RESEARCH ARTICLE

Single Nucleotide Polymorphisms in STAT3 and STAT4 and Risk of Hepatocellular Carcinoma in Thai Patients with Chronic Hepatitis B

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Abstract

Hepatitis B virus (HBV) infection is the leading cause of hepatocellular carcinoma (HCC) development. Recent studies demonstrated that single nucleotide polymorphisms (SNPs) rs2293152 in signal transducer and activator of transcription 3 (STAT3) and rs7574865 in signal transducer and activator of transcription 4 (STAT4) are associated with chronic hepatitis B (CHB)-related HCC in the Chinese population. We hypothesized that these polymorphisms might be related to HCC susceptibility in Thai population as well. Study subjects were divided into 3 groups consisting of CHB-related HCC (n=192), CHB without HCC (n=200) and healthy controls (n=190). The studied SNPs were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The results showed that the distribution of different genotypes for both polymorphisms were in Hardy-Weinberg equilibrium (P>0.05). Our data demonstrated positive association of rs7574865 with HCC risk when compared to healthy controls under an additive model (GG versus TT: odds ratio (OR) =2.07, 95% confidence interval (CI)=1.06-4.03, P=0.033). This correlation remained significant under allelic and recessive models (OR=1.46, 95% CI=1.09-1.96, P=0.012 and OR=1.71, 95% CI=1.13-2.59, P=0.011, respectively). However, no significant association between rs2293152 and HCC development was observed. These data suggest that SNP rs7574865 in STAT4 might contribute to progression to HCC in the Thai population.

Keywords: Chronic hepatitis B - hepatocellular carcinoma - STAT3 - STAT4 - single nucleotide polymorphisms

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Introduction

Liver cancers have been considered as the most cancer-related death. Accounting for 85% of all primary liver cancers is hepatocellular carcinoma (HCC) (Perz et al., 2006; Subramaniam et al., 2013). HCC is also the fifth most common malignancy worldwide (Yang and Roberts, 2010; Subramaniam et al., 2013). The occurrence of HCC is often correlated with several risk factors such as chronic alcohol consumption, aflatoxin B1 exposure and persistent infection with hepatitis viruses, including hepatitis B virus (HBV) and hepatitis C virus (HCV) (Ramakrishna et al., 2013; Subramaniam et al., 2013). Additionally, host genetic factors such as signal transducer and activator of transcription (STAT) polymorphisms may contribute to the risk of hepatic carcinogenesis, as accumulated evidences reported that STAT polymorphisms were associated with HBV-related HCC progression (Clark et al., 2013; Jiang et al., 2013; Xie et al., 2013; Kim et al., 2015).

STAT3, a key molecule of the Janus kinase (JAK)/STAT signaling pathway, has been shown to play pivotal role in the transcription of genes important for inflammation, survival, proliferation and invasion of HCC (Sansone and Bromberg, 2012; Subramaniam et al., 2013). STAT3 activation is tightly controlled to prevent dysregulation of gene transcription in normal cells, whereas constitutively activated STAT3 plays a crucial role in transcriptional gene involved in oncogenic transformation (Subramaniam et al., 2013). Moreover, STAT3 can be activated by HBV X protein (Lee and Yun, 1998; Wang et al., 2012). The activated STAT3 specifically binds HBV enhancer 1, leading to stimulation of HBV gene expression (Waris and Siddiqui, 2002; Wang et al., 2009). Results from recent study have demonstrated that single nucleotide polymorphism (SNP) rs2293152 in STAT3 was significantly associated with an increased risk of HCC in comparison to the subjects without HCC in Chinese population. The impact of this SNP was greater in women when compared to men (Xie et al., 2013).

In addition to STAT3 protein, STAT4 is also a member of STAT family proteins (Subramaniam et al., 2013). STAT4 regulates transcription and expression of various

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genes including type II interferon (IFN-γ) through signal transmission from interleukin-12, interleukin-23 and type I interferon (IFN-α or IFN-ß) (Thierfelder et al., 1996; Nguyen et al., 2002; Yu et al., 2009; O'Shea et al., 2013). In liver, IFN-γ is an essential cytokine for hepatocyte apoptosis and liver regeneration (Horras et al., 2011). The balance of IFN-γ activation through STAT4 can affect both antiviral and antitumor activities (Saha et al., 2010; Horras et al., 2011). Previous study based on genome-wide association study (GWAS) in Chinese cohort found that SNP rs7574865 in STAT4 was significantly associated with HCC susceptibility. The G allele in this SNP was considered as a risk allele for HCC development, and also significantly associated with lower mRNA levels of STAT4 in HCC tissues compared to adjacent non-tumor tissues (Jiang et al., 2013). This reported SNP has been confirmed to correlate with HBV-related HCC in Vietnamese population (Clark et al., 2013) and was associated with the risk of chronic hepatitis B (CHB) in Korean cohort (Kim et al., 2015). As a result, we hypothesized that the SNPs rs2293152 in STAT3 and rs7574865 in STAT4 might contribute to HCC progression in CHB carriers. Thus, the aim of this study was to determine the association between these SNPs and the risk of HCC among Thai population.

Materials and Methods

To investigate the associations between the SNPs rs2293152 in STAT3 and rs7574865 in STAT4 with susceptibility to HCC, we randomly selected blood samples from pool of CHB patients who followed-up at King Chulalongkorn Memorial Hospital (Bangkok, Thailand). Study subjects were divided into three groups, including 192 CHB-related HCC patients, 200 CHB patients without HCC and 190 healthy controls.

The diagnosis of CHB was confirmed by positive for hepatitis B surface antigen (HBsAg) for at least 6 months. Additionally, HCC were diagnosed 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. Healthy controls recruited from National Blood Centre Thai Red Cross Society (Bangkok, Thailand) were tested negative against HBV and/or HCV infection and had no history of liver disease. The study protocol was conducted with the approval of the Institutional Review Board, Faculty of Medicine, Chulalongkorn University. All participants signed an informed consent before recruited into this study.

DNA preparation and genetic analysis

To determine genotypes of SNPs rs2293152 and rs7574865, genomic DNA was extracted form peripheral blood mononuclear cells (PBMCs) using phenol-chloroform-isoamyl alcohol protocol, as described in a previous study (Sopipong et al., 2013). Following this step, concentration of genomic DNA was measured by Nanodrop spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA). The studied SNPs were genotyped based on polymerase chain reaction -restriction fragment length polymorphism (PCR-RFLP) method (Sato et al., 2009; Hu et al., 2010). The following primers were used for PCR amplification: forward 5'-TCCCCTGTGATT CAGATCCC-3' and reverse 5'-CATTCCCACATCTCTGCTC

C-3' for rs2293152 (Sato et al., 2009), whereas forward 5'- AAAGAAGTGGGATAAAAA

G A A G T T T G - 3 ' a n d r e v e r s e 5 ' - CCACTGAAATAAGATAACCACTGT-3' for rs7574865 (Hu et al., 2010). The PCR reaction was performed in 25 μl of reaction mixture containing 50-500 ng/μl of genomic DNA, 0.25 μM of forward and reverse primers, 0.2 mM of dNTPs mixture, 1.5 mM of MgCl2 and 0.65 units of Taq DNA polymerase. The polymorphic region was amplified with the following procedure: initial denaturation at 95 °c for 5 min, followed by 40 cycles of denaturation at 95 °c for 30 s, annealing at 59 °c for 30 s and extension at 72 °c for 30 s and final extension at 72 °c for 5 min in the PCR Mastercycler Gradient (Eppendorf, Germany). Positive and negative controls were performed together in each amplification. The PCR products were digested with restriction enzyme HpaII (Thermo Scientific, USA) and HpaI (Thermo Scientific, USA) for rs2293152 and rs7574865, respectively. The digests were electrophoresed on 3% agarose gel (Invitrogen, USA) and stained with ethidium bromide (AMRESCO, USA) to visualize with an ultraviolet transilluminator (Vilber Lourmat, Hong Kong). Figure 1 demonstrates PCR-RFLP profiles of the SNPs.

Statistical analysis

Quantitative variables were shown as mean ± standard deviation and assessed the intergroup differences using Student's t-test. Categorical variables were expressed as the number and percentage and calculated the significance of differences between groups by Fisher's exact or chi-square test. The intergroup comparisons were carried out using GraphPad prism software (http://www.graphpad.com/quickcalcs/). Deviation from Hardy-Weinberg equilibrium (HWE) was tested in all groups of study subjects using Pearson's Chi-square as carried out in online software (http://ihg.gsf.de/ihg/snps.html). P-values were considered to be in HWE when there were more than 0.05. Associations of different genetic models with HCC risk were assessed under additive, allelic, dominant and

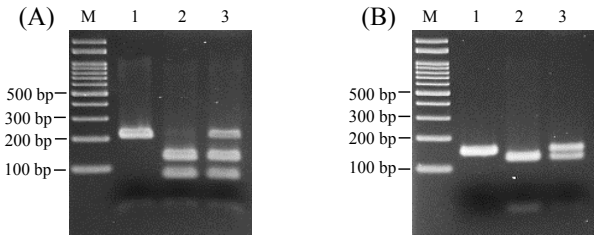

Figure 1. PCR-RFLP Profiles of the Studied SNPs. (A) Panel of profiles for rs2293152 in STAT3 after digestion with HpaII enzyme: lane M, 100-bp DNA ladder; lane 1, genotype GG; lane 2, genotype CC; lane 3, genotype GC. (B) Electrophoresis pattern of different genotypes for rs7574865 in STAT4 digested with restriction enzyme HpaI: lane M, 100-bp DNA ladder; lane 1, genotype GG; lane 2, genotype TT; lane 3, genotype GT

recessive models. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated by MedCalc statistical software Version 12.7.7 (http://www.medcalc.org/calc/odds_ratio.php). P-values below 0.05 were considered statistical significance.

Results

Demographic data of the participants

The clinical characteristics of all participants are shown in Table 1. The data showed that the HCC group was older than the CHB group and healthy controls. The proportion of male was highest in the HCC group (85.42%) whereas proportion of females was highest in healthy controls (41.05%). In addition, serum aspartate aminotransferase (AST) and alanine transaminase (ALT) levels in the HCC group were significantly higher than in the CHB group (P<0.001 and 0.023, respectively).

Association of rs2293152 with HCC development

All the SNPs in this cohort were in HWE (P>0.05) as shown in Table 2. This information indicated that no sample bias existed among the polymorphisms. We next determined the correlation of rs2293152 with HCC development. The results showed that there was no significant difference in genotype distribution among the HCC group compared with the CHB group and healthy controls (CC versus GG: OR=0.93, 95% CI=0.53-1.63, P=0.796 and OR=0.88, 95% CI=0.49-1.56, P=0.660, respectively). Additionally, the present study found the similar trend in comparison with allele frequency (OR=0.97, 95% CI=0.73-1.29, P=0.849 and OR=0.94, 95% CI=0.71-1.25, P=0.671, respectively). Furthermore, the positive association was not found between SNP rs2293152 and HCC development when compared to the CHB group (dominant model: OR=1.04, 95% CI=0.67-1.61, P=0.857 and recessive model: OR=0.88, 95% CI=0.54-1.42, P=0.598), as well as in comparison to healthy controls (dominant model: OR=0.94, 95% CI=0.60-1.47, P=0.783 and recessive model OR=0.90, 95% CI=0.55-1.47, P=0.668) (Table 3).

After combined the CHB group with healthy controls as the non-HCC group, our results showed that there was no significant difference in genotype or allele frequencies between the HCC and non-HCC groups [additive model (CC versus GG): OR=0.90, 95% CI=0.55-1.48, P=0.691

Table 1. Characteristics of Participants in this Study

(n=192)	CHB-related HCC	CHB without HCC (n=200)	Healthy controls (n=190)	P values
Age (years)	57.64 ± 9.80	45.84 ± 14.04	47.83 ± 5.31	<0.001*,†,‡
Gender				<0.001*,†,‡
Male (%)	164 (85.42)	140 (70.00)	112 (58.95)	
Female (%)	28 (14.58)	60 (30.00)	78 (41.05)	
AST (IU/L)	87.04 ± 76.01	35.75 ± 35.15	ND	<0.001*
ALT (IU/L)	59.32 ± 55.57	45.31 ± 62.58	ND	0.023*

HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; ALT, Alanine transaminase; AST, Aspartate aminotransferase; ND, no data; *The P-value represents comparison between HCC and CHB without HCC; † The P-value represents comparison between HCC and healthy controls; ‡ The P-value represents comparison between HCC and all controls

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

Gene	Study subject	Genotype	Observed amount	Expected amount	Chi-square (P value)†
STAT3 rs2293152	CHB related HCC	GG	56	56.88	0.799
		GC	97	95.25	
		CC	39	39.88	
	CHB without HCC	GG	60	57.78	0.528
		GC	95	99.44	
		CC	45	42.78	
	Healthy controls	GG	53	53.16	0.963
		GC	95	94.68	
		CC	42	42.16	
	All participants	GG	169	167.79	0.841
		GC	287	289.41	
		CC	126	124.79	
STAT4 rs7574865	CHB related HCC	TT	19	20.02	0.736
		GT	86	83.96	
		GG	87	88.02	
	CHB without HCC	TT	24	24.85	0.792
		GT	93	91.30	
		GG	83	83.85	
	Healthy controls	TT	28	32.02	0.228
		GT	100	91.96	
		GG	62	66.02	
	All participants	TT	71	76.13	0.357
		GT	279	268.73	
		GG	232	237.13	

†Pearson's goodness-of-fit chi-square

and allelic model: OR=0.96, 95% CI=0.75-1.22, P=0.725, respectively] (Table 3). These results provided evidence that rs2293152 in STAT3 might not be associated with HCC development in Thai population.

Association of rs7574865 with HCC development

In order to determine the association of SNP rs7574865 in STAT4 with HCC susceptibility, we first compared its distribution between the HCC and CHB groups. The results revealed that there was no significant difference in genotype frequencies (GG versus TT: OR=1.32, 95% CI=0.68-2.59, P=0.414). Similar results were also demonstrated regarding allelic, dominant and recessive models (OR=1.14, 95% CI=0.85-1.54, P=0.382; OR=1.24, 95% CI=0.66-2.35, P=0.506 and OR=1.17, 95% CI=0.78-1.74, P=0.447, respectively). These results implied that the SNP might not contribute to HCC development in patients with HBV infection. Furthermore, a similar trend of association was found when compared between the HCC group and all controls (additive model (GG versus TT): OR=1.64, 95% CI=0.91-2.96, P=0.099; allelic model: OR=1.29, 95% CI=1.00-1.67, P=0.054; dominant model: OR=1.40, 95% CI=0.80-2.44, P=0.235 and recessive model: OR=1.40, 95% CI=0.99-1.99, P=0.060) (Table 3).

Next, we analyzed the association between the HCC group and healthy controls. Under additive model, the results showed that the GG genotype was more frequently distributed in the HCC group than in healthy controls (OR=2.07, 95% CI=1.06-4.03, P=0.033), suggesting that the GG genotype might be associated with an increased risk of HCC. In addition, G allele was found more frequently in HCC patients when compared with healthy controls (OR=1.46, 95% CI=1.09-1.96, P=0.012), indicating that G allele was considered as a risk allele for HCC development. Similarly, we observed a similar trend of this association in recessive model (OR=1.71, 95% CI=1.13-2.59, P=0.011) (Table 3).

Discussion

STAT3 has been reported as a vital link between inflammation and development of HCC (He et al., 2010). It can be activated by many cytokines and growth factors such as interleukin-12 epidermal growth factor and hepatocyte growth factor (Hirano et al., 2000; Takeda and Akira, 2000). Moreover, tumor aggressiveness is also related to STAT3 activation (He and Karin, 2011). Indeed, STAT3 polymorphism at rs2293152 was first identified as genetic susceptibility to HCC in Chinese cohort (Xie et al., 2013). In our case-control study, G and C alleles were observed as the major and minor alleles, respectively. Interestingly, this observation was rather different to the allele frequencies documented in Chinese population as G and C alleles were minor and major alleles, respectively (Xie et al., 2013). These findings provide important evidence that the allele distribution of SNP rs2293152 has ethnographical heterogeneity among East-Asian (Thai and Chinese) cohorts.

SNP rs2293152 is located within intron 11 of STAT3 (Sato et al., 2009). It is classified as a synonymous polymorphism, which does not introduce any change to amino acid sequence (Hu et al., 2014). This SNP, however,

Table 3. Genotype and Allele Frequencies of SNPs rs2293152 on STAT3 and rs7574865 on STAT4 with HCC Risk

SNPs Genotype and Allele	CHB -related HCC (n=192)	CHB without HCC (n=200)	Healthy controls (n=190)	HCC vs. CHB OR (95% CI)	HCC vs. CHB P values	HCC vs. Healthy controls OR (95% CI)	HCC vs. Healthy controls P values	HCC vs. All controls† OR (95% CI)	HCC vs. All controls† P values
STAT3 rs2293152									
Additive model									
GG	56 (29.17%)	60 (30.00%)	53 (27.89%)	1	-	1	-	1	-
GC	97 (50.52%)	95 (47.50%)	95 (50.00%)	1.09 (0.69-1.74)	0.703	0.97 (0.60-1.55)	0.887	1.03 (0.69-1.54)	0.885
CC	39 (20.31%)	45 (22.50%)	42 (22.11%)	0.93 (0.53-1.63)	0.796	0.88 (0.49-1.56)	0.66	0.90 (0.55-1.48)	0.691
Allelic model									
Major (G)	209 (54.43%)	215 (53.75%)	201 (52.89%)	1	-	1	-	1	-
Minor (C)	175 (45.57%)	185 (46.25%)	179 (47.11%)	0.97 (0.73-1.29)	0.849	0.94 (0.71-1.25)	0.671	0.96 (0.75-1.22)	0.725
Dominant model									
GG	56 (29.17%)	60 (30.00%)	53 (27.89%)	1	-	1	-	1	-
GC+CC	136 (70.83%)	140 (70.00%)	137 (72.11%)	1.04 (0.67-1.61)	0.857	0.94 (0.60-1.47)	0.783	0.99 (0.68-1.45)	0.962
Recessive model									
GG+GC	153 (79.69%)	155 (77.50%)	148 (77.89%)	1	-	1	-	1	-
CC	39 (20.31%)	45 (22.50%)	42 (22.11%)	0.88 (0.54-1.42)	0.598	0.90 (0.55-1.47)	0.668	0.89 (0.58-1.36)	0.583
STAT4 rs7574865									
Additive model									
TT	19 (9.90%)	24 (12.00%)	28 (14.74%)	1	-	1	-	1	-
GT	86 (44.79%)	93 (46.50%)	100 (52.63%)	1.17 (0.60-2.28)	0.649	1.27 (0.66-2.43)	0.475	1.22 (0.68-2.19)	0.505
GG	87 (45.31%)	83 (41.50%)	62 (32.63%)	1.32 (0.68-2.59)	0.414	2.07 (1.06-4.03)	0.033	1.64 (0.91-2.96)	0.099
Allelic model									
Minor (T)	124 (32.29%)	141 (35.75%)	156 (41.05%)	1	-	1	-	1	-
Major (G)	260 (67.71%)	259 (64.75%)	224 (58.95%)	1.14 (0.85-1.54)	0.382	1.46 (1.09-1.96)	0.012	1.29 (1.00-1.67)	0.054
Dominant model									
TT	19 (9.90%)	24 (12.00%)	28 (14.74%)	1	-	1	-	1	-
GT+GG	173 (90.10%)	176 (88.00%)	162 (85.26%)	1.24 (0.66-2.35)	0.506	1.57 (0.85-2.93)	0.152	1.40 (0.80-2.44)	0.235
Recessive model									
TT+GT	105 (54.69%)	117 (58.50%)	128 (67.37%)	1	-	1	-	1	-
GG	87 (45.31%)	83 (41.50%)	62 (32.63%)	1.17 (0.78-1.74)	0.447	1.71 (1.13-2.59)	0.011	1.40 (0.99-1.99)	0.06

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

has been speculated to alter the function of STAT3 and lead to an activation of inflammatory signaling pathway (Sato et al., 2009), as several evidences have demonstrated the effect of intron SNPs on the functional change of target proteins (Kimchi-Sarfaty et al., 2007; Sauna et al., 2007). In the study conducted in Chinese population, rs2293152 was not found to be associated with the risk of HCC when compared between the HCC and CHB without HCC groups. However, the SNP rs2293152 was significantly associated with an increased risk of HCC when compared with all controls (CHB patients without HCC and healthy subjects) (GG versus CC: adjusted odds ratio=1.30, 95% CI=1.04-1.62, P=0.019). Moreover, this relation was entirely found among female population (Xie et al., 2013). In the present study, however, our evidence did not support the role of rs2293152 in HCC susceptibility, which was not consistent with the above-mentioned Chinese cohort (Xie et al., 2013). However, additional studies are required to confirm these findings and elucidate the underlying mechanisms of which modulate HCC risk through alteration of STAT3 activity.

STAT4 is an important cytosolic factor involving in transmitting signals stimulated by several cytokines to induced INF-γ production (Thierfelder et al., 1996; Nguyen et al., 2002; Yu et al., 2009; O'Shea et al., 2013). The polymorphisms of STAT4 have been reported to be contributed to autoimmune disease and HCC susceptibility (Remmers et al., 2007; Hirschfield et al., 2009; Jiang et al., 2013). In fact, previous data have identified SNP rs7574865 in STAT4 as the genetic susceptibility locus for HBV-associated HCC in Chinese cohort based on a three-stage GWAS (Jiang et al., 2013). Thus, additional studies in other ethnic populations are needed to confirm this finding. In this study, we found that the frequency of G allele was higher than T allele, indicating that the major allele of this locus was G allele, whereas T allele was considered as a minor allele. This trend of allele distributions also reported in the Chinese study (Jiang et al., 2013). Furthermore, the similar allele frequency distributions were found in other reports such as Vietnamese (Clark et al., 2013) and Korean cohorts (Kim et al., 2015). These results suggest that the distribution of allele frequencies for this SNP has homogeneity among various Asian populations.

Based on three-stage GWAS, it was shown that there was a trend of higher frequency for risk G allele in 5,480 HCC patients compared to 6,319 CHB carriers when carried out a meta-analysis (G versus T allele; OR=1.22, 95% CI=1.15-1.29, Pmeta=1.66 × 10-11) (Jiang et al., 2013). This association was also marginally demonstrated in the Vietnamese cohort (T versus G allele: OR=0.84, 95% CI=0.7-0.99, P=0.048) (Clark et al., 2013). However, the odds ratios of both reports were rather small that exhibited slight difference between groups studied. In this study, we could not obtain an association between SNP rs7574865 and HCC risk. Apart from our findings, the association of this locus with HBV-induced HCC was not statistically significant in two other replication cohorts from Chinese Han (506 HCC versus 772 CHB carriers) (Chen et al., 2013) and Korean (287 HCC versus 671 CHB carriers) populations (Kim et al., 2015). The possible explanation could be the fact that the effects of SNP rs7574865 on HCC risk might be difficult to observe among studies with relative small sample sizes.

SNP rs7574865 is localized on intron 3 of STAT4 and as a non-coding region. It is apparent that it may affect the STAT4 gene expression at the level of transcription or splicing of mRNA (Korman et al., 2008). In the study, we found that SNP rs7574865 was significantly associated with HCC risk when comparison HCC patients with healthy controls in additive model (OR=2.07, 95% CI=1.06-4.03, P=0.033), which is consistent with previous study in Korean population (OR=1.33, 95% CI=1.02-1.73, P=0.04). In the three-stage GWAS, it was reported that subjects with homozygous GG genotype had the lowest level of STAT4 mRNA compared to those with TG and TT genotypes. Also, lower mRNA level was detected in tumorous tissues when compared with paired adjacent non-tumor tissues (Jiang et al., 2013). However, the genotype differences of this locus were not found to be associated with STAT4 gene expression in Korean report (Kim et al., 2015). Accordingly, further functional studies are still needed for a better understand the mechanism of HCC development in related to SNP rs7574865.

Although being a cross-sectional retrospective study with a relative small sample size, our report had some strength that should be mentioned. First, the distributions of the SNPs were in Hardy-Weinberg Equilibrium. Second, in order to investigate the potential association of the studied SNPs with HCC risk, the non-HCC group was comprised of two distinct groups including patients with CHB and healthy controls. In summary, our results revealed that rs7574865 polymorphism in STAT4 might contribution to HCC susceptibility in healthy individuals. Further prospective studies are required to confirm these observations and to evaluate the mechanisms of STAT3 and STAT4 by which influence HCC development.

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