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Association between Signal Transducer and Activator of Transcription 4 Genetic Polymorphisms and the Spontaneous Clearance of Hepatitis B Surface Antigen: A Large Population Case Control Study in China

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ABSTRACT

Aim: Several host factors mediating immune response influence susceptibility to Hepatitis B Virus (HBV) infection, ability to clear the virus, and maintenance of a chronic state. Signal Transducer and Activator of Transcription 4 (STAT4) variations are correlated with the risk of developing autoimmune diseases. However, there have been few studies to assess the relationship between STAT4 variations and Hepatitis B surface Antigen (HBsAg) clearance in adults infected with HBV. Our aim was to evaluate the association between genetic variants in STAT4 and HBsAg clearance in a large sample size population.

Methods: This case control study included Chronic Hepatitis B (CHB) (*n* = 1.688), HBsAg Clearance after Treatment (TC) (*n* = 170), HBV Uninfected (HC) (*n* = 1.012), and HBsAg Spontaneous Clearance (SC) (*n* = 1,052) patients. In the CHB group, patients were categorized into four subgroups: the Immune Tolerant (IT), Immune Active (IA), Inactive (IC), and Immune Reactivation (IR) phases, with 97, 855, 198, and 538 patients in each subgroup, respectively.

Results: We found that the G allele in STAT4 rs7574865 was more frequent in the CHB and TC groups, compared with the SC group, whereas the STAT4 rs7574865 GG genotype was more frequent in the CHB and TC group, compared with the SC group in the dominant model. However, there was no statistical significance in genotype between TC and CHB, nor between the IT, IA, IC, and IR groups.

Conclusions: The prevalence of the minor allele rs7574865 T was higher in subjects with spontaneously cleared HBV infections than in CHB patients.

INTRODUCTION

Hepatitis B Virus (HBV) infection is a major global health problem, though the effective HBV vaccine is widely used. HBV infects about 2.5 billion people worldwide and 300 million of those are chronically infected [1,2]. China has one of the highest HBV carrier prevalences in the world. It is estimated that there are approximately

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30 million Chronic HBV (CHB) patients in China, almost 5% - 6% of the entire population [3]. About 95% of adults can successfully clear HBV after being infected and 5% - 10% will develop CHB; even fewer progress to liver failure [4]. Several host and viral genetic factors are known to influence susceptibility to HBV infection, ability to clear the virus, and maintenance of a chronic state. Among these factors, host immunogenetic background has a significant role in mediating the clinical progression of viral hepatitis infections [5-8].

Signal Transducer and Activator of Transcription 4 (STAT4), a member of the STAT family, can be activated by several cytokines including IL 12, type I Interferon (IFN), and IL 23, as well as lead to T helper (Th) type 1 and 17 differentiation, monocyte activation, and production of IFN-γ [9]. In fact, more and more studies indicated that STAT4 variations are correlated with the risk of developing autoimmune diseases. The Single Nucleotide Polymorphism (SNP) rs7574865, which is in the third intron of the STAT4 gene, is associated with a higher risk of rheumatoid arthritis [10-13], Systemic Lupus Erythematosus (SLE) [14], type I diabetes [15], and systemic scleroderma [16]. Furthermore, it is reported that the G allele of rs7574865 can increase the risk of Hepatocellular Carcinoma (HCC) and decrease the efficacy of IFN treatment in CHB patients [17-19].

However, few studies have assessed the relationship between STAT4 variations and Hepatitis B surface Antigen (HBsAg) clearance in adults infected with HBV, and the results of these studies have been inconsistent. Our aim was to evaluate the association between the genetic variations in STAT4 and HBsAg clearance in HBV infected adults in a large sample size population.

METHODS

Patients

This case control study enrolled 1,858 of 2,398 patients with CHB infection, aged 18-70 years, who were followed up at our hospitals between March 2016 and December 2018. These patients were divided into two groups: CHB patients, and HBsAg Clearance after Treatment (TC). There were 1,688 and 170 patients in each group, respectively. In the CHB group, the patients were categorized into four subgroups according to the natural history [20]: immune Tolerant Phase (IT), Immune Active Phase (IA), Inactive Phase (IC), and Immune Reactivation Phase (IR) with 97, 855, 198, and 538 patients in each subgroup, respectively. TC was defined as CHB patients who received antiviral treatment for at least 1 year and had HBsAg clearance (Figure 1).

Major inclusion criteria for the CHB group included HBV monoinfection with detectable HBsAg at screening and for at least 6 months prior to the onset of the study. The evidence of cirrhosis or HCC by imaging, or a history of alcohol abuse were excluded.

Of the 3,425 healthy subjects, 2,064 were enrolled into this study as controls. These subjects were divided into the HBV uninfected (HC) group and HBsAg Spontaneous Clearance (SC) group with 1,012 and 1,052 individuals in each group, respectively. The HC group was defined as those

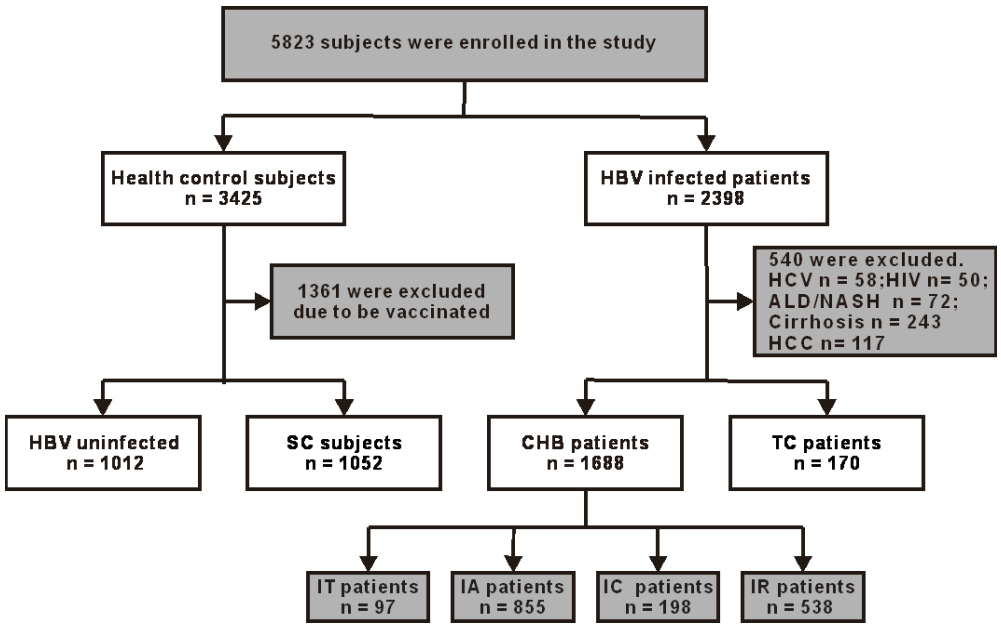

Figure 1 Patient disposition.
ALD: Alcoholic Liver Disease; CHB: Chronic Hepatitis B; HCC: Hepatocellular Carcinoma; IA: Immune-Active Phase; IC: Inactive CHB Phase; IR: Immune Reactivation Phase; IT: Immune-Tolerant Phase; NASH: Non-Alcoholic Steatohepatitis; SC: Hepatitis B Surface Antigen (Hbsag) Spontaneous Clearance; TC: Hbsag Clearance after Treatment.

in which all HBV markers were negative. SC subjects were defined as those who were negative for HBsAg, but positive for both the Hepatitis B surface Antibody (HBsAb) and Hepatitis B core Antibody (HBcAb). All control subjects had no other ailments and had normal liver function (Figure 1).

The study was in complied with the Helsinki Declaration. Our Center's Ethics Committee reviewed and approved the study design (KY2016-196) and all patients provided written informed consent.

Study design

All data of the subjects were collected at the baseline, including history, liver function, and White Blood Cell Count (WBC), Platelet Count (PLT), and HBV markers. In CHB infected patients, HBV DNA load and HBV genotype were tested.

DNA isolation and rs7574865 genotyping

Genomic DNA was extracted from 1 ml of peripheral whole blood from each subject using Qiagen DNA kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The STAT4 variants were genotyped using a real-time Polymerase Chain Reaction (PCR) in a 50 μl reaction system including 20ng of genomic DNA. The thermal cycling conditions applied were initial activation for 5 min at 95˚C, 40 cycles of denaturation at 94˚C for 30s, annealing at 57˚C for 30s, and extension at 72˚C for 30s. All the samples were analyzed by direct sequencing.

Statistical methods

All data were processed using Stata 16.0 software (Stata Corporation, College Station, TX, USA). A two-tailed $p < 0.05$ was considered statistically significant. Continuous variables were expressed as median (range) and categorical variables were expressed as number (%). Continuous variables were analyzed using the Student's t-test or Wilcoxon test. Categorical variables were analyzed using the chi-square test or Fisher's exact test. The logistic regression model was used to estimate the univariate and multivariate Odds Ratios (OR) of various factors related to HBsAg clearance after HBV infection.

RESULTS

Baseline characteristics of the subjects

In this study, 5,823 subjects were screened, and 3,922 met the inclusion criteria and were included (Figure 1). The proportion of male, age, and the values of Alanine Transaminase (ALT) and Aspartate Aminotransferase (AST) were higher in the CHB and TC groups than in the HC and SC groups. However, the values of WBC and PLT in the CHB and TC groups were lower than in the HC and SC groups. In CHB patients, the major HBV genotype was the C genotype, and the percentage of Hepatitis B e Antigen (HBeAg) positive patients was 56% (Table 1).

Association between STAT4 polymorphisms and HBsAg clearance

To evaluate the relationship between rs7574865 variation and HBsAg spontaneous clearance, the SC group was compared with the CHB, TC, and HC groups. The frequency of the G allele in STAT4 rs7574865 was higher in the CHB and TC groups, compared with the SC group (CHB *vs.* SC: 70% *vs.* 67%, $p = 0.018$; TC *vs.* SC: 72% *vs.* 67%, $p = 0.047$)

Table 1: The characteristics of the subjects in the study.

	HC	SC	CHB	TC
Total (n)	1.012	1.052	1.688	170
Male (%)	548 (54%)	548 (52%)	1,097 (65%)	132 (78%)
Age (years)	31 (18 - 69)	36 (20 - 65)	45 (18 - 68)	43 (24 - 65)
ALT (U/L)	20 (4 - 43)	20 (5 - 38)	47 (6 - 1586)	43 (9 - 112)
AST (U/L)	20 (9 - 37)	20 (6 - 31)	37 (11 - 899)	33 (12 - 117)
TBil (μmol/L)	16 (2-21)	16 (2-29)	22 (3-40)	20 (6 - 38)
ALB (g/L)	47 (36 - 54)	47 (39 - 57)	45 (36 - 54)	46 (40 - 55)
WBC ($\times 10^9$/L)	6.65 (3.83 - 9.92)	6.63 (3.97 - 9.53)	5.66 (3.61 - 9.90)	5.01 (2.41 - 9.40)
PLT ($\times 10^{12}$/L)	244 (116 - 451)	241 (111 - 447)	177 (91 - 375)	172 (77 - 378)
HBV genotype (B/C/unknown)	-	-	371/632/685 (22%/37%/41%)	-
HBsAg ($\log_{10}$IU/ml)	-	-	4.02 (1.23 - 4.72)	-
HBeAg positive (%)	-	-	952 (56%)	-
HBV DNA ($\log_{10}$IU/ml)	-	-	7.05 (0.00 - 9.46)	-

Values are expressed as median (range) or number of subjects (%).
HC: HBV Uninfected; HBsAg: Hepatitis B Surface Antigen; HBeAg: Hepatitis B e-Antigen; SC: Hepatitis B Surface Antigen (Hbsag) Spontaneous Clearance; CHB: Chronic Hepatitis B; TC: HBsAg Clearance after Treatment; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; TBil: Total Bilirubin; ALB: Albumin; WB: White Blood Cell Count; PLT: Platelet Count; HBV: Hepatitis B Virus; HBsAg: Hepatitis B Surface Antigen; HBeAg: Hepatitis B e-Antigen.

(Figure 2A). However, there was no significant difference between the HC and CHB group (Supplementary table 1).

The similar trend was observed in the dominant model. The STAT4 rs7574865 GG genotype was more frequent in the CHB and TC groups, compared with the SC group (CHB *vs.* SC: 49% *vs.* 44%, p = 0.023; TC *vs.* SC: 53% *vs.* 44%, p = 0.032; Figure 2B). However, there was no significant difference between the HC and CHB groups (Supplementary table 1), and no statistical significance in the recession model between the CHB, TC, and SC groups (Figure 2C).

Furthermore, the association between rs7574865 variation and HBsAg clearance after the treatment was analyzed, which indicated no statistical significance regarding the genotype between the TC and CHB groups (G allele: 72% *vs.* 70%, p = 0.359; the dominant model: 53% *vs.* 49%, p = 0.272; the recession model: 8% *vs.* 9%, p = 0.857; figure 2).

Association between STAT4 polymorphisms and HBV natural history

The relationship between the rs7574865 variation and HBV natural history was also analyzed. In the recession model, the frequency of rs7574865 TT genotype was higher in the IR group than the IT group (93% *vs.* 87%, p = 0.034; figure 3C). However, the trend was not similar in the dominant model and genotype allele between the IR and IT groups (Supplementary table 2). There was no statistical genotype significance between the IT, IA, IC, and IR groups (Supplementary table 2).

Predictors associated with HBsAg spontaneous clearance

The univariate and multivariate predictors, and OR for HBsAg spontaneous clearance are shown in table 2. According to the multivariate analysis, the independent predictors for HBsAg spontaneous clearance were STAT4 rs7574865 genotype, gender, age, ALT, total bilirubin, albumin, and PLT.

DISCUSSION

HBV persistence or clearance during viral infection is dictated by the host's complex immune response [21]. The relationship between the SNP of STAT4 and HBV clearance is a heated topic [17-19], Our study revealed a significant association between HBsAg spontaneous clearance and variations in STAT4. The prevalence of the minor allele rs7574865 T was higher in the subjects who naturally cleared HBV infection than in the CHB patients, which indicated that the subjects with rs7574865 T have a better chance of clearing the HBV infection spontaneously. However, there was no statistical significance in genotype between HBsAg

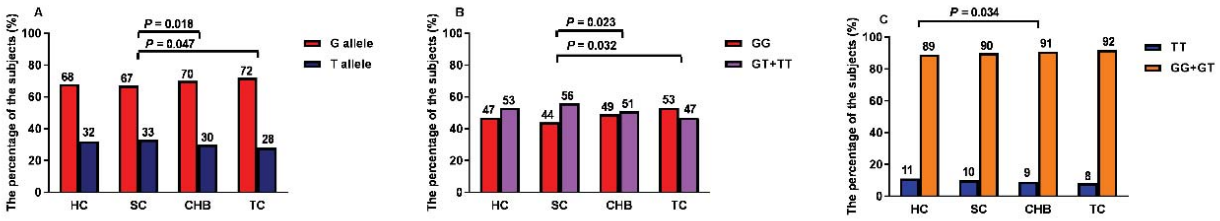

Figure 2 Association between rs7574865 variation in STAT4 and HBsAg clearance. (A) The frequency of allele; (B) The frequency of genotype in dominant model; (C) The frequency of genotype in recessive model. SC: Hepatitis B Surface Antigen (Hbsag) Spontaneous Clearance; TC: HBsAg clearance after treatment.

Table 2: Predictors for HBsAg Spontaneous clearance.

	Univariate Analysis		Multivariate Analysis	
	HR (95% Confidence Interval)	*p* Value	HR (95% Confidence Interval)	*p* Value
Gender	1.6895 (1.4442 - 1.9764)	<0.001	1.3318 (1.0487 - 1.6913)	0.019
Age	1.0695 (1.0613 - 1.0778)	<0.001	1.0558 (1.0455 - 1.0662)	<0.001
ALT	1.0586 (1.0510 - 1.0663)	<0.001	1.0492 (1.0378 - 1.0608)	<0.001
AST	1.1118 (1.0970 - 1.1267)	<0.001	1.0025 (0.9835 - 1.0218)	0.800
TBil	1.0450 (1.0347 - 1.0556)	<0.001	1.0381 (1.0229 - 1.0535)	<0.001
ALB	0.8301 (0.8073 - 0.8535)	<0.001	0.8035 (0.7688 - 0.8398)	<0.001
WBC	0.8267 (0.7907 - 0.8640)	<0.001	0.9942 (0.9809 - 1.0077)	0.397
PLT	0.9848 (0.9833 - 0.9863)	<0.001	0.9883 (0.9866 - 0.9902)	<0.001
rs7574865 polymorphism	0.8362 (0.7164 - 0.9761)	0.023	0.9102 (0.7404 - 0.9235)	0.038

ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; Tbil: Total Bilirubin; ALB: Albumin; WBC: White Blood Cell Count; PLT: Platelet Count; HR: Hazard Ratio.

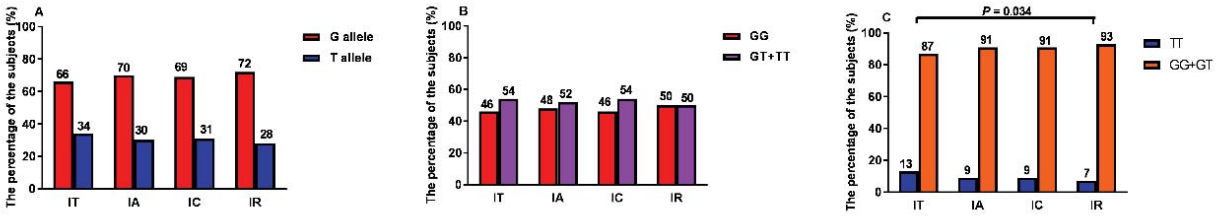

Figure 3 Association between rs7574865 variation in STAT4 and HBV natural history. (A) The frequency of allele; (B) The frequency of genotype in dominant model; (C) The frequency of genotype in recessive model. IA: Immune-Active Phase; IC: Inactive CHB phase; IR: Immune Reactivation Phase; IT: Immune-Tolerant Phase.

clearance after treatment and STAT4 variations, nor between the IT, IA, IC, and IR groups.

STAT4 is an important cytosolic factor, which is involved in transmitting signals stimulated by I type IFN and IL12, which induce INF-γ and activate the immune response [22-24]. Recently, several studies have indicated that the polymorphisms of STAT4 contribute to autoimmune diseases [25-27]. However, few studies have identified SNP rs7574865 in STAT4 as the genetic susceptibility locus for HBV spontaneous clearance, and results were inconsistent. Lu, et al. [28] found the association between SNP rs7574865 and HBV spontaneous clearance in a study including 288 subjects. However, in two studies, Liao, et al. [29,30] failed to find the relationship between SNP rs7574865 in STAT4 and HBV spontaneous clearance. The difference in the results of these studies may be attributed to the small sample size, as well as the different regions and races. In our large sample size study, which included 3,922 subjects, we confirmed that HBV spontaneous clearance is significantly associated with the rs7574865 variation in STAT4, regardless of the allele or dominant (recessive) model analysis. Furthermore, logistic regression analysis was used to eliminate confounders, and the results were still significant. Therefore, our study indicated that variations in STAT4 play a critical role in HBV spontaneous clearance.

Nowadays, the precise mechanism by which variations in STAT4 influence natural clearance of HBV is not clear. SNP rs7574865 is located on intron 3 of STAT4 and is a non-coding region. However, rs7574865 T was reported to express more STAT4 and proteins than rs7574865 G, [17,31-32] which produced more INF-γ. A study indicated that T cells from SLE patients carrying the STAT4 risk allele T displayed an augmented response to IL-12 and induced INF-γ production [18]. INF-γ is a critical cytosolic factor to control antiviral and immune response [33]. Therefore, we speculated that subjects infected with HBV with rs7574865 T may express more STAT4 genes and proteins, produce more INF-γ, and have a higher chance of clearing HBV naturally. However, there are more factors influencing HBsAg clearance after treatment, including drug types, duration of the treatment and the host factor [18,19]. Therefore, there is no association between HBsAg clearance after treatment and STAT4 variations.

Furthermore, the relationship between CHB patients in different natural history phases and SNP rs7574865 was analyzed. The results indicated that there was no significant association between genetic variations in STAT4 and CHB natural history. In addition, genetic variations in STAT4 were not correlated with the ALT, load of HBV DNA, and HBeAg positive in CHB patients, which is consistent with the results reported by Lu, et al. [28].

Although the sample size of the study is large, there are still limitations. This is a case control study, which has a weaker evidence power than the prospective study due to lack of follow-up information. Moreover, there were more confounders than in the prospective study, although logistic regression analysis was used. Therefore, matched case control studies are warranted to obtain more potent evidence.

In summary, SNP rs7574865 in STAT4 is correlated with HBV spontaneous clearance. Subjects with the T allele had a higher chance of clearing HBV naturally than patients with the G allele. However, there is no relationship between genetic variations in STAT4 and CHB natural history phases.

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Supplementary table 1: Impact of STAT4 rs7574865 polymorphism on HBV outcome.

	a	b	c	d	*p* value					
	HC (*n* = 1,012)	SC (*n* = 1,052)	CHB (*n* = 1,688)	TC (*n* = 170)	a vs. b	a vs. c	a vs. d	b vs. c	b vs. d	c vs. d
STAT4 polymorphism										
GG	474 (47%)	464 (44%)	819 (49%)	90 (53%)	0.269	0.103	0.272	0.056	0.098	0.538
GT	426 (42%)	480 (46%)	724 (43%)	66 (39%)						
TT	112 (11%)	108 (10%)	145 (9%)	14 (8%)						
Recessive model										
GG+GT	900 (89%)	944 (90%)	1,543 (91%)	156 (92%)	0.556	0.034	0.268	0.141	0.412	0.875
TT	112 (11%)	108 (10%)	145 (9%)	14 (8%)						
Dominant model										
GG	474 (47%)	464 (44%)	819 (49%)	90 (53%)	0.213	0.397	0.140	0.023	0.032	0.272
GT+TT	538 (53%)	588 (56%)	869 (51%)	80 (47%)						
Allele										
G	1,374 (68%)	1,408 (67%)	2,362 (70%)	246 (72%)	0.508	0.109	0.101	0.018	0.047	0.359
T	650 (32%)	696 (33%)	1,014 (30%)	94 (28%)						

Values are expressed as number of subjects (%).
HC: HBV Uninfected; SC: Hepatitis B Surface Antigen (Hbsag) Spontaneous Clearance; CHB: Chronic Hepatitis B; TC: Hbsag Clearance after Treatment.

Supplementary table 2: Impact of STAT4 rs7574865 polymorphism on chronic hepatitis B patients.

	a	b	c	d	*p* value					
	IT (*n* = 97)	IA (*n* = 855)	IC (*n* = 198)	IR (n = 538)	a *vs.* b	a *vs.* c	a *vs.* d	b *vs.* c	b *vs.* d	c *vs.* d
STAT4 polymorphism										
GG	45 (46%)	412 (48%)	92 (46%)	270 (50%)	0.349	0.491	0.107	0.908	0.446	0.526
GT	39 (40%)	367 (43%)	88(45%)	230 (43%)						
TT	13 (14%)	76 (9%)	18 (9%)	38 (7%)						
Recessive model										
GG+GT	84 (87%)	779 (91%)	180 (91%)	500 (93%)	0.148	0.257	0.034	0.928	0.226	0.358
TT	13 (13%)	76 (9%)	18 (9%)	38 (7%)						
Dominant model										
GG	45 (46%)	412 (48%)	92 (46%)	270 (50%)	0.737	0.991	0.491	0.662	0.468	0.371
GT+TT	52 (54%)	443 (52%)	106 (54%)	268 (50%)						
Allele										
G	129 (66%)	1,191(70%)	272 (69%)	770 (72%)	0.367	0.592	0.153	0.708	0.282	0.282
T	65 (34%)	519 (30%)	124 (31%)	306 (28%)						

Values are expressed as number of subjects (%).
IT: Immune-Tolerant Phase; IA: Immune-Tolerant Phase; IC: Inactive Chronic Hepatitis B Phase; IR: Immune Reactivation Phase.

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