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STAT4 genetic polymorphisms association with spontaneous clearance of hepatitis B virus infection

Yanjun Lu[1] · Yaowu Zhu[1] · Jing Peng[1] · Xiong Wang[1] · Feng Wang[1] · Ziyong Sun[1]

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Abstract STAT4 signal pathway plays an important role in IFN-γ-mediated antiviral activity. Recent studies show an association of STAT4 polymorphisms with hepatitis B virus (HBV) infection. We therefore investigated the influence of STAT4 polymorphisms on the susceptibility of spontaneous clearance of HBV in a Chinese Han population. Genomic DNA from 288 cases with chronic HBV infection and 288 controls who spontaneously recovered from HBV infection was analyzed for five SNPs in the STAT4 gene (rs7574865, rs7572482, rs7582694 rs11889341, and rs8179673).Our analysis revealed that all the minor alleles of the four SNPs (rs7574865, rs7582694, rs11889341, and rs8179673) had an association with overall decreased risk to HBV infection [$p = 0.040$, OR 0.762 (95 % CI 0.593–0.981); $p = 0.011$, OR 0.686 (95 % CI 0.535–0.878); $p = 0.023$, OR 0.751 (95 % CI 0.586–0.962); $p = 0.002$, OR 0.670 (95 % CI 0.521–0.861), respectively]. The major alleles of the four SNPs were found to be associated with increased risk of HBV-related cirrhosis and hepatocellular carcinoma. Furthermore, the haplotype GGGCT constructed from the five SNPs was found to have a highly significant association with chronic HBV infection when compared to the controls who spontaneously recovered from HBV infection [$p = 0.031$, OR 1.368 (95 % CI 1.028–1.818)]. These findings indicate that STAT4 minor allele may be associated with the spontaneous clearance of HBV, whereas the major allele may be associated with the progress of the HBV-related liver disease.

Keywords STAT4 · Hepatitis B virus · Single nucleotide polymorphism · Genotype

Introduction

Chronic hepatitis B (CHB) is one of the most common infectious liver diseases caused by hepatitis B virus (HBV) infection. More than two billion people alive today have been infected with HBV at some time in their life, about 95 % of individuals can successfully clear HBV, and only 5–10 % will develop CHB [1]. Among those CHB individuals, 20–30 % will develop liver cirrhosis and 5 % further progress to hepatocellular carcinoma (HCC) [2].

It has been shown that the host immune system may affect HBV clearance [3]. Viral clearance during HBV infection is associated with the entry of $CD8^+$ T cells into the liver and the production of IFN-γ [4]. Studies in HBV-transgenic mice have shown a crucial role for IFN-γ in the non-cytolytic mechanism of virus clearance [5]. It is well known that IFN-γ-mediated antiviral effect is associated with the Jak/STAT4 signal pathway activation, and STAT4 deficiency dramatically impairs IFN-α/β-dependent induction of IFN-γ during viral infection [6]. STAT4 gene is located on human chromosome 2q32.3 encoding a transcription factor involved in the signaling pathways of several cytokines, including interleukin (IL)-12, the type I interferons (IFN), and IL-23 [7]. Studies of viral infections, i.e., lymphocytic choriomeningitis virus (LCMV), influenza virus, and HSV-1 have revealed diverse roles for STAT4 in the antiviral

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✉ Ziyong Sun
tjszyong@163.com

[1] Department of Laboratory Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, No. 1095 Jie Fang Avenue, Wuhan 430030, China

response in the animal model [8–11]. Moreover, STAT4 also affects non-virus infection, and it has been demonstrated that STAT4 is essential for the generation of a protective innate immune response against *K. pneumoniae* in the lung and STAT4−/− animals have impaired pulmonary and blood Klebsiella clearance [12].

Although the role of STAT4 has been studied extensively in animal models of infectious diseases, little is known about its role in human infections. Most studies of human STAT4 have focused on correlations between variations in the STAT4 gene and the incidence of autoimmune diseases. The STAT4 single nucleotide polymorphism (SNP) rs7574865 was first revealed to be associated with increased risk of both rheumatoid arthritis and systemic lupus erythematosus [13], and then other SNPs, including rs11889341, rs8179673, and rs7582694, were associated with inflammatory bowel disease, Crohn's disease ulcerative colitis, and type 1 diabetes mellitus [14–16]. Recently, the STAT4 SNPs were found to be correlated with HSV-2-infection disease severity, for one of the SNPs, rs7572482, which is located in an intron close to the 5 un-transcribed region (UTR), the minor A allele variant correlated with the incidence of symptomatic infection [17]. The GWAS study shows that STAT4 was a genetic susceptibility loci for HBV-related HCC in the Chinese population, the risk allele G at STAT4 SNP rs7574865 is significantly associated with lower mRNA levels of STAT4 in both the HCC tissues and non-tumor tissues with HBV-related HCC [18].

Based on the above research, in the current study, we attempted to further investigate whether the STAT4 polymorphism SNPs (rs7574865, rs7572482, rs7582694, rs11889341, and rs8179673) are associated with HBV susceptibility and viral clearance in Chinese population. There is no direct evidence shown that these SNPs probably are functional SNPs and change the expression of STAT4 on molecular level. However, a few studies have revealed that among these SNPs, the SNP rs7574865 genotype was significantly associated with mRNA levels of STAT4 in both the disease of HCC and inflammatory bowel disease (IBD) [18, 19], the rs7572482 is tightly linked with rs897200 which directly affects STAT4 expression [20]. The other SNPs have strong linkage disequilibrium (LD) with rs7574865 in previous research [21].

Materials and methods

Ethics statement

The study conformed to guidelines set forth by the Declaration of Helsinki and was approved by the ethics committee of the Tongji Medical College, Huazhong University of Science and Technology. Written informed consent was obtained from each patient.

Study population

A total of 576 individuals comprising 288 cases with chronic HBV infection and 288 controls who spontaneously recovered from HBV infection were included for the analysis. Patients with chronic HBV infection were diagnosed by the repeated detection of HBsAg over a period of 6 months, and controls who spontaneously recovered from HBV infection were HBsAg(−), anti-HBs(+), and anti-HBc(+). All the subjects were recruited at Tongji Hospital, from March 2013 to November 2013 and had been screened by an automated quantitative CMIA, Architect HBsAg assay (Architect i2000, Abbott Diagnostics, Abbott Park, IL) for HBsAg, anti-HBs, and anti-HBc. The alanine amino-transferase (ALT) of all the subjects was used to assess liver function, which was measured with the Roche Cobas 8000 automated biochemistry analyzer (Roche Diagnostics, Pleasanton, CA). Abnormal liver function was defined as ALT value >41 IU. Serum HBV-DNA level for all subjects was quantified with a quantitative real-time PCR analyzer (Roche LightCycler 480; Roche Diagnostics, Pleasanton, CA) and commercial reagents (PG Biotech; Shenzhen PG Biotech Co, Ltd., Guangdong, China). The demographic characteristics and the testing results of all the subjects were shown in Table 1. All blood samples were negative for hepatitis C virus and human immunodeficiency virus. Presence of other chronic liver disease, such as autoimmune hepatitis, toxic hepatitis, or primary biliary cirrhosis was excluded.

Genotyping

Genomic DNA was extracted from whole blood as previously described [22]. The SNPs in STAT4 were genotyped using predesigned TaqMan® allelic discrimination assays in a ViiA 7 real-time polymerase chain reaction (PCR)

Table 1 Characteristics of participants in CHB and resolved HBV subjects in Han Chinese

SNP	Case	Control	*p*
Total	288	288	
Gender (M/F)	135/153	137/151	0.933
Age	42.1 ± 12.4	43.9 ± 13.9	0.103
ALT > 41	123	21	0.000
ALB (35–52)	39.9 ± 6.8	39.4 ± 6.0	0.371
TB (3.4–20.5)	19.4 ± 27.0	11.4 ± 7.9	0.000
Serum HBV-DNA	+	−	

Table 2 Prevalence of five SNPs in CHB and resolved HBV subjects in Han Chinese

SNP	Control ($n = 288$)	Case ($n = 288$)	OR (95 % CI)	p
rs7574865				
Analyzed sample	283	265		0.040
GG	114	122		
GT	132	125		
TT	37	18		
G allele	360	369		0.035
T allele	206	161	0.762 (0.593–0.981)	
rs7572482				
Analyzed sample	282	263		0.026
AA	91	83		
AG	152	121		
GG	39	59		
A allele	334	287		0.121
G allele	230	239	1.209 (0.951–1.538)	
rs7582694				
Analyzed sample	287	288		0.003
GG	114	146		
GC	134	119		
CC	39	23		
G allele	362	411		0.011
C allele	212	165	0.686 (0.535–0.878)	
rs11889341				
Analyzed sample	281	284		0.073
CC	112	136		
CT	131	123		
TT	38	25		
C allele	355	395		0.023
T allele	207	173	0.751 (0.586–0.962)	
rs8179673				
Analyzed sample	279	278		0.007
TT	107	140		
TC	134	116		
CC	38	22		
T allele	348	396		0.002
C allele	210	160	0.670 (0.521–0.861)	

Table 3 Genotype frequencies in STAT4 SNPs and association with HBV-DNA level of CHB

SNP	CHB			p
rs7574865	GG	GT	TT	
HBV $> 5\times10^2$	60	68	9	0.706
HBV $< 5\times10^2$	62	57	9	
rs7572482	AA	AG	GG	
HBV $> 5\times10^2$	43	59	33	0.661
HBV $< 5\times10^2$	40	62	26	
rs7582694	GG	CG	CC	
HBV $> 5\times10^2$	75	64	12	0.926
HBV $< 5\times10^2$	71	55	11	
rs11889341	CC	CT	TT	
HBV $> 5\times10^2$	69	66	13	0.895
HBV $< 5\times10^2$	67	57	12	
rs8179673	TT	CT	CC	
HBV $> 5\times10^2$	72	62	12	0.931
HBV $< 5\times10^2$	68	54	10	

system from Applied Biosystems (Foster City, CA, USA). All reagents required for the TaqMan assay were obtained from Applied Biosystems including universal master mix, amplifying primers, and probes. One allelic probe was labeled with FAM dye and the other with VIC dye. PCR was run in the TaqMan universal master mix at a probe concentration of 20×. The reaction was performed in a total reaction volume of 25 mL including 20 ng of genomic DNA. The reaction plates were heated for 2 min at 50 °C and for 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 90 s. The fluorescence intensity of each well was subsequently read, and fluorescence data files from each plate were analyzed by automated software.

Statistical analysis

All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). A x2-test was used to compare the distribution of genotypes among patients and controls. The association between the STAT4 SNPs and the disease status was expressed in odds ratio (OR) and their 95 % confidence intervals (CI). A $p < 0.05$ was considered

Table 4 Genotype frequencies in STAT4 SNPs and association with HBV-related liver disease of CHB

SNP	CHB			*p*
rs7574865	GG	GT	TT	
CHB	77	95	15	0.040
cirrhosis + HCC	45	30	3	
rs7572482	AA	AG	GG	
CHB	60	83	44	0.679
cirrhosis + HCC	23	38	15	
rs7582694	GG	CG	CC	
CHB	94	88	21	0.017
cirrhosis + HCC	52	31	2	
rs11889341	CC	CT	TT	
CHB	88	89	23	0.019
cirrhosis + HCC	48	34	2	
rs8179673	TT	CT	CC	
CHB	92	86	20	0.035
cirrhosis + HCC	48	30	2	

Table 5 Genotype frequencies in STAT4 SNPs and association with liver function of CHB

SNP	CHB			*p*
rs7574865	GG	GT	TT	
normal	75	70	10	0.660
abnormal	47	55	8	
rs7572482	AA	AG	GG	
normal	53	67	35	0.480
abnormal	30	54	24	
rs7582694	GG	CG	CC	
normal	77	67	12	0.829
abnormal	69	52	11	
rs11889341	CC	CT	TT	
normal	72	73	11	0.302
abnormal	64	50	14	
rs8179673	TT	CT	CC	
normal	76	68	12	0.776
abnormal	64	48	10	

to be statistically significant. The distributions of genotype for SNPs were analyzed for deviation from Hardy–Weinberg Equilibrium (HWE) using x2 analysis. A cut-off *p* value of 0.05 was set for HWE. The haplotype analysis of these SNPs was determined using the SHEsis analysis platform, as described previously [23].

Results

STAT4 polymorphisms are associated with spontaneous clearance of HBV

In the present case–control study, 288 cases with CHB and 288 controls who naturally cleared HBV infection were recruited and five SNPs of STAT4 (rs7574865, rs7572482, rs11889341, rs7582694, and rs8179673) were analyzed. The successful genotype call rates were shown in Table 2. All the SNPs were found to be in HWE both in cases and controls (data not shown).

Significant differences were found in the genotypic distribution of four SNPs, rs7574865, rs7572482, rs7582694, and rs8179673 between control and CHB groups. The minor allele frequencies (MAF) of rs7574865-T, rs7282694-C, rs11889341-T, and rs8179673-C were found to be more frequent in the controls than CHB. Although the allele frequency of rs7572482-G was not statistically different between the two groups, the genotypic distribution of the rs7572482 was significantly different between the two groups. As the controls recruited from people who naturally cleared HBV infection, these data indicate that the minor allele of these SNPs in STAT4 may be associated with spontaneous clearance of HBV and their major alleles may be associated with HBV persistent infection in the cases.

Association between STAT4 polymorphisms and HBV disease progress

We further investigated whether the above-mentioned SNPs in STAT4 were associated with HBV virus level. A total of 288 cases with CHB were divided into two groups according to the HBV-DNA levels (>500 IU/ml). Association analysis showed that none of these SNPs in STAT4 were associated with HBV-DNA levels in the two subgroups (as shown in Table 3), suggesting that these SNPs in STAT4 may not be associated with the HBV-DNA level after the HBV infection.

We also analyzed the association between these SNPs and HBV-related liver disease progress. In all patients with CHB, 10 patients with hepatitis cirrhosis and 75 patients with hepatocellular carcinoma were sorted as the disease progress group, the others as the control group, and

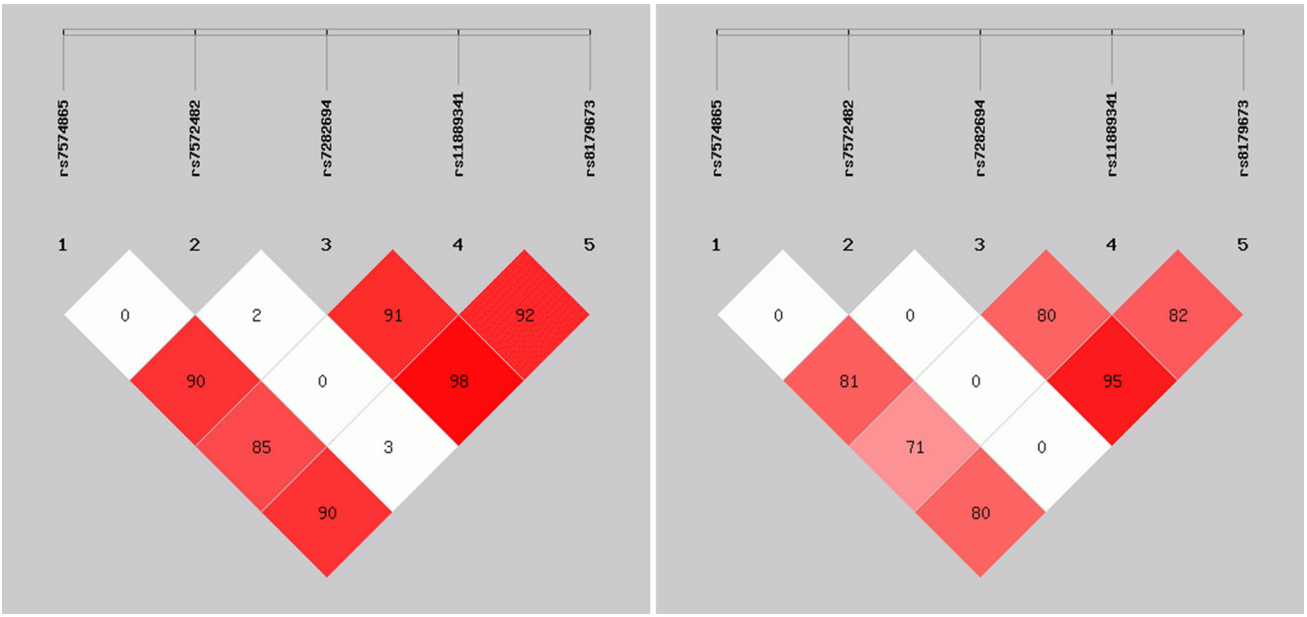

Fig. 1 Pair-wise linkage disequilibrium analysis of five STAT4 SNPs

Table 6 Frequencies of STAT4 haplotypes in all CHB patients and controls

Haplotype	Genotype frequency		*p*	OR (95 % CI)
	Control ($n = 288$)	Case ($n = 288$)		
GAGCT	0.365	0.352	0.586	1.076 (0.827–1.400)
GGGCT	0.242	0.280	0.031	1.368 (1.028–1.818)
TACTC	0.199	0.147	0.102	0.758 (0.544–1.057)
TGCTC	0.148	0.102	0.084	0.716 (0.489–1.047)

association analysis showed that the genotypes of the SNPs rs7574865, rs7282694, rs11889341, and rs8179673 were significantly different between the two groups (as shown in Table 4). The allele frequencies of rs7574865-G, rs11889341-G, rs11889341-C, and rs8179673-T were higher in the disease progress group than in the control group. Collectively, the above results indicate that the SNPs of STAT4 were not only associated with the spontaneous clearance of HBV, but also correlated with the HBV-related liver disease progress.

We also analyzed the association between the genotype of the SNPs and liver function in patients with CHB, and the genotypic distribution of the five SNPs was not significantly different between the normal liver function and abnormal liver function subgroups (as shown in Table 5).

Haplotype analysis

Linkage disequilibrium (LD) information of these five SNPs for our study groups was shown in Fig. 1. Haplotype analysis was also performed to assess the effect of the combination of these SNPs on HBV chronicity and clearance of HBV. Of the 25 possible haplotypes, four common haplotypes (with overall haplotype frequencies >0.03) were identified. As shown in Table 6, the haplotype GGGCT was found to be more frequently in the patients with CHB compared to the controls who naturally cleared HBV infection, suggesting this haplotype was significantly correlated with higher risk of persistent HBV infection.

Discussion

Viral persistence or clearance during HBV infection is dictated by the host complex immune response [24]. It has been shown that in the transgenic mouse model, adoptively transferred HBV-specific $CD8^+$ T cells inhibited HBV replication via an IFN-γ-dependent mechanism [25]. IFN-γ secretion is induced and maintained by STAT4-mediated signaling pathway. In the present study, we analyzed the association of STAT4 polymorphism and HBV infection outcome in a natural history setting.

In our study, we genotyped the five SNPs of STAT4 (rs7574865, rs7572482, rs7582694, rs11889341, and rs8179673) in a case-control study comprising subjects who spontaneously cleared HBV or had persistent infection. Our data found that the four SNPs (rs7574865,

rs7582694, rs11889341, and rs8179673) in STAT4 were significantly correlated with HBV infection. The prevalence of the minor alleles rs7574865-T, rs7282694-C, rs11889341-T, and rs8179673-C was higher in controls who naturally cleared HBV infection than in the patients with CHB, while the MAF of rs7572482-G was not significantly correlated with HBV infection. Highly linked SNPs rs7582694, rs11889341, and rs8179673 with rs7574865 in STAT4 found in haplotype analysis, consistently, rs7282694-C, rs11889341-T, and rs8179673-C showed similar results in our study. Our results suggest that SNPs of STAT4 may be correlated with HBV clearance.

The susceptibility SNP rs7574865 is located within intron 3 of STAT4, a noncoding region. It is suspected that it may influence the gene expression of STAT4 at the level of transcription or splicing variation [26]. A recent study has reported that the expression level of STAT4 in peripheral blood mononuclear cells is correlated with the risk allele of STAT4 rs7574865 [27]. STAT4 may contribute to affect HBV infection by several possible mechanisms. The main STAT4-activating cytokines are IL-12 and IL-23 leading to Th1 and Th17 differentiation with IFN-γ and IL-17 production, which are key players in a pro-inflammatory immune response [7]. IL-12 may have an important role for viral clearance in chronic HBV infection [28]. STAT4 is the major downstream transcription factor of IL-12; this finding supports that IL-12 mediated viral clearance by STAT4 signaling.

A recent study has shown that G allele at rs7574865 is associated with increased risk of HCC [18], and our data also show that the genotypes of the SNPs rs7574865, rs7282694, rs11889341, and rs8179673 are significantly different between patients with HBV-related liver disease progress (patients with hepatitis cirrhosis or HCC) and patients with CHB (non-progress). However, none of the SNPs in our study show significant association with HBV-DNA levels in patients with CHB. It has been speculated that when HBV infected the host body, the complex immunity of the host will be activated, finally cause HBV-related liver disease progress and HCC.

In the current study, the four SNPs in STAT4 (rs7574865, rs7282694, rs11889341, and rs8179673) showing the similar findings which were correlated with HBV spontaneously clearance in the Chinese Han population may be explained by the strong LD between the STAT4 SNPs [21]. Furthermore, one of the haplotypes constructed with the five SNPs showed increased risk with HBV infection. Our data revealed significant association with spontaneous clearance of HBV infection and STAT4 polymorphism.

The current study is limited because there are relatively small number of patients, and because some of the phenotypes (ALT) examined were related to disease activity, and therefore may have fluctuated naturally or as a result of treatment, another limitation is the lack of complete information regarding the causal polymorphisms and their exact functional roles.

In summary, our results identified STAT4 SNPs as a disease-susceptible gene in spontaneous clearance of HBV and the progress of the HBV-related liver disease. Further studies will be required to investigate how the different genotypes affect the expression and regulate STAT4 in the liver.

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