

## RESEARCH ARTICLE

# Association of Single Nucleotide Polymorphism rs1053004 in *Signal Transducer and Activator of Transcription 3 (STAT3)* with Susceptibility to Hepatocellular Carcinoma in Thai Patients with Chronic Hepatitis B

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### Abstract

The single nucleotide polymorphism (SNP) rs1053004 in *Signal transducer and activator of transcription 3 (STAT3)* was recently reported to be associated with chronic hepatitis B (CHB)-related hepatocellular carcinoma (HCC) in a Chinese cohort. This study was aimed at investigating whether the SNP might also contribute to HCC susceptibility in the Thai population. Study subjects were enrolled and divided into 3 groups including CHB-related HCC (n=211), CHB without HCC (n=233) and healthy controls (n=206). The SNP was genotyped using allelic discrimination assays based on *TaqMan* real-time PCR. Data analysis revealed that the distribution of different genotypes was in Hardy-Weinberg equilibrium ( $P>0.05$ ). The frequencies of allele T (major allele) in HCC patients, CHB patients and healthy controls were 51.4%, 58.6% and 61.4%, respectively, whereas the frequencies of C allele (minor allele) were 48.6%, 41.4% and 38.6%. The C allele frequency was higher in HCC when compared with CHB patients (odds ratio (OR)=1.34, 95% confidence interval (CI)=1.02-1.74,  $P=0.032$ ). The genotype of SNP rs1053004 (CC versus TT+TC) was significantly associated with an increased risk when compared with CHB patients (OR=1.83, 95% CI=1.13-2.99,  $P=0.015$ ). In addition, we observed a similar trend of association when comparing HCC patients with healthy controls (OR=1.77, 95% CI=1.07-2.93,  $P=0.025$ ) and all controls (OR=1.81, 95% CI=1.19-2.74,  $P=0.005$ ). These findings suggest that the SNP rs1053004 in *STAT3* might contribute to HCC susceptibility and could be used as a genetic marker for HCC in the Thai population.

**Keywords:** Chronic hepatitis B - hepatocellular carcinoma - *STAT3* - single nucleotide polymorphism - Thailand

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### Introduction

Signal transducer and activator of transcription (STAT) proteins are inflammatory mediators which transduce signal across the cytoplasm and function as transcription factors in the nucleus (Yu et al., 2009). Activations of the STAT proteins through growth factors and cytokine receptors have been implicated in initiation and progression of cancer (Yu and Jove, 2004; Yu et al., 2009). The STAT protein family comprises seven members, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Yu and Jove, 2004; Subramaniam et al., 2013). Among STAT family protein, the *STAT3* has gained a considerable attention because it relates to hepatocarcinogenesis, by which regulates numerous genes involved in many biological and cellular processes, including survival, angiogenesis, proliferation and inflammation of the hepatocytes (Subramaniam et

al., 2013). Chronic hepatitis B (CHB) is known to be a major risk factor for the development of hepatocellular carcinoma (HCC) (Gao et al., 2012). It has been shown that viral genetic variations such as genotypes and mutations may be responsible for different disease profiles in CHB infection (Xie et al., 2013). Moreover, previous studies indicated that *STAT3* can be activated by the X protein of HBV and activated *STAT3* can also bind with the HBV enhancer 1 to activate gene expression, suggesting the interplay between *STAT3* and X protein in promoting HCC development (Waris and Siddiqui, 2002).

In addition to the functions of *STAT3* and HBV infection, several lines of evidence have indicated that single nucleotide polymorphisms (SNPs) in *STAT3* gene are associated with susceptibility to malignant development or response to therapy in several types of cancer such as cervical, gastric and renal cancers (Wang et al., 2011; Eto et al., 2013; Yuan et al., 2014). Along with

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the above-mentioned evidences, it would be interested that the SNPs in *STAT3* gene may potentially contribute to the susceptibility of HCC. Recently, a case-control study in Chinese cohort identified a positive association between SNP rs1053004 in *STAT3* gene and HCC risk in a large cohort. This effect was particularly observed in female, though did not reach statistical significance after Bonferroni correction (Xie et al., 2013). This SNP is located on 3'-untranslated region (UTR) in *STAT3* gene which is the binding site of targeted mRNAs and regulates the expression of *STAT3* protein by mRNA degradation (Foshay and Gallicano, 2009; Xie et al., 2013). However, the mechanism by which the SNP contributes to the function of *STAT3* protein is not well defined.

Despite different studies have revealed that polymorphisms in *STAT3* gene were associated with susceptibility to carcinogenesis, there has been limited data regarding the association of the SNP rs1053004 and HCC risk. Thus, the aim of this study was to investigate whether the SNP were related to HCC susceptibility in Thai patients with CHB.

## Materials and Methods

### Study subjects

All subjects in the present study were classified into 3 groups, including 211 individuals with CHB-related HCC, 233 individuals CHB without HCC and 206 healthy controls. Blood samples used in this study were randomly selected from a pool of patients who were seen and followed-up at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between January 2011 and February 2013. The diagnosis of CHB was based on positive for hepatitis B surface antigen (HBsAg) for at least 6 months. The diagnosis of HCC was based on typical imaging studies and/or histology (fine needle aspiration or surgical resection) in accordance with the guidelines of American Association for the Study of Liver Diseases (AASLD) (Bruix and Sherman, 2005). Patients who were seropositive for HCV or human immunodeficiency virus (HIV) were excluded. The healthy controls were blood donors at National Blood Centre Thai Red Cross Society (Bangkok, Thailand), who were tested negative against HBV and/or HCV infection and had no history of

liver disease. All participants were signed the informed consent for this study. The study protocol was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University.

### Genotyping

The genotypic analyses were done on genomic DNA which was isolated from 100  $\mu$ l of peripheral blood mononuclear cells (PBMCs) using phenol-chloroform-isoamyl alcohol extraction as described previously (Sopipong et al., 2013). DNA concentration and purity were subsequently measured using a nanodrop spectrophotometer (NanoDrop 2000c, Thermo scientific, Waltham, MA, USA). The SNP rs1053004 was identified using a real-time PCR protocol based on the *TaqMan* MGB probe for allelic discrimination assays. The reactions were operated in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). The reaction was performed in a total volume of 10  $\mu$ l, briefly, 1X PCR buffer minus Mg, 0.2 mM dNTPs mixture, 1.5 mM MgCl<sub>2</sub>, 0.2 units of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1X primers and probes mix (*TaqMan* SNP Genotyping Assays [ID: C\_1795285\_1]; Applied Biosystems), and 1  $\mu$ l of genomic DNA (50-500 ng/ $\mu$ l). The thermocycling conditions were conducted according to the manufacturer's instructions. Fluorescent signals (FAM and VIC) were acquired at the end of each cycle. To ensure the genotyping quality, positive controls for each allele and negative controls were included in each experiment. Allelic discrimination was analyzed using StepOne™ software (version 2.2, Applied Biosystems).

### Statistical analysis

Clinical data were presented as mean $\pm$ standard deviation for quantitative variables, or as percentages for categorical variables. Intergroup comparisons were evaluated by Student's t test for quantitative variables, and Fisher's exact or chi-square test which appropriate for categorical variables. The intergroup comparisons were carried out using GraphPad prism software (<http://www.graphpad.com/quickcalcs/>). Hardy-Weinberg equilibrium (HWE) was tested using Pearson's Chi-square as implemented in online software (<http://ihg.gsf.de/ihg/snps.html>). Associations of different genetic models with

**Table 1. The Clinical Characteristics of All Participants in this Study**

	CHB related HCC (n=211)	CHB without HCC (n=233)	Healthy controls (n=206)	P value HCC vs. CHB	P value HCC vs. healthy controls
Age (years)	55.50 $\pm$ 9.58	53.81 $\pm$ 11.25	48.60 $\pm$ 5.78	0.091	<0.001
Gender (%)				0.409	<0.001
Male	172 (81.52)	182 (78.11)	122 (59.22)		
Female	39 (18.48)	51 (21.89)	84 (40.78)		
AST (IU/L)	93.75 $\pm$ 111.60	34.37 $\pm$ 26.90	ND	<0.001	
ALT (IU/L)	64.57 $\pm$ 77.30	41.36 $\pm$ 46.79	ND	<0.001	
Albumin (mg/dL)	3.58 $\pm$ 0.61	4.33 $\pm$ 0.55	ND	<0.001	
TB (mg/dL)	1.16 $\pm$ 0.86	0.76 $\pm$ 1.10	ND	<0.001	
ALP (mg/L)	171.04 $\pm$ 132.54	82.03 $\pm$ 40.33	ND	<0.001	
HBV DNA level(log <sub>10</sub> IU/mL)	4.58 $\pm$ 2.00	4.48 $\pm$ 1.68	ND	0.689	

HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; AST, Aspartate aminotransferase; ALT, Alanine transaminase; TB, Total bilirubin; ALP, Alkaline phosphatase; ND, no data

HCC risk were assessed under allelic, additive, dominant and recessive models. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated using MedCalc statistical software Version 12.7.7 ([http://www.medcalc.org/calc/odds\\_ratio.php](http://www.medcalc.org/calc/odds_ratio.php)). P-values lower than 0.05 were considered as statistically significant.

## Results

### Baseline characteristics of the participants

The characteristics of all participants were summarized in Table 1. Data included age, gender, serum levels of aspartate aminotransferase (AST), alanine transaminase (ALT), albumin, total bilirubin (TB), alkaline phosphatase (ALP) and HBV DNA levels. In this retrospective analysis, we found that there was no significant difference in age and gender distribution between HCC and CHB patients (P=0.091 and 0.409, respectively) and we observed the same trend in HBV DNA levels (P=0.689). The mean AST level in the HCC group was significantly higher than that of the CHB group, whereas the serum level of ALT was high in CHB patients rather than in HCC patients, implying that AST and ALT levels had an inverse

proportion among HCC and CHB groups. There were also significantly different in the mean of albumin, TB and ALP levels between the HCC and CHB groups.

### Associations of the STAT3 SNP with HBV-related HCC

The genotype frequencies were not deviated from Hardy-Weinberg Equilibrium in HCC patients, CHB patients and healthy controls as well as all the study participants (P>0.05), as shown in Table 2. The genotype distributions and allele frequencies of SNP rs1053004 in STAT3 were presented in Table 3. The frequencies of T allele in HCC patients, CHB patients and healthy controls were 51.42%, 58.58% and 61.41%, respectively, whereas the frequencies of C allele were 48.58%, 41.42% and 38.59%, respectively. The frequency of C allele was significantly higher in HCC patients when compared with CHB patients (odds ratio (OR)=1.34, 95% confidence interval (CI)=1.02-1.74, P=0.032), indicating that this allele might correlate with an increased risk of HCC whereas T allele was considered as a protective allele for HCC development. We found the similar results under additive (OR=1.97, 95% CI=1.12-3.46, P=0.018) and recessive models (OR=1.83, 95% CI=1.13-2.99, P=0.015).

We compared HCC patients with healthy controls to assess the association of the SNP rs1053004 with HCC risk in individuals who were not infected with HBV. The results showed that the C allele was significantly associated with an increased risk of HCC (OR=1.50, 95% CI=1.14-1.98, P=0.004) (Table 3). This association remained significant under additive (OR=2.29, 95% CI=1.29-4.05, P=0.005), dominant (OR=1.69, 95% CI=1.12-2.57, P=0.013) and recessive models (OR=1.77, 95% CI=1.07-2.93, P=0.025), implying that this SNP might contribute to HCC development among normal individuals. In addition, when compared HCC patients with all controls (CHB patients and healthy controls), we observed the same trend of association under additive (OR=2.12, 95% CI=1.31-3.44, P=0.002), dominant (OR=1.47, 95% CI=1.02-2.12, P=0.038) and recessive models (OR=1.81, 95% CI=1.19-2.74, P=0.005).

**Table 2. The Comparisons between Observed and Expected Genotypes from Hardy-Weinberg Equilibrium (HWE)**

Study subject	Genotype	Observed amount	Expected amount	Chi-square (P value) <sup>†</sup>
CHB related HCC	TT	55	55.79	0.827
	TC	107	105.41	
	CC	49	49.79	
CHB without HCC	TT	73	79.97	0.06
	TC	127	113.07	
	CC	33	39.97	
Healthy controls	TT	77	77.68	0.841
	TC	99	97.64	
	CC	30	30.68	
All participants	TT	205	212.33	0.241
	TC	333	318.35	
	CC	112	119.33	

<sup>†</sup>Pearson's goodness-of-fit chi-square

**Table 3. Genotype and Allele Frequencies of SNP rs1053004 in STAT3 Gene with HCC Risk**

STAT3 rs1053004	CHB related HCC (n=211)	CHB without HCC (n=233)	Healthy controls (n=206)	HCC vs. CHB		HCC vs. Healthy controls		HCC vs. All controls <sup>†</sup>	
				OR (95% CI)	P values	OR (95% CI)	P values	OR (95% CI)	P values
<b>Allelic model</b>									
Major (T)	217 (51.42%)	273 (58.58%)	253 (61.41%)	1	-	1	-	1	-
Minor (C)	205 (48.58%)	193 (41.42%)	159 (38.59%)	1.34 (1.02-1.74)	0.032	1.50 (1.14-1.98)	0.004	1.41 (1.12-1.78)	0.004
<b>Additive model*</b>									
TT	55 (26.07%)	73 (31.33%)	77 (37.38%)	1	-	1	-	1	-
TC	107 (50.71%)	127 (54.51%)	99 (48.06%)	1.12 (0.72-1.73)	0.614	1.51 (0.97-2.35)	0.066	1.29 (0.88-1.90)	0.193
CC	49 (23.22%)	33 (14.16%)	30 (14.56%)	1.97 (1.12-3.46)	0.018	2.29 (1.29-4.05)	0.005	2.12 (1.31-3.44)	0.002
<b>Dominant model</b>									
TT	55 (26.07%)	73 (31.33%)	77 (37.38%)	1	-	1	-	1	-
TC+CC	156 (73.93%)	160 (68.67%)	129 (62.62%)	1.29 (0.86-1.96)	0.222	1.69 (1.12-2.57)	0.013	1.47 (1.02-2.12)	0.038
<b>Recessive model</b>									
TT+TC	162 (76.78%)	200 (85.84%)	176 (85.44%)	1	-	1	-	1	-
CC	49 (23.22%)	33 (14.16%)	30 (14.56%)	1.83 (1.13-2.99)	0.015	1.77 (1.07-2.93)	0.025	1.81 (1.19-2.74)	0.005

\*HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; CI, confidence interval; OR, odds ratio; \*rs1053004 alleles are reported in reverse orientation to genome; <sup>†</sup>CHB patients and Healthy controls

## Discussion

STAT3 is a member of STAT family protein which is activated by growth factors and cytokine receptors (Yu et al., 2009; Subramaniam et al., 2013). It has been shown that several tumor-related viruses, including HBV, are related to the activation of STAT3 (Migone et al., 1995; Sun and Steinberg, 2002; Choudhari et al., 2007; Muromoto et al., 2009). Indeed, constitutively activated STAT3 is crucial for compensating hepatocyte turnover and leads to advanced fibrosis and cirrhosis, which are the key clinical risk factors for HCC development (Subramaniam et al., 2013). Despite the important functions of STAT3 in hepatocarcinogenesis, there are limited data regarding the association of SNPs in STAT3 gene with HCC susceptibility. Recently, a case-control study in Chinese population suggested that SNP rs1053004 might influence with CHB induced HCC (Xie et al., 2013). This study is the first report that attempted to demonstrate the association of this SNP with HCC risk in Thai population. In this analysis, we found that the distribution of T allele among healthy controls was more frequent than C allele (T allele: 61.41% and C allele: 38.59%), indicating that the T allele was the major allele, whereas the C allele was considered as the minor allele in our cohort. These findings were consistent with the data conducted in the recent Chinese cohort (T allele: 65.63% and C allele: 34.37%) (Xie et al., 2013), suggesting that the SNP has homogeneity in the allele distribution among East-Asian (Thai and Chinese) populations. Interestingly, in contrast to our report, the frequency of C allele was higher than T allele in Tunisian Arabs (Middle East) (Messaoudi et al., 2013a; 2013b).

SNP rs1053004, localized on 3'-UTR of STAT3, regulates expression of STAT3 protein by altering mRNA degradation and decreased STAT3 activity (Foshay and Gallicano, 2009; Xie et al., 2013). To investigate the correlation of SNP rs1053004 in STAT3 gene with HCC susceptibility, we assessed the association of specific genotype with the risk of HCC under additive model by taking TT genotype as a reference (OR=1.00). Our study found that CC genotype was significantly associated with an increased risk of HCC in approximately two folds when compared with CHB patients (OR=1.97, 95% CI=1.12-3.46, P=0.018) and all subjects without HCC (OR=2.12, 95% CI=1.31-3.44, P=0.002). In contrast to our findings, the data from the Chinese cohort indicated that the SNP was not significantly associated with the risk of HCC as compared with all subjects without the cancer (OR=1.07, 95% CI=0.91-1.25, P=0.435) (Xie et al., 2013). Interestingly, rs1053004 CC genotype tended to be associated with a reduced risk of HCC in Chinese females, although did not reach statistical significance (OR=0.49, 95% CI=0.25-0.97). In addition, multiplicative interaction of the SNP (CC versus TT) with sex (male versus female) was significantly associated with an increased risk of HCC in that report (Xie et al., 2013). This dissimilarity between studies remains unclear but might be related to differences between ethnicities. This information emphasizes the necessity to conduct further such association studies in ethnically diverse populations.

Worldwide, HCC ranks as third-leading cause of cancer-related death (Shi et al., 2014). In Thailand, HCC is the most common cancer in males rather than in females (Chittmitrapap et al., 2013; Thongbai et al., 2013). This gender disparity might be partly contributed to exposure with environmental risk factors, such as smoking and heavy alcohol consumption (Jee et al., 2004). In addition, the sex differences in HCC risk might also be related to hormone signaling. Previous studies have demonstrated that STAT3 signaling is activated by estrogen hormone, while androgen receptor-mediated gene expression is activated via interleukin-6/STAT3 signaling (Chen et al., 2000; Bjornstrom and Sjoberg, 2002). In addition, estrogen has been shown to play a protective role in HCC development through inhibition of STAT3 function and nuclear factor- $\kappa$ B signaling, the key process of which is related to STAT3 signaling pathway (Yu et al., 2009). In contrast, androgen is able to promote the development of certain types of cancers, such as prostate and HCC (Nguyen et al., 2002; Hou et al., 2013).

It should be mentioned that the present study had some strength. These included age and gender between HCC and CHB patients were well matched and the distribution of the SNP genotypes in all subject groups was in Hardy-Weinberg Equilibrium. However, this study was conducted in a tertiary hospital and selection bias of the patients might be possible. Second, some clinical data such as HBV genotypes and mutations, as well as alcohol or tobacco consumptions, were not available thus they were excluded from the analysis. Third, although this study demonstrated a positive association between SNP rs1053004 and HCC risk, we did not link the SNP genotype with STAT3 expression. Further experiments are needed to reveal the underlying mechanism by which SNP rs1053004 affects the activity of STAT3.

In conclusion, our study provided important evidence that SNP rs1053004 in STAT3 gene was significantly associated with HCC risk in Thai individuals. Thus, identification of the SNP may be useful as a novel genetic marker of HCC development in our population. Further case-control studies with increased numbers of samples will be required to reduce the confounding factors and confirm the association of the SNP with the risk of HCC in Thai population.

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