



# Association of viral load and autophagy-related genes polymorphisms with hepatitis B virus pre-core/core mutations in chronic hepatitis B virus Iraqi patients

Abdulhussain Kadhim Jwaziri<sup>1</sup>, Maryam Esghaei<sup>1</sup>, Mohammad Hadi Karbalaie Niya<sup>1,2</sup>, Mohsen Mehrjoo<sup>3</sup>, Hadi Abd Zaid Sayah<sup>4</sup>, Hossein Keyvani<sup>1\*</sup>

<sup>1</sup>Department of Virology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

<sup>2</sup>Gastrointestinal and Liver Diseases Research Center, Iran University of Medical Sciences, Tehran, Iran

<sup>3</sup>Department of Biochemistry and Genetics, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran

<sup>4</sup>Department of Gastroenterology and Hepatology, Al-Husain Hospital, Kerbala, Iraq

## \*Correspondence to

Hossein Keyvani,

Email: Keyvanlab@yahoo.com,

Keyvani.h@iums.ac.ir

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## Abstract

**Introduction:** Chronic hepatitis B (CHB) is a global concern due to its association with cirrhosis and hepatocellular carcinoma (HCC) development. The interplay between viral load, the immune system, and host factors is critical in tumorigenesis. Autophagy is a significant contributor to immune system function, since vitamin D plays an important role in this context.

**Objectives:** The objective of this study was to assess the association between ATG5 (rs506027 and rs510432) and ATG16L1 (rs2241880 and ATG16 rs2241879) polymorphisms, viral load, and vitamin D with HBV pre-C/C mutations in Iraqi patients with CHB.

**Patients and Methods:** In this cross-sectional study, a total of 134 CHB patients were evaluated for ATG polymorphisms, viral load, and vitamin D levels. Blood samples were collected after obtaining ethical consent, and the mutations were analyzed using polymerase chain reaction (PCR) followed by Sanger sequencing. Serum samples from CHB patients were used for viral load and vitamin D assessment.

**Results:** The evaluation of patients revealed that 67 (44.6%) were male and 83 (55.4%) were female, with a mean age of 36±12.7 years and a mean duration of infection of 5.2±4.8 years. Mutations in pre-C/C were observed in 20% (27/134) of the patients. There was a significant association between all evaluated ATG polymorphisms and pre-C/C mutants ( $P < 0.05$ ). Furthermore, there was an association between viral load and mutations in pre-C/C ( $P = 0.03$ ), while no statistically significant difference was found between vitamin D levels and pre-core/core mutants or viral load.

**Conclusion:** Our study demonstrates a higher frequency of ATG5 (rs506027 and rs510432) and ATG16L1 (rs2241880 and ATG16 rs2241879) polymorphisms, as well as a higher viral load in Iraqi CHB patients with HBV pre-C/C mutations.



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## Introduction

The hepatitis B virus (HBV) is distributed all around the world. HBV is a causative agent for severe and chronic hepatitis. Chronic hepatitis B (CHB) is affected 292 million people worldwide. Recent studies highlight the importance of CHB in Iraq (1). CHB is considered a significant health concern due to cirrhosis and hepatocellular carcinoma (HCC) development. The main target for HBV infection treatment in CHB patients is to avoid HCC development (2). One of the markers for HBV replication and active infection in CHB patients is HBeAg. Some viral strains represent HBeAg-negative mutants. This mutation is due to the HBV pre-core/core (preC/C) region mutations. Previous studies

## Key point

The present study underscores a higher occurrence of ATG5 (rs506027 and rs510432) and ATG16L1 (rs2241880 and ATG16 rs2241879) genetic variations, along with an elevated viral load, in Iraqi patients with chronic hepatitis B (CHB) who possess hepatitis B virus (HBV) pre-core/core (pre C/C) mutations compared to CHB patients without any mutations in the pre-core/core region.

revealed that mutations in pre-C/C can alter the natural course of virus infection and effect viral replication or persistence, which might be critical in HBV pathogenesis (3).

The interactions between the immune system and other host factors are critical for HCC development in CHB patients.

In this case, alterations in some genes such as signal transducer and activator of transcription 3 (STAT3), STAT4, chemokine (C-X-C motif) ligand 2 (CXCL2), and interleukin-1 beta (IL-1 $\beta$ ) are described (4-6).

One of the main cellular maintenance mechanisms is autophagy. Autophagy plays an essential role in immune system function. One main function of autophagy is innate immune responses and antigen presentation (7). Autophagy is a considerable element in tumor microenvironments too (8). This tight association between autophagy flux and the immune system highlights clinical applications for immunotherapy or other immune system-related therapeutic approaches (9,10).

Autophagy flux is a combination of different proteins. Polymorphisms in the coding gene for these proteins could alter the autophagy pathway or responses (11,12). Autophagy-related genes (ATGs) are major genes in the autophagy system. Two of the most essential ATGs in different diseases are considered as ATG16L1 and ATG5 (11,12).

In this regard, these genes could alter the autophagy response and affect the immune system, which will be the pathogenesis mechanism in different diseases. Previous studies disclose the role of ATG5 rs506027 G>A (11), rs510432 T>C and ATG16L1 rs2241880 A>G (13) and rs2241879 G>A (14) in different malignant or non-malignant diseases.

Vitamin D is a critical element in immune system development and function. Vitamin D deficiency is associated with many diseases (15,16). Vitamin D is shown to be a critical factor in the development of HCC or cirrhosis in CHB patients (17). The HBV viral load in CHB patients results from the interaction of multiple factors. The higher viral load in CHB patients increases the risk of HCC development during the following years (18).

## Objectives

The aim of the current study was to evaluate the association of ATG5 (rs506027 and rs510432), ATG16L1 (rs2241880 and ATG16 rs2241879) polymorphisms, viral load, and vitamin D with HBV pre-C/C mutations in Iraqi CHB patients.

## Patients and Methods

### Study design

Patients were included in this study without specific demographic criteria. Inclusion criteria encompassed individuals with CHB, serologically confirmed to have HBV infection, with or without clinical manifestations of HBV infection. Exclusion criteria involved patients lacking ethical consent, those with HCC, co-infection with HIV, other types of viral hepatitis apart from HBV, and individuals with acute HBV infection.

### Sample collection and genome extraction

Samples were collected from various centers in Iraq between 2022 and 2023. A total of 134 CHB patients were evaluated in the current study. CHB cases were confirmed by a positive HBsAg serological result for over six months, with no severe infection manifestations and no other viral hepatitis agents.

Phlebotomy was conducted on the patients after obtaining ethical consent. We collected 5 mL of blood in ethylenediaminetetraacetic acid (EDTA) anti-coagulant tubes. Then we separated plasma and peripheral blood mononuclear cells (PBMCs). PBMCs were isolated using the Ficoll and density gradient method. After PBMC separation, samples were stored at -20 °C until extraction.

DNA extraction was performed from both plasma and PBMC samples. The quality of extracted DNA was evaluated using the NanoDrop spectrophotometer by measuring optical density (OD) at 260 nm and 280 nm. The plasma and PBMC samples were extracted using the commercially available extraction kit "AddBio genomic extraction" (AddBio, South Korea) following the manufacturer's protocol.

### Pre-core/core mutations evaluation

Pre-core/core mutations were evaluated using conventional polymerase chain reaction (PCR) followed by Sanger sequencing. The PCR amplified two fragments; one of 605 bp and another of 490bp, from the pre-core/core region, and the conducted primers are listed in Table 1. The PCR mixture included 20  $\mu$ L of Master Mix, 1.5  $\mu$ L of each primer, 10  $\mu$ L of template, and adjusted to 40  $\mu$ L with water. The thermal program consisted of initial

**Table 1.** The PCR primers for ATGs polymorphisms and preC/C mutations evaluation

	Gene	Primer	
Variant evaluation	ATG 5 rs506027 rs510432	Forward	5'-AGCTTGATCGCAACATCCCTT-3'
		Reverse	5'-ATGCCTGCTAAGACTTCTGGATA-3'
	ATG16L1 rs2241880 and rs2241879	Forward	5'-ACAGGTTAGTGTGCAGGAGA-3'
		Reverse	5'-CAGTCAGCTCTGCCATTACA-3'
Pre-C/C	Primer 1	Forward	5'-AGGAGGCTGTAGGCATAAATTGG-3'
		Reverse	5'-GTTGTTCTTCTAGGGGACCTGCC-3'
	Primer 2	Forward	5'-TGCCTTCTGACTTCTTTCC-3'
		Reverse	5'-TGAGGTTCCCGAGAGGGA-3'

ATG 5, Autophagy related 5; ATG16L1, Autophagy related 16 like 1.

denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 0.5 minutes, 52 °C for 0.5 minutes, and 72 °C for one minute, followed by a final extension at 72 °C for 10 minutes.

#### ATG polymorphisms assessment

The ATG5 (rs506027 and rs510432) and ATG16L1 (rs2241880 and ATG16 rs2241879) were evaluated in all enrolled patients. Polymorphisms were assessed using conventional PCR followed by Sanger sequencing. The PCR mixture and temperature program included Master Mix 20 µL, 1.5 µL of each primer, and 10 µL of template, and brought to a total volume of 40 µL with PCR grade water. The thermal program consisted of initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 0.5 minutes, 59.5 °C for 0.5 minutes, and 72 °C for one minute, followed by a final extension at 72 °C for 10 minutes. Additionally, primer sequences are provided in Table 1. The PCR products were assessed using agarose gel electrophoresis, which was directly used for sequencing.

#### Viral load evaluation

Chronic hepatitis B serum samples were employed for viral load assessment. The HBV viral load was evaluated using the Real Best DNA HBV quantitative kit (Vector-Best, Russia) following the manufacturer's protocol. Based on the manufacturer's instructions, quantitative real-time PCR was performed on Rotor-Gene 6000 (Corbett Research, Australia).

#### Vitamin D status

All CHB serum samples were conducted to evaluate vitamin D status. Vitamin D was assessed using the 25-OH vitamin D kit (AccuBind, MonoBind, USA) following the manufacturer's protocol.

#### Statistical analysis

All statistical analyses were performed using SPSS version 22 (IBM SPSS, USA). A statistically significant difference was considered when  $P < 0.05$ . The parameters were evaluated using the Mann-Whitney U and chi-square tests based on variables.

## Results

#### Demographic features

The patient evaluation revealed that 67 (44.6%) of the patients were male, and 83 (55.4%) were female. The mean age of the included patients was  $36 \pm 12.7$  and the mean duration of infection was  $5.2 \pm 4.8$  years. Patients were categorized into two groups based on the presence of pre-core/core nucleotide mutations.

#### HBV pre-core/core mutations

Our results indicated the presence of mutations in 20% of the investigated patients (27 out of 134 patients based on the amino acid sequence). The included patients were

divided into two groups based on the presence of pre-core/core mutations.

#### ATG polymorphisms in CHB patients

ATG polymorphisms were evaluated in the CHB patients, divided into two groups based on the presence of pre-core/core mutations. The amplified section in ATG16 and ATG5 identified two nucleic acid substitutions for each. PCR analysis yielded acceptable sequences in 124 patients. Information regarding the association between ATG polymorphisms and preC/C mutants is provided in Table 2. A significant association was observed between mutations in ATGs and preC/C mutants ( $P < 0.05$ ). All pre-core/core mutant samples represented a higher frequency of homozygous or heterozygous mutations in all four evaluated polymorphisms.

#### Viral load and vitamin D status

The vitamin D status was evaluated between two groups of pre-core/core mutants and non-mutants. The vitamin D level is divided into three stages including normal ( $>30$  ng/mL), insufficient (20-30 ng/mL), and deficient ( $<20$  ng/mL). The mean vitamin D level was  $25.7 \pm 20.2$  ng/mL. There was no statistically significant difference between the vitamin D mean levels or stage with pre-core/core mutations. More information is provided in Table 3. Regardless of pre-core/core mutants, there was a high frequency of vitamin D deficiency (55%) and insufficient (20%) in evaluated patients. Furthermore, the HBV viral load assessment could not represent any statistically significant difference between other variables (vitamin D, age, gender, and disease duration), except pre-core/core mutants or non-mutant patients ( $P = 0.03$ ). The evaluated viral load represents the mean viral load of  $100047 \pm 213516$  IU/mL in CHB patients. The viral load information is presented in Table 3.

**Table 2.** The ATGs polymorphisms in chronic hepatitis B patients based on the presence of preC/C mutations

Variable	Total	Pre-C/C		P value
		Yes	No	
ATG5 rs506027	G 94 (75.8%)	20	74	0.001
	A 3 (2.4%)	2	1	
	AG 27 (21.8%)	7	20	
ATG5 rs510432	T 90 (72.6%)	20	70	0.001
	C 4 (3.2%)	3	1	
	TC 30 (24.2%)	9	21	
ATG16 rs2241880	A 94 (75.8%)	18	76	0.01
	G 2 (1.6%)	2	0	
	AG 28 (22.6%)	7	21	
ATG16 rs2241879	G 97 (78.2%)	20	77	0.03
	A 3 (2.4%)	3	0	
	GA 24 (19.4%)	7	17	

**Table 3.** Vitamin D and viral load status based on the presence of Pre-C/C mutants

Vitamin D					
Pre-C/C mutant	Vitamin D status			Mean (ng/mL)	P value
	Deficient	Insufficient	Normal		
No	44%	15%	20%	28.5±20.3	0.46
Yes	11%	5 %	5%	25.1±20.22	
Viral load					
Pre-C/C mutant	Mean Viral load (IU/mL ± SD)			Pvalue	
No	48114±67540			0.03	
Yes	130113±261366				
Total	100047±213516				

## Discussion

### ATG polymorphisms and HBV pre-C/C mutations

The study sheds light on the higher prevalence of ATG5 (rs506027 and rs510432) and ATG16L1 (rs2241880 and ATG16 rs2241879) polymorphisms, as well as a higher viral load in Iraqi CHB patients with HBV Pre-C/C mutations, compared to CHB patients without mutations in the pre-core/core region. These findings provide valuable insights for future investigations into the role of autophagy in CHB patients.

Several studies have delved into the significance of ATG polymorphisms in various diseases. Wisetsathorn et al (21) assessed 114 CHB patients, revealing an association between ATG16L1 rs2241880 and ATG5 rs77859116 with an increased risk of HCC development. Furthermore, Sharma et al (22) reported a higher frequency of rs2241880 in HBV-infected cases with cirrhosis or HCC than in healthy controls. Li et al evaluated rs573775, rs510432, rs573775, rs510432, rs573775, and rs510432 polymorphisms in over 200 HCC and CHB patients in China, highlighting the role of ATG5 rs510432 in HCC development risk in CHB patients (19).

In line with our study, Li and colleagues also emphasized the importance of ATG5 rs6568431 and rs548234 in the CHB patient population (23). Additionally, the role of ATG16L1 rs2241880 polymorphisms has been examined in other diseases (12,14), consistent with our findings of a higher prevalence of ATG5 and ATG16L1 mutations in CHB patients. It is important to note the limited available research on the frequency of these polymorphisms in different diseases, especially CHB and HCC, highlighting the need for further investigations in this field.

### Vitamin D and viral load

The study also addresses the role of vitamin D in different diseases, given its ability to influence immune system function, which holds promise in understanding and potentially preventing chronic conditions (15,16,25). Vitamin D is implicated in HBV viral load, a critical element in predicting the future risk of HCC development in CHB patients (18). A meta-analysis concluded by Hu et al represent that CHB patients display lower vitamin D levels compared to healthy individuals, with lower vitamin D status being more frequent in patients with high viral

load. Other studies suggest vitamin D as a marker for HCC development (18,26).

Our findings align with these observations, highlighting higher viral load in CHB patients carrying pre-C/C mutations than in non-mutant patients. This observation confirms the influence of pre-C/C mutations on viral replication (27), further supporting our findings.

## Conclusion

This study emphasizes a greater prevalence of ATG5 (rs506027 and rs510432), ATG16L1 (rs2241880 and ATG16 rs2241879) polymorphisms, and a higher viral load in Iraqi CHB patients with HBV pre-C/C mutations, providing valuable leads for future research on autophagy in CHB patients.

## Limitations of the study

The study's limitations include the relatively limited number of patients for polymorphism and pre-C/C mutation or vitamin D evaluation, which may account for discrepancies with some other conducted studies, particularly concerning vitamin D status. Additionally, the cross-sectional design of the study is a notable limitation. Longitudinal cohort studies to track patient outcomes and complications would benefit future investigations.

## Authors' contribution

**Conceptualization:** Hossein Keyvani, Abdulhussain Kadhim Jwaziri, Maryam Esghaei.

**Data curation:** Maryam Esghaei, Mohammad Hadi Karbalaie Niya.

**Formal analysis:** Abdulhussain Kadhim Jwaziri.

**Funding acquisition:** Hossein Keyvani.

**Investigation:** Abdulhussain Kadhim Jwaziri, Mohammad Hadi Karbalaie Niya, Mohsen Mehrjoo, Hadi Abd Zaid Sayah.

**Methodology:** Hossein Keyvani, Abdulhussain Kadhim Jwaziri, Maryam Esghaei.

**Supervision:** Hossein Keyvani, Hadi Abd Zaid Sayah.

**Writing-original draft:** Abdulhussain Kadhim Jwaziri, Mohammad Hadi Karbalaie Niya, Mohsen Mehrjoo.

## Conflicts of interest

The authors declare that they have no competing interests.

## Ethical issues

The research followed the Declaration of Helsinki. The institutional ethical committee at Iran University of Medical Sciences approved all study protocols (Ethical code# IR.IUMS.FMD.REC.1401.221).



Furthermore, written informed consent was taken from all participants. This study was extracted from the Ph.D., thesis of Abdullhussain Kadhim Jawaziri at the Department of Medical virology at Iran University of Medical Sciences (Thesis#1400-3-104-22-331). Additionally, ethical issues (including plagiarism, data fabrication, and double publication) have been completely observed by the authors.

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