal transducer and activator of transcription 3 (STAT3) in Hepatocytes by HBV. The purpose of this research is to evaluate the single nucleotide polymorphism (SNP) rs1053004 in the STAT3 gene in CHB patients and individuals who suffer from HCC. In this research, 33 patients CHB-related HCC, 50 patients infected with chronic hepatitis B infection (CHB) without HCC and 50 healthy individuals were investigated for the presence of rs1053004 in the STAT3 gene according to the PCR-based differentiation of alleles test. Data analysis presented a different and significant distribution of alleles and genotypes ($p<0.05$). When the HCC and CHB groups were compared from the point of the frequency of alleles, the frequency of the C allele and CC genotype in the HCC group were higher CHB and control groups. Analysis of our data in the genotype model (CC vs. TT + TC) showed, this meaningful relationship remained between the HCC group and the three groups of CHB, healthy and all controls. These results illustrate that perhaps rs1053004 polymorphisms in the STAT3 gene participated in the progression of hepatitis B to HCC in Iranian people.

Key words: Hepatocellular carcinoma; Chronic hepatitis B; Single nucleotide polymorphism; STAT3.

Introduction

The liver cancer can be seen in two primary and secondary forms. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer which is different from the secondary liver cancer that occurs due to the malignant metastasis from other organs to the liver (1). About 90% of the primary liver cancers are of the type HCC. HCC is the third cause of death among other cancers. The highest incidence of HCC is found in the African Sahara and Southeast Asia and also, the incidence rate of this disease in men is higher than in women and the highest age group involved in this cancer is between 30 and 50 (2). Different factors play a role in developing HCC; the most important factors include infection with the hepatitis B or C virus, exposure to the Aflatoxin, hemochromatosis and alpha 1-antitrypsin deficiency. In addition to what is mentioned above, the host genetic factors can also play a role in the progression of the disease to HCC. One of these genetic factors is the STAT3 (the signal transducer and activator of transcription 3) polymorphisms (3). STAT proteins consist of seven organs, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (4). Among these seven organs, STAT3 is related to activating a number of intracellular signal transition pathways involved in tumor creation (5). The gene encoding the STAT3 protein is located on the chromosome number 17(6). In natural and normal cells, activation of STAT3 is carefully controlled to prevent the misplaced activations of the planned genes. Many of the activities of STAT3 have been proved in a large number of cancer cells and primary tumors, such as 50% of the cancers of hepatocellular, leukemia, lymphoma, multiple myeloma, the cancers of the prostate, breast, chest, lung and the cancers of head and neck (5). STAT3 is activated by means of connecting a number of ligands to their cell surface receptors, these ligands are included in EGF, IL6 and LIF, all of which are from the family of cytokines (7). Chronic hepatitis B (CHB) is known as one of the most important risk factors for HCC and approximately 25% of CHB cases lead to hepatocyte carcinoma(8). HBV (Hepatitis B virus) genome encodes a trans-activator protein which is called HBX. HBX protein has an important role in increasing the transcription of viral genes and host hepatocyte genes, by activating the STAT3 signal transduction pathway (9). HBX increases the phosphorylation of tyrosine connected to JAK (Janus kinase). JAK is an intracellular tyrosine kinase, which phosphorylated STAT3, as a result of extra cellular stimulation. Activating STAT3 increases the transcription of the nuclear genes of the infected cells and this function will ultimately result in HCC (10, 11). In one research, it has been shown that STAT3 activated

by HBX, is able to connect to the enhancer-1 part of the HBV virus genome and by following this connection, increasing expression of viral genes can be observed (12). Polymorphisms are not usually the cause of health or disease, however, they make a difference with regard to being infected with disease, the severity of disease and how the body responds to treatment. Many studies report, a wide range relationships with regard to diseases, such as the cancers of cervical, stomach and kidney with polymorphisms. Polymorphisms of the STAT3 gene cause functional and practical changes in target proteins. They also cause disruption in adjusting the gene expression (13, 14). STAT3 polymorphisms are capable of reacting with the pres2 start codon part in the mutation genome of the hepatitis B virus and as a result of this reaction, the activity of STAT3 will increase and the disease eventually progresses to HCC (15). These polymorphisms which are located in the 3'-UTR part of the STAT3 gene, have an important role in adjusting the expression of the STAT3 gene (16). In spite of the important role of the STAT3 protein in malignant development of the liver cells, limited information exists about the polymorphisms of this protein and how it correlates to becoming infected with the HCC disease. So far, no researches have been provided in Iran about the polymorphisms of STAT3 and its relationship with HCC. So the purpose of this research is to evaluate the single nucleotide polymorphism (SNP) rs1053004 in the STAT3 gene in CHB patients and individuals who suffer from HCC.

Materials and Methods

In this case-control study, totally 133 people were examined. For this research, paraffin-embedded blocks were analyzed from 33 CHB related HCC patients referring to Afzalipour Hospital in Kerman and Namazi Hospital in Shiraz during April 2008 to March 2016, blood samples from 50 CHB without HCC patients who referred to Afzalipour Hospital in Kerman during 2015-2016 and blood samples from 50 healthy people were examined. Patients with CHB must be positive for virus surface antigen for at least 6 months and their HBSAg should be confirmed by ELISA. The diagnosis of HCC was confirmed based on imaging or histological studies (17). Healthy people were selected from blood donors at Kerman Blood Transfusion Center. The healthy control group should be free from any indicators of the hepatitis B disease, not suffer from other infectious diseases and autoimmune diseases, and they should not be using any immunosuppressive drugs. People with positive HIV and HCV, pregnant women, people who had an infection or surgery during two weeks before the test were excluded from the study. In the patient groups, people with positive HIV and HCV were excluded from the studies. This project was approved by the research center ethics committee of the Kerman University of Medical Sciences.

DNA extraction

In order to analyze the viral load and STAT3 polymorphisms, DNA was extracted from blood samples and tissue samples. For two groups of healthy people and CHB patients, 2 to 3 cc of blood was collected from each person in tubes containing EDTA. After centrifuging the samples, their PBMCs were separated and lysed by Red Blood Cell Lysing Buffer. PBMCs samples were frozen in normal saline buffer at PH=7.4 and a temperature of -20°C until the next steps of the experiments were performed. Liver biopsy paraffin samples with 3 to 5 incisions, of patients with HCC were provided, the incisions were then deparaffinized by xylene. After heating them for 10 min at a temperature of 60°C, they were centrifuged for 1 min at 10,000 rpm and the superficial fluid was removed. This step was repeated three times. Afterwards, 500 µl of absolute ethanol was added and re-centrifuged for 1 min. Samples were dried with an open lid for 5 min at a temperature of 50°C. In the next step, 200-400 µl of the tissue lysing buffer was added to each micro tube [4 M Urea, 200 mMTris, 20 mMNaCl, 200 mM EDTA; PH=7.4 (25°C)]. Afterwards, 200-400 µl of protein kinase K was added and after slightly stirring, was cooled at 60°C for 1 min and incubated for one night at 37°C. The next day, 200µl of binding buffer [6 M Guanidine- Hcl, 10mM Urea, 10mM Tris-Hcl, 20% Tritonx-100 (v/v); PH=4.4 (25°C)] was added and the micro tubes were shaken slowly. DNA was extracted using RIBO-prep nucleic acid extraction kit (Slovak Republic), according to the manufacturer's instruction . The extracted DNA was stored in micro tubes containing 70µl of elution buffer at 70°C until the next steps of the experiments. For determining the viral load of RTA Viral DNA Isolation Kit (Cat No: 09006; RTA Laboratories, Turkey), according to the kit instructions and using the RT-PCR technique, the number of viral DNA copies in the pre-stored samples was analyzed.

Determining polymorphism genotypes

For analyzing the rs1053004 alleles (T/C) in the STAT3 gene, the Master Mix kit of the Amplicon Company and with the Taq Man-based RT-PCR technique was used. For testing, two types of primers and probes were designed(18). Each of the probes was considered for one allele and was marked with a specific color. Primers and probes were used for Real-Time PCR:
forward: ACAGGTGAGGCAGAACAGC and reverse: AGAGGCAGCCCATCCAG, also, probe-1: Fam-CTGGCTAGCTTGCCTCTCC-BHQ1 and probe-2: Joe-CTGGCTAGCTTGCCTCTCC-BHQ1.

The reactions were performed in a total volume of 25 µl. In short, 1 x of the magnesium-free PCR buffer, 0.2 mM of the dNTPs mixture, 1.5 mM of MgCl2, 0.2 unit of Taq polymerase DNA and 1x of the primer and probe mixture were used to determine the SNP genotypes.

Statistical analysis method

Considering the fact that the data in the control group and the patient group were obtained in quantitative-continuous form, after collection, the data were entered in the SPSS v 18. For the quantitative variables, the mean ± standard deviation was used and for the non-parametric variables, percentage was used. The comparisons between groups, Alleles and genotype frequencies were performed using the Chi-square test and odds ratio. In all tests of significance level, P value was considered less than 0.05.

Table 1. Patient Characteristics.

Characteristics	CHB related HCC (n=33)	CHB without HCC (n=50)	Healthy Controls (n=50)	P Values
Age, years, mean ±SD	42.38±13.83	38.04±13.99	34.86±15. 28	<0.001*,†,‡
Gender				
Male n(%)	28(84.8%)	33(66%)	32(64%)	<0.001*,†,‡
Female n(%)	5(15.2%)	17(34%)	18(36%)	
HBV DNA Level(log10 IU/ml), mean ±SD	421.06±168.67	275.90±171.08	ND	<0.05*

HCC: hepatocellular carcinoma, CHB: chronic hepatitis B, ND: no data. *Between HBV-related HCC and CHB without HCC. †Between HBV-related HCC and healthy controls. ‡Between HBV-related HCC and all of the controls (CHB without HCC and healthy controls).

Results

In this research, after analysis, 133 people were divided into three groups, including: 33 patients with CHB related HCC, 50 patients CHB without HCC and 50 healthy control individuals. A summary of demographic data of these people is briefly shown in Table 1. The samples were analyzed with the average age and standard deviation of 42.38±13.83 for the HCC group, 38.04±13.99 for the CHB group and 34.86±15.28 for the healthy group. Among the 133 people who were analyzed, 93(69.92%) were men and 40(30.07%) were women. The relationship between the gender in three groups was meaningful, so that the percentage of men in the HCC group was higher than that of the CHB group and the healthy control group (P<0.001). In addition, the data analysis represented that the age of people in the HCC group was higher than the other two groups (P<0.001). In this analysis, there was a meaningful statistical level between the viral load in the HCC and CHB groups (P<0.05). Furthermore, the viral load in the HCC patients was more than in patients with CHB (421.06±168.67 and 275.90±171.08 respectively) (Table1).

The frequency of alleles and the distribution of genotypes between the three groups of HCC, CHB and healthy controls were compared (Table 2). The frequency of the T allele in the HCC, CHB and healthy control groups was 37.87%, 62% and 78%, respectively. While the frequency of the C allele in these three groups was 62.13%, 38% and 22%, respectively. Therefore, T allele was considered as the major allele and C allele as the minor allele. When the HCC group was compared to the CHB group, the frequency of C allele was significantly higher in the HCC group (OR=2.85, 95% CI=0.59-1.70, P=0.019); we observed similar results in the additive model (OR=4.50, 95% CI=0.44-2.00. P=0.007) and the recessive model (OR=6.300, 95% CI=0.382.39, P=0.000). Additionally, the HCC group was compared to the healthy control group (OR=6.20, 95% CI=0.352.22, P=0.000). This relationship that remained as the additive model (OR=7.03, 95% CI=0.443.09, P=0.004), the dominant model (OR=4.08, 95% CI=0.421.73, P=0.003) and the recessive model (OR=0.72, 95% CI=0.243.32, P=0.000) were also analyzed. Data analysis indicated that C allele may be associated with the risk of being infected with HCC. When the HCC group was compared with all controls (including the CHB group and the healthy control group) (OR= 4.08 ,95% CI=0.35-1.45, P=0.001), we observed similar meaningful results in the additive model (OR=3.75, 95% CI=0.41-1.55, P=0.011), the dominant model (OR= 2.49, 95% CI=0.49-1.24, P=0.030) and the recessive model (OR=0.11, 95% CI=0.46-4.12, P=0.030) (Table2). According to these results, it is indicated that T allele is a protective allele against the progress of HCC and C allele is a progressive allele for being infected with HCC.

In Table 3, the relationship between age and rs10553004 has been evaluated. The subjects of study were divided into two groups of less than 45 and more than 45 years old. There was a significant relationship between people over 45 years old and rs10553004 (P=0.001). According to the data analysis, in the group of people who are more than 45 years old, C allele had the most frequency (70.83%). Consequently, in people who are more than 45 years old and have the CC genotype, the probability of progressing the chronic hepatitis B disease to carcinoma in them can be more than the others.

Discussion

Liver cancer has the highest percentage of death among other cancers (19). Hepatocellular carcinoma has the highest incidence in Asia and Africa. These areas are considered as the endemic areas from the point of infection with hepatitis B and hepatitis C viruses and the chronic hepatitis can sometimes lead to liver cancer (20). 80% of cases of HCC infection in the world are related to HBV virus infection (21). One of the factors which have a role in converting the chronic infection of hepatitis B into liver is to increase the expression of the

Table 3. Associations of STAT3 SNPrs1053004 with age and HCC Risk.

	STAT3 (rs1053004)	CHB related HCC (n=33)	CHB without HCC (n=50)	Healthy Controls (n=50)	P values
Age, number (%)					
45>					0.211
	TT	1 (3.70%)	12 (44.44%)	14 (51.85%)	
	CC	1 (16.66%)	3 (50.00%)	2 (33.33%)	
	TC	0 (0.00%)	13 (76.47%)	4 (23.52%)	
45<					0.000
	TT	9 (25.71%)	8 (22.85%)	18 (51.42%)	
	CC	17 (70.83%)	5 (20.83%)	2 (8.33%)	
	TC	5 (20.83%)	9 (37.50%)	10 (41.66%)	

Table 2. Genotype and Allele Frequencies of SNP rs1053004 in STAT3 Gene with HCC Risk.

STAT3 rs1053004	CHB related HCC (n=33)	CHB Without HCC (n=50)	Healthy controls (n=50)	HCC vs CHB		HCC vs. Healthy Controls		HCC vs. All Controls	
				OR (95% CI)	P Values	OR (95% CI)	P Values	OR (95% CI)	P Values
Allelic model									
Major (T)	25(37.87%)	62(62%)	78(78%)	1	-	1	-	1	-
Minor (C)	41(62.13%)	38(38%)	22(22%)	2.85 (0.59-1.70)	0.019	6.20 (0.352.22)	0.000	4.08 (0.35-1.45)	0.001
Additive model									
TT	10(30.30%)	20(40%)	32(64%)	1	-	1	-	1	-
TC	5(15.15%)	22(44%)	14(28%)	0.10 (0.34-3.37)	0.000	5.40 (0.301.62)	0.005	3.38 (0.35-1.12)	0.028
CC	18(54.55%)	8(16%)	4(8%)	4.50 (0.44-2.00)	0.007	7.03 (0.443.09)	0.004	3.75 (0.41-1.55)	0.011
Dominant model									
TT	10(30.30%)	20(40%)	32(64%)	1	-	1	-	1	-
TC+CC	23(69.70%)	30(60%)	18(36%)	1.53 (0.76-1.17)	0.368	4.08 (0.421.73)	0.003	2.49 (0.49-1.24)	0.03
Recessive model									
TT+TC	18(54.54%)	8(16%)	4(8%)	1	-	1	-	1	-
CC	15(45.46%)	42(84%)	46(92%)	6.300(0.38-2.39)	0.000	0.72 (0.243.32)	0.000	0.11(0.46-4.12)	0.03

HCC: hepatocellular carcinoma, CHB: chronic hepatitis B, CI: confidence interval, OR: odds ratio.

STAT3 protein by the HBV virus, and the activation of STAT3 in hepatocytes is an essential factor in developing tumor in the liver (22, 23). Improper activity and increase of STAT3 can be seen in many malignancies, including HCC (24). On the other hand, polymorphisms located at the 3'-UTR of the STAT3 protein have an adjusting role in the expression of this protein and in many cases, these polymorphisms can increase the activity of the JAK/STAT intracellular signal pathway, which is related to increasing the expression of the cell genes and finally, the progress of the cancer (25). In spite of the important role of the STAT3 protein and its polymorphisms in providing HCC, scarce information exists about this protein and providing the HCC and, in the other hand, chronic infection with HBV is one of the most important risk factors of liver cancer in Iran (24). Therefore, this research has been done with the purpose of analyzing the distribution of rs1053004 genotypes and alleles of the STAT3 gene and its relationship with HCC in three groups of Iranian people including the HCC patients, the CHB patients and healthy people. This research is the first study undertaken to analyze the relationship between the STAT3 polymorphisms with HCC in the Iranian population. In this analysis, we considered that the distribution of T allele among the healthy control individuals was more repetitive than C allele (T allele: 78% and C allele: 22%), which indicates that T allele can be the major allele and the protective allele against the HCC, while C allele had the most frequency in the HCC group (C allele: 62.13%). Furthermore, when the HCC group was compared to the CHB group in terms of genotypes, the CC genotype of the rs10553004 was significantly related to an increased risk of HCC infection (OR=4.50, 95% CI=0.44-2.00, P=0.007). This relationship was also seen when the HCC group was compared to all issues without HCC. (OR=3.75, 95% CI=0.41-1.55, P=0.011) According to the results, there was a meaningful relationship between liver cancer and gender, so that the liver cancer had a higher incidence in men than in women ($p<0.001$).This difference in the incidence of cancer among men and women can happen because of the risk of environmental factors such as alcohol consumption or smoking in men (26), and also, because of the hormonal differences, so estrogen in women has a protective role against the effects and activities of the STAT3, while androgens in men are the cause of the progression of some of the cancers, such as liver and prostate cancers (27). In a part of the study, the age of the people and its relationship with HCC were analyzed. According to data analysis, there was a significant relationship between age and HCC, so that HCC appears at the older ages ($p<0.001$). Additionally, in analyzing the relationship between two age groups of less and more than 45 years old with rs10553004, data analysis indicated that the potential of being infected with liver cancer in people with more than 45 years old who have the CC genotype is greater ($p=0.001$). Many mutations and gene polymorphisms and their relationship with many proteins are related to age and occur at specific ages. In this research, the reasons for this difference between the two age groups with polymorphism have not been provided. In a research by Nguyen and Hou, it was shown that the incidence of HCC in men is more than in women, which is consistent with our results (28). In a case-control study provided by Xie et al, on the relationship between HCC and rs1053004 in the Chinese population, the distribution of the T allele was observed more frequently among the healthy controls than the C allele (T allele: 65.63% and C allele: 34.37%). But, unlike the data obtained from our research, in the research of Xie, when the HCC group was compared to the issues without HCC, there was not any significant relationship between rs1053004 and the potential of being infected with HCC (OR=1.07, 95% CI=0.91-1.25, P=0.435). In the Xie study, there was a meaningful relationship between the CC genotype versus the TT genotype in the male gender versus the female gender, indicating that the CC genotype in men can be related with the risk of being infected with HCC (OR=2.26, 95% CI=1.10-4.65, P=0.027) (15). In a research provided by Chantra et al, when the HCC group was compared to patients with CHB (OR=1.97, 95% CI=1.12-3.46, P=0.018) and all issues without HCC (OR=2.12, 95% CI=1.31-3.44, P=0.002), there was a significant relationship between the CC genotype and the increased risk of being infected with HCC, which is similar to our results. In the Chantra research, like our results, T allele had more frequency in the healthy groups than C allele (T allele: 61.41% and C allele: 38.59%) and T allele was considered as the major allele and C allele was considered as the minor allele (29). Unlike our data, in a research on the Tunisian Arab population, the frequency of C allele in healthy people was higher than the C allele. This difference between the studies may be due to the racial differences (30). This information demonstrates that more researches in populations which are racially different need to be done.

In conclusion, the present research for the first time represents that there is a meaningful relationship between rs1053004 of the STAT3 gene and the potential of being infected with HCC in Iranian population. The results of this research may be affected by the study limitations, including the low number of the studied population; therefore, for analyzing the presence of this polymorphism in patients with HCC, studies with a larger sample volume and in the different geographical areas in Iran are proposed for better conclusion. It is also better to investigate other polymorphisms of STAT3 gene and prove their role in the liver cancer. If the relationship of STAT3 polymorphisms with the liver cancer is confirmed in other studies, it may be possible to do more effective therapies at the right time, with timely diagnosis of the people who are infected with this disease.

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