Autophagy-Related 5 Gene rs510432 Polymorphism Is Associated with Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus Infection

Na Li, Xiude Fan, Xiaoyun Wang, Huan Deng, Kun Zhang, Xiaoge Zhang, Qunying Han, Yilv, and Zhengwen Liu

Department of Infectious Diseases, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, People's Republic of China; Department of Hepatobiliary Surgery, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, People's Republic of China; Institute of Advanced Surgical Technology and Engineering, Xi'an Jiaotong University, Xi'an, Shaanxi, People's Republic of China

ABSTRACT

Background: Despite the identification of autophagy-related protein 5 (ATG5) as a molecule involved in the activated autophagy machinery during hepatitis B virus (HBV) infection and hepatocarcinogenesis, the consequences of ATG5 mutation carriage for patients with chronic HBV infection remain unclear. This study examined the association of ATG5 polymorphisms with HBV-related diseases including hepatocellular carcinoma (HCC).

Patients and Methods: Two functionally relevant polymorphisms ATG5 rs573775 and rs510432 were genotyped by ligase detection reaction-polymerase chain reaction in 403 patients with chronic HBV infection (171 chronic hepatitis, 119 cirrhosis and 113 HCC) and 196 healthy controls. Univariate and multivariate logistic regression was performed to evaluate factors associated with HCC.

Results: The rs573775 genotype and allele frequencies had no significant differences between patients with different clinical diseases. However, HCC patients had significantly higher frequency of rs510432 genotype AA (odds ratio [OR] 2.185, 95% confidence interval [CI] 1.042–4.581, P = 0.037, P value by Bonferroni correction [Pc] = 0.074) and allele A (OR 1.435, 95% CI 1.023–2.013, P = 0.036) than chronic hepatitis patients. In multivariate analyses, rs510432 allele A-containing genotypes (AA+GA) were independently associated with cirrhosis in comparison to chronic hepatitis (OR 1.927, 95% CI 1.011–3.017, P = 0.032). The rs510432 genotypes AA+GA were also independently associated with HCC in comparison to chronic hepatitis (OR 2.583, 95% CI 1.025–6.348, P = 0.032) or chronic HBV infection without HCC (OR 2.632, 95% CI 1.067–6.482, P = 0.032).

Conclusion: These results indicate that rs510432 genotypes AA+GA are associated with disease progression and HCC risk in chronic HBV infection, providing novel evidence for a role of ATG5 in the pathogenesis of HBV-related HCC.

Abbreviations: HBV: hepatitis B virus; HCC: hepatocellular carcinoma; TNFSF10: tumor necrosis factor superfamily member 10; ATG5: autophagy-related protein 5; DNA: deoxyribonucleic acid; LDR-PCR: ligase detection reactions-polymerase chain reaction; PCR: polymerase chain reaction; SLE: systemic lupus erythematosus; BD: Behçet's disease; IL-10: interleukin-10; LPS: lipopolysaccharide; PBMC: peripheral blood mononuclear cells; CWP: coal workers' pneumoconiosis; TNF-α: tumor necrosis factor-α

KEYWORDS

Hepatitis B virus infection; hepatocellular carcinoma; ATG5; polymorphism
Introduction

Chronic hepatitis B virus (HBV) infection is associated with chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) and remains a severe public health issue worldwide (Schweitzer et al., 2015). The natural course of chronic HBV infection is a complex dynamic process of the interaction between the virus and the host immune responses (European Association for the Study of the Liver, 2017). The pathogenic mechanisms of chronic HBV infection in relation to the various clinical outcomes remain largely to be investigated.

Autophagy is regarded as a cellular process directed at recycling cellular proteins and eliminating intracellular microorganisms. Increasing evidence suggests that autophagy has diverse physiological functions and deficiency in autophagy has been implicated in a variety of diseases including HBV-associated liver diseases. Studies have shown that HBV can induce and enhance the autophagic process and this autophagic response can enhance HBV replication and is involved in the pathogenesis of HBV infection (Sir et al., 2010; Tian et al., 2011; Xie et al., 2018). Autophagy may influence the efficiency priming of virus-specific CD8+ T cells (Uhl et al., 2009) which have been demonstrated to play a pivotal role in both the virus control and liver damage of chronic HBV infection (Maini et al., 2000). Autophagy is also involved in the HBV evasion from tumor necrosis factor superfamily member 10 (TNFSF10)-mediated antiviral immunity (Shin et al., 2016). Dysregulation of autophagy has also been shown to be significantly correlated with HBV infection and HBV-associated HCC (Kunanopparat et al., 2016; Lan et al., 2014).

Autophagy-related protein 5 (ATG5), an essential component of the autophagic machinery encoded by Autophagy-related 5 gene (ATG5), is involved in the crucial phase of autophagy, autophagosome formation (Vij et al., 2016). In terms of HBV and HBV-related diseases, ATG5 is involved in the activated autophagy machinery during HBV infection to enhance HBV replication (Li et al., 2011) and plays an essential role for HBV capsid biogenesis (Döring and Prange, 2015; Döring et al., 2018). Dysregulation of immune response is involved in pathogenesis of cirrhosis and HCC (Elwan et al., 2018). ATG5 appears to block innate antiviral immune responses (Jounai et al., 2007) and its expression is involved in the autophagic influence on the efficiency of priming of virus-specific CD8+ T cells (Uhl et al., 2009). ATG5 mRNA expression was significantly increased in HBV-infected hepatic cells (HepG2.2.15 cells) and its protein levels were increased in tumor liver tissues mainly from HCC patients with HBV infection (Kunanopparat et al., 2016). ATG5 is involved in the decreased apoptosis of HCC cells and increased HCC tumor growth (Yang et al., 2018) and its expression shows a significant correlation with HBV infection and overall survival rate of HCC patients (Lan et al., 2014).

In view of the significant involvement of autophagy and ATG5 in HBV replication and HBV-related diseases including HCC, we hypothesize that ATG5 gene polymorphisms, especially the functionally relevant polymorphisms, may be associated with chronic HBV infection and the development of HBV-related liver diseases. A study in Thai population indicated that polymorphisms of the ATG5 gene might be involved in HBV-related HCC (Wisetsathorna et al., 2017). However, the possible associations of ATG5 polymorphisms with chronic HBV infection and HBV-related HCC have not been addressed in other
ethnic populations so far. Therefore, the present study determined ATG5 polymorphisms in patients with chronic HBV infection of various liver diseases and analyzed the associations with clinical diseases in Chinese Han population.

**Patients and methods**

**Study population**

Adults with chronic HBV infection were enrolled from the First Affiliated Hospital of Xi’an Jiaotong University from March 2009 to May 2013. Fasting blood was collected in the morning. Three milliliter coagulation blood was used for serum separation by centrifugation and the serum samples were frozen at −20°C until use and 2 mL of EDTA-anticoagulated whole blood was frozen at −30°C for the extraction of human genomic DNA. The diagnosis of the patients was in accordance with the diagnostic criteria of guidance (Terrault et al. 2018). Other liver diseases (hepatitis A, hepatitis C and hepatitis E, drug-induced liver injury, steatohepatitis, alcoholic hepatitis, autoimmune hepatitis, and Wilson’s disease), diseases associated with metabolism disorders (diabetes, acquired immunodeficiency syndrome and hyperthyroidism), and complications with severe cardiovascular and respiratory diseases as well as renal impairment were excluded. Patients under 18 years of age were excluded. A history of antiviral therapy (interferon or nucleos(t)ides) and pregnancy were also excluded in the patients with chronic HBV infection. Individuals who had no history of hepatitis with normal liver function and had no other diseases were included as healthy controls. The study recruited 403 patients with chronic HBV infection (male/female, 283/120; mean age, 40.37 ± 13.68 [18–78] years) and 196 healthy controls (male/female, 127/69; mean age, 38.58 ± 14.29 [18–76] years). All the subjects are of Chinese Han ethnicity, were permanent residents of Shaanxi Province and had no close kinship. The study was approved by the Ethics Committee of the First Affiliated Hospital of Xi’an Jiaotong University and performed in accordance with the Declaration of Helsinki. All subjects completed the informed consent and voluntarily participated in the study.

**Genotyping of rs573775 and rs510432 polymorphisms**

ATG5 rs573775 (a C/T single-nucleotide variation on human chromosome 6) and rs510432 (an A/G single-nucleotide variation on human chromosome 6) polymorphisms were selected given their potential relevance of function demonstrated by previous studies (López et al., 2013; Martin et al., 2012; Shao et al., 2017; Zheng et al., 2015).

Human genomic DNA was extracted by commercial DNA Extraction Kit (Beijing Tiangen Biochemical Technology Co., Ltd., Beijing, China). Genotyping of rs573775 and rs510432 was performed using high-temperature ligase detection reaction-polymerase chain reaction (PCR).

The primers were designed using oligo6.0 and primer5.0 software and synthesized by Shanghai Biotechnology Co., Ltd (Shanghai, China). The polymorphic regions of the ATG5 gene were amplified by a multiplex PCR with specific primers (Supplementary Table 1) which produced a 285 and 314 bp product, respectively.
For multiplex PCR, the reaction (10 µL) contained 0.5 µL 1× GC-I buffer (TaKaRa Bio, Dalian, China), 0.5 µL Mg²⁺ (3.0 mM) (TaKaRa Bio, Dalian, China), 0.5 µL dNTP (0.3 mM) (TaKaRa Bio, Dalian, China), 0.5 µL 1 U HotStar Taq Polymerase (Qiagen Inc.), 1 µL of sample DNA (5–10 ng/µL), 2 µL of multiplex PCR primers (3 µM for rs573775 and 1 µM for rs510432, respectively) and 5 µL ddH₂O. PCR programs were as follows: 1 cycle of 95°C for 2 min; 11 cycles of 94°C for 20 s, 65°C for 40 s, and 72°C for 1.5 min; 24 cycles of 94°C for 20 s, 59°C for 30 s, and 72°C for 1.5 min; and 1 cycle of 72°C for 2 min and 4°C for forever. 2,720 Thermal Cycler (Applied Biosystems, Inc. Carlsbad, USA) and Gel Imaging & Analysis System (Applied Biosystems, Inc. Carlsbad, USA) were used for analysis. Multiplex PCR product was purified by addition of 5 U of SAP (Promega, Madison, USA) and 2 U of Exonuclease I (EXO-I, Epicentre, Madison, USA) to 10 µL of PCR product, incubation at 37°C for 1 h, and then inactivation at 75°C for 15 min.

For ligation reaction, the primers for rs573775 and rs510432 were shown in Supplementary Table 1. The ligation reaction was carried out in a volume of 10 µL containing 1 µL of 10× ligation buffer (TaKaRa Bio, Dalian, China), 0.2 µL of high temperature ligase (New England Biolabs, Ipswich, MA, USA), 0.4 µL (1 µM) of 5’ ligation primer mixture (TaKaRa Bio, Dalian, China), 0.4 µL (2 µM) of 3’ ligation primer mixture (TaKaRa Bio, Dalian, China), 2 µL of purified multiplex PCR products and 6 µL of ddH₂O. The ligation program was as follows: 38 cycles of 94°C for 1 min and 56°C for 4 min; and 1 cycle of 4°C forever. A volume of 0.5 µL of the diluted ligation product, 0.5 µL of Liz500 SIZE STANDARD (Applied Biosystems, Carlsbad, CA, USA) and 9 µL of Hi-Di (Applied Biosystems, Carlsbad, CA, USA) were thoroughly mixed, denatured at 95°C for 5 min and then analyzed by ABI 3730 XL sequencer (ABI, USA). The data were analyzed by GeneMapper 4.1 (Applied Biosystems, CA, USA).

**Power analysis**

At the 5% significance level and a minor allele frequencies (MAFs) of 0.05 and 0.15, there is ≥80% power for detecting a dominant effect with an odds ratio (OR) of 1.2 and 1.5, respectively, for chronic hepatitis, an OR of 1.6 and 1.1, respectively, for liver cirrhosis, and an OR of 1.9 and 1.2, respectively, for HCC (Supplementary Table 2). The Genetic Power Calculator (Purcell et al., 2003) was used for power calculations, setting ‘prevalence’ of chronic hepatitis to 42.4%, liver cirrhosis to 29.5%, and HCC to 28.0% with the available sample size of patients with chronic HBV infection, D-prime to 1, and type 1 error rate to 0.05.

**Statistical analysis**

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc. Chicago). The t-test or χ² test was used to compare the general data of patients with chronic HBV infection, and healthy controls. Genotype frequencies were tested for Hardy–Weinberg equilibrium. The χ² test was used for the difference in genotype and allele frequencies between patients with chronic HBV infection, and healthy controls. Univariate and multivariate analyses with logistic regression were used to analyze independent risk factors associated with HBV-related cirrhosis or HCC. P < 0.05 was
considered statistically significant. The Bonferroni procedure was applied to account for multiple testing where appropriate.

Results

Demographics and Hardy-Weinberg equilibrium of the genotypes of ATG5 rs573775 and rs510432 polymorphisms in the study population

The study enrolled 403 patients with chronic HBV infection (male/female, 283/120; mean age, 40.37 ± 13.68 [18–78] years) and 196 healthy controls (male/female, 127/69; mean age, 38.58 ± 14.29 [18–76] years). There were no significant differences in age and gender between the two groups (both P > 0.05). The genotype distribution of the genotypes of ATG5 rs573775 and rs510432 in patients with chronic HBV infection and healthy controls were all in Hardy–Weinberg equilibrium (Supplementary Table 3).

Genotype and allele frequencies of ATG5 polymorphisms in chronic HBV infection and healthy controls

The genotype and allele frequencies of rs573775 and rs510432 between patients with chronic HBV infection and healthy controls had no significant differences (Supplementary Table 4).

Genotype and allele frequencies of ATG5 polymorphisms in chronic HBV-infected patients with different clinical diseases

The clinical diagnosis of the 403 patients with chronic HBV infection included 171 chronic hepatitis, 119 liver cirrhosis, and 113 HCC. Calculation of sample size and power showed that the minimum effect size in which we had ≥80% power at 5% significance level and a MAF of 0.05 and 0.15 for chronic hepatitis, cirrhosis, and HCC was 167, 103, and 100, respectively (Supplementary Table 2).

The genotype and allele frequencies of rs573775 had no significant differences between patients with chronic hepatitis, liver cirrhosis, and HCC (Table 1). In regard to rs510432, patients with HCC had higher frequency of rs510432 genotype AA (20.4% vs. 14.6%, OR 2.185, 95% CI 1.042–4.581, P = 0.037, P value by Bonferroni correction [Pc] = 0.074) and allele A (49.6% vs. 40.6%, OR 1.435, 95% CI 1.023–2.013, Pc = 0.036) than patients with chronic hepatitis (Table 1).

Haplotype frequencies of ATG5 rs573775 and rs510432 polymorphisms in patients with chronic HBV infection and healthy controls

Comparison of ATG5 haplotype (rs573775–rs510432) frequencies in patients with chronic HBV infection and healthy controls showed that patients with chronic HBV infection had significantly lower haplotypes C-G (24.6% vs. 34.4%, OR 0.678, 95% CI 0.508–0.905, Pc = 0.024) and T-G (1.9% vs. 24.8%, OR 0.076, 95% CI 0.044–0.134, Pc ≤ 0.001) but higher
Table 1. Genotype and allele frequencies of ATG5 rs573775 and rs510432 polymorphisms in patients with different clinical diseases.

|                  | CH (n = 171) | LC (n = 119) | HCC (n = 113) | P   | OR (95% CI) | P   | OR (95% CI) | P   | OR (95% CI) |
|------------------|--------------|--------------|---------------|-----|-------------|-----|-------------|-----|-------------|
| **rs573775**     |              |              |               |     |             |     |             |     |             |
| Genotype         |              |              |               |     |             |     |             |     |             |
| CC               | 68 (39.8)    | 58 (48.7)    | 53 (46.9)     | Reference | Reference | Reference | Reference | Reference |
| CT               | 82 (47.9)    | 53 (44.6)    | 51 (45.1)     | 0.269 | 0.758 (0.463–1.239) | 0.377 | 0.798 (0.483–1.317) | 0.850 | 1.053 (0.617–1.799) |
| TT               | 21 (12.3)    | 8 (6.7)      | 9 (8.0)       | 0.070 | 0.447 (0.184–1.084) | 0.169 | 0.550 (0.233–1.299) | 0.690 | 1.231 (0.443–3.423) |
| **Allele**       |              |              |               |     |             |     |             |     |             |
| C                | 218 (63.7)   | 169 (71.0)   | 157 (69.5)    | Reference | Reference | Reference | Reference | Reference |
| T                | 124 (36.3)   | 69 (29.0)    | 69 (30.5)     | 0.068 | 0.718 (0.503–1.025) | 0.158 | 0.773 (0.540–1.106) | 0.717 | 1.076 (0.723–1.603) |
| **rs510432**     |              |              |               |     |             |     |             |     |             |
| Genotype         |              |              |               |     |             |     |             |     |             |
| GG               | 57 (33.3)    | 32 (26.9)    | 24 (21.2)     | Reference | Reference | Reference | Reference | Reference |
| GA               | 89 (52.1)    | 63 (52.9)    | 66 (58.4)     | 0.400 | 1.261 (0.735–2.164) | 0.052 | 1.761 (0.993–3.125) | 0.299 | 1.397 (0.743–2.628) |
| AA               | 25 (14.6)    | 24 (20.2)    | 23 (20.4)     | 0.136 | 1.710 (0.842–3.471) | 0.037 | 2.185 (1.042–4.581) | 0.573 | 1.278 (0.586–2.785) |
| **Allele**       |              |              |               |     |             |     |             |     |             |
| G                | 203 (59.4)   | 127 (53.4)   | 114 (50.4)    | Reference | Reference | Reference | Reference | Reference |
| A                | 139 (40.6)   | 111 (46.6)   | 112 (49.6)    | 0.152 | 1.276 (0.914–1.782) | 0.036 | 1.435 (1.023–2.013) | 0.529 | 1.124 (0.781–1.618) |

Data are presented as n (%). CH: chronic hepatitis; LC: liver cirrhosis; HCC: hepatocellular carcinoma; OR: odds ratio; 95% CI: 95% confidence intervals. \( P_c \) (P value by Bonferroni correction) = 0.074. \( P_c = 0.036. \)
haplotype T-A (30.6% vs. 0%, OR 1.462, 95% CI 1.378–1.552, \(p_c \leq 0.001\)) than healthy controls (Table 2).

**Haplotype frequencies of ATG5 rs573775 and rs510432 polymorphisms in chronic HBV-infected patients with different clinical diseases**

Comparison of ATG5 haplotype (rs573775–rs510432) frequencies in patients with different clinical diseases showed that haplotype C-G frequency in chronic hepatitis patients was higher than HCC patients (26.0% vs. 21.2%, OR 1.567, 95% CI 1.015–2.418, \(P = 0.042\), Table 3).

**Associations of ATG5 polymorphisms with cirrhosis and HCC in chronic HBV infection by univariate and multivariate analyses**

Univariate and multivariate analyses were performed to identify whether ATG5 polymorphisms were factors associated with cirrhosis or HCC in chronic HBV infection. In comparison with chronic hepatitis, univariate analysis showed that age and albumin level were associated with cirrhosis (Table 4). Multivariate analysis showed that rs510432 genotype and age were independently associated with cirrhosis, with cirrhosis patients having higher rs510432 genotypes allele A-containing genotypes (AA+GA) (OR 1.927, 95% CI 1.011–3.017, \(P = 0.032\), Table 4).

In comparison with chronic hepatitis, univariate analysis showed that gender, age, HBV DNA, alanine aminotransferase (ALT), and albumin levels and rs510432 genotype were associated with HCC (Table 5). In multivariate analysis, ATG5 rs510432 genotype, together with gender, age and HBV DNA, was independently associated with HCC, with HCC patients having higher rs510432 genotypes AA+GA (OR 2.583, 95% CI 1.025–3.911, \(P = 0.006\), Table 5).

In comparison with cirrhosis, univariate analysis showed that gender, age and albumin were associated with HCC (Table 6). In multivariate analysis, gender and age were independent factors associated with HCC while rs510432 genotype GG was not shown to be an independent factor associated with HCC (OR 0.841, 95% CI 0.376–1.275, \(P = 0.088\), Table 6).

In comparison with all the patients with chronic HBV infection without HCC (chronic hepatitis and cirrhosis), univariate analysis showed that gender, age, HBV DNA, ALT, and albumin were associated with HCC (Table 7). ATG5 rs510432 genotype, together with gender and age, was independently associated with HCC, with HCC patients having higher rs510432 genotypes AA+GA carriers (OR 2.632, 95% CI 1.067–3.482, \(P = 0.032\), Table 7).
Table 3. Haplotype frequencies of ATG5 rs573775 and rs510432 polymorphisms in patients with different clinical diseases.

| Haplotype (rs573775–rs510432) | CH (n = 171) | LC (n = 119) | HCC (n = 113) | P     | OR (95% CI) | P     | OR (95% CI) | P     | OR (95% CI) |
|-------------------------------|-------------|-------------|-------------|------|-------------|------|-------------|------|-------------|
| C-A                          | 129 (37.7)  | 108 (45.4)  | 109 (48.2)  | Reference | Reference | Reference | Reference | 0.291 | 1.283 (0.808–2.037) |
| C-G                          | 89 (26.0)   | 61 (25.6)   | 48 (21.2)   | 0.343 | 1.222 (0.807–1.848) | 0.042 | 1.567 (1.015–2.418) | 0.967 | 0.991 (0.643–1.527) |
| T-G                          | 114 (33.3)  | 66 (27.7)   | 66 (29.2)   | 0.068 | 1.446 (0.973–2.150) | 0.061 | 1.459 (0.982–2.169) | 0.967 | 0.991 (0.643–1.527) |
| T-A                          | 10 (3.0)    | 3 (1.3)     | 3 (1.4)     | 0.112 | 2.791 (0.749–10.398) | 0.109 | 2.817 (0.756–10.493) | 0.991 | 0.991 (0.196–5.018) |

Data are presented as n (%). CH: chronic hepatitis; LC: liver cirrhosis; HCC: hepatocellular carcinoma; OR: odds ratio; 95% CI: 95% confidence intervals.
Table 4. Univariate and multivariate analyses of risk factors associated with cirrhosis in comparison with chronic hepatitis.

| Variable                  | CH (n = 171) | LC (n = 119) | Univariate analysis (P) | Multivariate analysis (OR, 95% CI) | P     |
|---------------------------|-------------|-------------|-------------------------|-----------------------------------|-------|
| Gender (M/F)              | 105/66      | 80/39       | 0.310                   | 2.766 (0.097–3.016)               | 0.085 |
| Age (mean, years)         | 31.93 ± 12.32 | 44.35 ± 10.86 | <0.001                   | 1.928 (1.011–3.025)               | 0.007 |
| HBV DNA (log IU/ml)       | 5.84 ± 1.87 | 5.31 ± 1.63 | 0.127                   |                                   |       |
| ALT (IU/L)                | 70 (10–3629) | 50 (9–590) | 0.096                   |                                   |       |
| AST (IU/L)                | 61 (13–4082) | 58 (14–1022) | 0.103                   |                                   |       |
| TBIL (μmol/L)             | 18.2 (2–656.8) | 27.4 (6.5–470) | 0.097                   |                                   |       |
| Albumin (g/L)             | 38.1 (18.7–50.8) | 31.2 (18.8–50.7) | 0.036                   | 0.725 (0.008–1.123)               | 0.336 |
| rs573775 CC vs. CT+TT     | 68/103      | 58/61       | 0.129                   | 1.235 (0.062–1.923)               | 0.307 |
| rs510432 AA+GA vs. GG     | 114/57      | 87/32       | 0.242                   | 1.927 (1.011–3.017)               | 0.032 |

HBV: hepatitis B virus; CH: chronic hepatitis; LC: liver cirrhosis; OR: odds ratio; 95% CI: 95% confidence intervals; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBIL: total bilirubin.

Table 5. Univariate and multivariate analyses of risk factors associated with HCC in comparison with chronic hepatitis without HCC in chronic HBV infection.

| Variable                  | CH (n = 171) | HCC (n = 113) | Univariate analysis (P) | Multivariate analysis (OR, 95% CI) | P     |
|---------------------------|-------------|-------------|-------------------------|-----------------------------------|-------|
| Gender (M/F)              | 105/66      | 98/15       | <0.001                  | 3.092 (1.361–4.994)               | <0.001|
| Age (mean, years)         | 31.93 ± 12.32 | 48.93 ± 10.85 | <0.001                  | 4.673 (1.258–5.671)               | <0.001|
| HBV DNA (log IU/mL)       | 5.84 ± 1.87 | 4.91 ± 1.48 | <0.001                  | 0.926 (0.037–0.995)               | 0.041 |
| ALT (IU/L)                | 70 (10–3629) | 54 (7–765) | <0.001                  | 0.722 (0.165–1.038)               | 0.128 |
| AST (IU/L)                | 61 (13–4082) | 71 (15–1348) | 0.095                   |                                   |       |
| TBIL (μmol/L)             | 18.2 (2–656.8) | 26.23(1.48–727.24) | 0.417                   |                                   |       |
| Albumin (g/L)             | 38.1 (18.7–50.8) | 33.3 (21.1–51.29) | <0.001                  | 0.822 (0.016–1.095)               | 0.258 |
| rs573775 CC vs. CT+TT     | 68/103      | 53/60       | 0.234                   | 0.619 (0.032–1.157)               | 0.307 |
| rs510432 GA+AA vs. GG     | 114/57      | 89/24       | 0.027                   | 2.583 (1.025–3.911)               | 0.006 |

HBV: hepatitis B virus; CH: chronic hepatitis; HCC: hepatocellular carcinoma; OR: odds ratio; 95% CI: 95% confidence intervals; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBIL: total bilirubin.

Table 6. Univariate and multivariate analyses of risk factors associated with HCC in comparison with cirrhosis in chronic HBV infection.

| Variable                  | LC (n = 119) | HCC (n = 113) | Univariate analysis (P) | Multivariate analysis (OR, 95% CI) | P     |
|---------------------------|-------------|-------------|-------------------------|-----------------------------------|-------|
| Gender (M/F)              | 80/39       | 98/15       | <0.001                  | 1.937 (1.072–3.571)               | <0.001|
| Age (mean, years)         | 44.35 ± 10.86 | 48.93 ± 10.85 | 0.002                   | 1.252 (1.037–2.007)               | <0.001|
| HBV DNA (log IU/mL)       | 5.31 ± 1.63 | 4.91 ± 1.48 | 0.052                   |                                   |       |
| ALT (IU/L)                | 50 (9–590) | 54 (7–765) | 0.762                   |                                   |       |
| AST (IU/L)                | 58 (14–1022) | 71 (15–1348) | 0.330                   |                                   |       |
| TBIL (μmol/L)             | 27.4 (6.5–470) | 26.23(1.48–727.24) | 0.056                   |                                   |       |
| Albumin (g/L)             | 31.2 (18.8–50.7) | 33.3 (21.1–51.29) | 0.030                   | 1.127 (0.921–1.976)               | 0.132 |
| rs573775 CC vs. CT+TT     | 58/61       | 53/60       | 0.780                   | 0.792 (0.604–1.976)               | 0.134 |
| rs510432 AA+GA vs. GG     | 87/32       | 89/24       | 0.315                   | 0.841 (0.376–1.275)               | 0.088 |

HBV: hepatitis B virus; LC: liver cirrhosis; HCC: hepatocellular carcinoma; OR: odds ratio; 95% CI: 95% confidence intervals; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBIL: total bilirubin.
This study investigated the rs573775 and the rs510432 polymorphisms in the ATG5 gene in patients with chronic hepatitis, liver cirrhosis and HCC associated with chronic HBV infection. The genotypes of rs573775 and rs510432 were not shown to have significant differences between patients with chronic HBV infection and healthy controls. However, in relation to clinical diseases, HCC patients had higher rs510432 AA genotype and significantly higher A allele frequencies than chronic hepatitis patients. Patients with chronic HBV infection had significantly lower rs573775–rs510432 haplotypes C-G and T-G but higher haplotype T-A than healthy controls. Chronic hepatitis patients had higher haplotype C-G than HCC patients. The rs510432 AA+GA genotypes were shown to be an independent factor associated with cirrhosis in comparison with chronic hepatitis. The rs510432 AA+GA genotypes were also shown to be an independent factor associated with HCC in comparison with chronic hepatitis or chronic HBV infection without HCC. The insignificance of rs510432 polymorphism in HCC in relation to cirrhosis may be related to the small number of patients with cirrhosis. These results suggested that rs510432 allele A and AA+GA were factors predisposing to the disease progression from chronic hepatitis to the development of cirrhosis and HCC in chronic HBV infection.

ATG5 rs573775 polymorphism has been shown to influence systemic lupus erythematosus (SLE) susceptibility in Spanish (López et al., 2013) and Italian populations (Ciccacci et al., 2018). This polymorphism has also been shown to influence the susceptibility to Behçet’s disease (BD) in Chinese Han population (Zheng et al., 2015). The allele T of rs573775 was shown to confer a higher risk of developing SLE in Spanish (López et al., 2013) and Italian populations (Ciccacci et al., 2018) but a lower risk of developing BD in Chinese Han population (Zheng et al., 2015). Functionally, the T allele is associated with high interleukin (IL)-10 producer genotype in SLE patients of Spanish population (López et al., 2013) and the TT genotype is associated with increased ATG5 expression by lipopolysaccharide (LPS) stimulated peripheral blood mononuclear cells in Chinese Han BD patients and the level of ATG5 mRNA in active BD patients was significantly increased (Zheng et al., 2015). However, the present study did not observe an association between rs573775 polymorphism and HBV-related liver diseases including HCC in Chinese Han

---

**Table 7. Univariate and multivariate analyses of risk factors associated with HCC in comparison with chronic HBV infection without HCC.**

| Variable | HBV infection without HCC (n = 290) | HCC (n = 113) | Univariate analysis (P) | Multivariate analysis OR (95% CI) | P |
|----------|--------------------------------------|---------------|-------------------------|----------------------------------|---|
| Gender (M/F) | 185/105 | 98/15 | | 2.231 (1.203–3.556) | <0.001 |
| Age (mean, years) | 37.03 ± 13.23 | 48.93 ± 10.85 | | 3.662 (1.167–5.233) | <0.001 |
| HBV DNA (logIU/ml) | 5.63 ± 1.79 | 4.91 ± 1.48 | | 0.769 (0.036–1.972) | 0.146 |
| ALT (IU/L) | 55.95 (9–3629) | 54 (7–765) | | 0.912 (0.015–1.068) | 0.228 |
| AST (IU/L) | 60 (13–4082) | 71 (15–1348) | | – | – |
| TBIL (μmol/L) | 21.6 (2–656.8) | 26.23(1.48–727.24) | | – | – |
| Albumin (g/L) | 36.7(18.7–50.8) | 33.3 (21.1–51.29) | | 0.953 (0.122–1.169) | 0.532 |
| rs573775 CC vs. CT+TT | 126/164 | 53/60 | | 1.523 (0.097–1.679) | 0.423 |
| rs510432 AA+GA vs. GG | 201/89 | 89/24 | 0.058 | 2.632 (1.067–3.482) | 0.032 |

HBV: hepatitis B virus; HCC: hepatocellular carcinoma; OR: odds ratio; 95% CI: 95% confidence intervals; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBIL: total bilirubin.
population. This may be a reflection of the disease differences in relation to the susceptibility with genetic background of the host.

ATG5 rs510432 polymorphism was found to be associated with the development of asthma in white/Caucasian (Martin et al., 2012), the risk of coal workers’ pneumoconiosis in a Chinese population (Yuan et al., 2017a) and the progression and mortality of sepsis in Chinese patients (Shao et al., 2017). The rs510432 was also shown to be associated with response to therapy with anti-tumor necrosis factor (TNF)-α drug adalimumab in Crohn’s disease patients (Deželak et al., 2016). Furthermore, rs510432 polymorphism is found to be related to cancers. For example, rs510432 was found to be associated with stage and non-brisk tumor infiltrating lymphocytes in melanoma (White et al., 2016). Among epidermal growth factor receptor-mutant patients with advanced lung adenocarcinoma, rs510432 was found to contribute to disease prognosis and to be associated with primary or acquired resistance to gefitinib (Yuan et al., 2017b). A recent study in Thai population indicated that ATG5 rs510432 polymorphism might be involved in HBV-related HCC (Wisetsathorna et al., 2017). The present study showed that rs510432 was associated with the disease progression of chronic HBV infection, especially the development of HBV-related HCC. These findings provide novel information for the role of ATG5 rs510432 polymorphism in disease predisposition of viral infection and carcinogenesis.

The rs510432 is located in the 5’ untranslated region, 335 bp upstream of the transcription start site of human ATG5 gene. It has been shown to be functionally relevant and confers significant effect on promoter activity. The rs510432 G allele had higher promoter activity than the A allele and associated with the increased gene expression of ATG5 in asthmatics (Martin et al., 2012) while the rs510432 allele A appeared to be related to lower promoter activity in sepsis (Shao et al., 2017). The rs510432 polymorphism was also indicated to influence the expression levels of ATG5 which was decreased with the severity of sepsis (Shao et al., 2017). The mononuclear cell of rs510432 allele A carriers exhibited decreased levels of ATG5 expression which led to enhanced releases of TNF-α and IL-1β under LPS stimulation in vitro (Shao et al., 2017). ATG5 is involved in HBV-associated HCC (Kunanopparat et al., 2016) and autophagy suppresses tumorigenesis of HBV-associated HCC (Lan et al., 2014). The significant association of rs510432 allele A and AA+GA with HCC found in the present study is consistent with the functional relevance of the genotypes. The lower promoter activity related to rs510432 allele A may lead to the decreased expression of ATG5, compromising the inhibitory effect of ATG5 in autophagy on tumorigenesis in chronic HBV infection and predisposing the development of HCC.

This study was performed in Chinese Han patients with a relatively small sample size. There are other polymorphisms in ATG5 which have not been investigated in this study. Therefore, further studies in large sample sizes of patients and controls and different ethnic populations to examine more ATG5 polymorphisms are needed to confirm the findings in this study and to clarify the contribution of ATG5 polymorphisms to HBV-associated diseases including HCC. Notably, there are other host genetic polymorphisms such as HLA class II alleles which have also been found to be associated with HBV infection (Matei et al., 2018). Therefore, further studies are also needed to investigate the potential effects of various host genetic polymorphisms on chronic HBV infection and the development of HBV-related HCC.

In conclusion, this study showed that ATG5 rs573775 appeared to have no significant association with chronic HBV infection and HBV-related liver diseases. However, rs510432 was associated with the disease progression in chronic HBV infection, especially
the development of HBV-related HCC, highlighting a likely important role of ATG5 rs510432 in chronic HBV infection and hepatocarcinogenesis. These associations may be critical to understanding the role of autophagy in chronic HBV infection and HBV-related HCC. Further investigations in large patient populations are warranted to characterize the contribution of ATG5 rs510432 and other polymorphisms to the disease progression of chronic HBV infection and the development of HBV-related HCC as well as the responses to antiviral therapy or to anticancer therapy in HCC patients.

Acknowledgments

We thank Dr Yan Li, Dr Xiaoyan Zeng, Dr Fan Gao, Dr Yawen Wang, Dr Guoyu Zhang, Dr Man Li, and Dr Zhu Li from the First Affiliated Hospital of Xi’an Jiaotong University for their help during this study.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The study was supported by the National Natural Science Foundation of China (Grant no. 81371798), the Science Innovation Foundation of the First Affiliated Hospital of Xi’an Jiaotong University (Youth Projec, Grant no. 2016QN-11), and the Natural Science Basic Research Foundation of Shaanxi Province (Youth Project, Grant no. 2017Q8033). The funders were involved neither in the design and conduction of the study nor in the analysis of data.

Competing interests

All authors declare that they have no competing interests.

Ethical approval

All procedures were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards and approved by the Ethics Committee of the First Affiliated Hospital of Xi’an Jiaotong University.

Informed consent

Informed consent was obtained from all participants included in the study.

References

Ciccacci C, Perricone C, Alessandri C, et al. (2018). Evaluation of ATG5 polymorphisms in Italian patients with systemic lupus erythematosus: contribution to disease susceptibility and clinical phenotypes. Lupus, 27(9), 1464–1469. doi: 10.1177/0961203318776108.
Deželak M, Repnik K, Koder S, et al. (2016). A prospective pharmacogenomic study of Crohn’s disease patients during routine therapy with anti-TNF-α drug adalimumab: contribution of
ATG5, NFKB1, and CRP genes to pharmacodynamic variability. OMICS, 20(5), 296–309. doi: 10.1089/omi.2016.0005.

Döring T, Prange R. (2015). Rab33B and its autophagic Atg5/12/16L1 effector assist in hepatitis B virus naked capsid formation and release. Cell Microbiol, 17(5), 747–764. doi: 10.1111/cmi.12398.

Döring T, Zeyen L, Bartusch C, Prange R. (2018). Hepatitis B virus subverts the autophagy elongation complex Atg5-12/16L1 and does not require Atg8/LC3 lipidation for viral maturation. J Virol, 92(7). doi: 10.1128/JVI.01513-17.

Elwan N, Salem ML, Kobtan A, et al. (2018). High numbers of myeloid derived suppressor cells in peripheral blood and ascitic fluid of cirrhotic and HCC patients. Immunol Invest, 47(2), 169–180. doi: 10.1080/08820139.2017.1407787.

European Association for the Study of the Liver. (2017). EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol, 67(2), 370–398. doi: 10.1016/j.jhep.2017.03.021.

Jounai N, Takeshita F, Kobiyama K, et al. (2007). The Atg5 Atg12 conjugate associates with innate antiviral immune responses. Proc Natl Acad Sci U S A, 104(35), 14050–14055. doi: 10.1073/pnas.0704014104.

Kunanopparat A, Kimkong I, Palaga T, et al. (2016). Increased ATG5-ATG12 in hepatitis B virus-associated hepatocellular carcinoma and their role in apoptosis. World J Gastroenterol, 22(37), 8361–8374. doi: 10.3748/wjg.v22.i37.8361.

Lan SH, Wu SY, Zuchini R, et al. (2014). Autophagy suppresses tumorigenesis of hepatitis B virus-associated hepatocellular carcinoma through degradation of microRNA-224. Hepatology, 59(2), 505–517. doi: 10.1002/hep.26659.

Li J, Liu Y, Wang Z, et al. (2011). Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. J Virol, 85(13), 6319–6333. doi: 10.1128/JVI.02627-10.

Maini MK, Boni C, Lee CK, et al. (2000). The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med, 191(8), 1269–1280. doi: 10.1084/jem.191.8.1269.

Martin LJ, Gupta J, Jyothula SS, et al. (2012). Functional variant in the autophagy-related 5 gene promotor is associated with childhood asthma. PLoS One, 7(4), e33454. doi: 10.1371/journal.pone.0033454.

Matei HV, Vica ML, Siserman CV. (2018). Association between HLA class II alleles and hepatitis B virus infection in Transylvania, Romania. Immunol Invest, 47(7), 735–744. doi: 10.1080/08820139.2018.1489832.

Na T, Asf L, Bj M, et al. (2018). Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance. Hepatology, 67(4), 1560–1599. doi: 10.1002/hep.29800.

Purcell S, Cherny SS, Sham PC. (2003). Genetic power calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics, 19(1), 149–150. doi: 10.1093/bioinformatics/19.1.149.

Schweitzer A, Horn J, Mikolajczyk RT, et al. (2015). Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet, 386(10003), 1546–1555. doi: 10.1016/S0140-6736(15)61412-X.

Shao Y, Chen F, Chen Y, et al. (2017). Association between genetic polymorphisms in the autophagy-related 5 gene promotor and the risk of sepsis. Sci Rep, 7(1), 9399. doi: 10.1038/s41598-017-09978-5.

Shin GC, Kang HS, Lee AR, Kim KH. (2016). Hepatitis B virus-triggered autophagy targets TNFRSF10B/death receptor 5 for degradation to limit TNFSF10/TRAIL response. Autophagy, 12(12), 2451–2466. doi: 10.1080/15548627.2016.1239002.

Sir D, Tian Y, Chen WL, et al. (2010). The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. Proc Natl Acad Sci USA, 107 (9), 4383–4388. doi: 10.1073/pnas.0911373107.
Tian Y, Sir D, Kuo CF, et al. (2011). Autophagy required for hepatitis B virus replication in transgenic mice. J Virol, 85(24), 13453–13456. doi: 10.1128/JVI.06064-11.

Uhl M, Kepp O, Jusforgues-Saklani H, et al. (2009). Autophagy within the antigen donor cell facilitates efficient antigen cross-priming of virus-specific CD8+ T cells. Cell Death Differ, 16(7), 991–1005. doi: 10.1038/cdd.2009.8.

Vij A, Randhawa R, Parkash J, Changotra H. (2016). Investigating regulatory signatures of human autophagy related gene 5 (ATG5) through functional in silico analysis. Meta Gene, 9, 237–248. doi: 10.1016/j.mgene.2016.07.012.

White KA, Luo L, Thompson TA, et al. (2016). Variants in autophagy-related genes and clinical characteristics in melanoma: a population-based study. Cancer Med, 5(11), 3336–3345. doi: 10.1002/cam4.929.

Wisetsathorna S, Tantithavorna V, Hirankarnb N, et al. (2017). Gene polymorphisms of autophagy machinery and the risk of hepatitis B virus-related hepatocellular carcinoma in a Thai population. Science Asia, 43, 362–368. doi: 10.2306/scienceasia1513-1874.2017.43.362.

Xie M, Yang Z, Liu Y, Zheng M. (2018). The role of HBV-induced autophagy in HBV replication and HBV related-HCC. Life Sci, 205, 107–112. doi: 10.1016/j.lfs.2018.04.051.

Yang J, He Y, Zhai N, et al. (2018). MicroRNA-181a inhibits autophagy by targeting Atg5 in hepatocellular carcinoma. Front Biosci (Landmark Ed), 23, 388–396.

Yuan J, Han R, Esther A, et al. (2017a). Polymorphisms in autophagy related genes and the coal workers’ pneumoconiosis in a Chinese population. Gene, 632, 36–42. doi: 10.1016/j.gene.2017.08.017.

Yuan J, Zhang N, Yin L, et al. (2017b). Clinical Implications of the autophagy core gene variations in advanced lung adenocarcinoma treated with gefitinib. Sci Rep, 7(1), 17814. doi: 10.1038/s41598-017-18165-5.

Zheng M, Yu H, Zhang L, et al. (2015). Association of ATG5 gene polymorphisms with Behçet’s disease and ATG10 gene polymorphisms with VKH syndrome in a Chinese han population. Invest Ophthalmol Vis Sci, 56(13), 8280–8287. doi: 10.1167/iovs.15-18035.