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Evaluation of Cytotoxic T-Lymphocyte Antigen-4 (+49A/G) Gene Polymorphism in Chronic Hepatitis B Virus Infection

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Abstract

Background Chronic hepatitis B (CHB) infection is associated with the depletion of T cells, resulting in weak or absent virus specific T cells reactivity, which is described as 'exhaustion'. This exhaustion is characterized by impaired cytokine production and sustained expression of multiple coinhibitory molecules. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is one of many coinhibitory molecules that can attenuate T cell activation by inhibiting stimulation and transmitting inhibitory signals to T cells.

Objective To explore the effect of CTLA-4+49A/G single nucleotide polymorphism (SNP) on the progression CHB in Iraqi patients.

Methods Blood serum and genomic DNA was isolated from 90 patients with CHB. Tetra-Primer Amplification Refractory System-Polymerase Chain Reaction (ARMS-PCR) was used for amplification and genotyping of CTLA-4 gene using specific primers, and plasma hepatitis B virus (HBV) viral load was investigated by real time PCR, in addition to estimate the hepatitis B e antigen (HBeAg) and anti-HBe by enzyme-linked immunosorbent assay (ELISA).

Results AA genotype was more frequent among uncomplicated than complicated CHB (44.83% versus 28.12%) with a significant difference (OR= 0.315, 95%CI=1.0-0.99, p= 0.048).

Conclusion These data strongly suggested the persistence role of CTLA-4+49 polymorphism against HBV among Iraqi patients.

Keywords CTLA 4, SNP, ARMS-PCR

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List of abbreviations: ARMS-PCR = Amplification Refractory System-Polymerase Chain Reaction, Chronic Hepatitis B = CHB, CTLA-4 = Cytotoxic T-lymphocyte antigen-4, Enzyme-linked immunosorbent assay = ELISA, Hepatitis B virus = HBV, Polymerase chain reaction = PCR, SNP = single nucleotide polymorphism

Introduction

Chronic hepatitis B (CHB) infection has been an important global health problem. Cirrhosis related complications and hepatocellular carcinoma (HCC) are found in 25-40% of the patients with CHB infection [(1)]. Hepatitis B virus (HBV) does not kill liver cells directly, the host immunity recognizes the virus as a foreign antigen which leads to activate immune system and destroy infected liver cells, resulting in an inflammation and necrosis of liver tissue. However, this process occurs intermittently during the course of chronic HBV infection [(2)]. Persistence of hepatitis B surface antigen (HBsAg) beyond 6 months is considered as chronic hepatitis [(3)]. Immune tolerance is controlled by multiple mechanisms [(4)], including: regulatory T (T-reg) cells [(5)] and inhibitory receptors [(6)]. Cytotoxic-T lymphocyte-associated antigen-4 (CTLA-4) functions at a key "checkpoint" in immune tolerance, CTLA-4 (which also known as CD152) [7] expressed transiently on CD4+ and CD8+ T cell and constitutively on CD4+ and CD25+ T-reg cells [8]. Several studies using neutralizing monoclonal antibody to block CTLA-4 on T-reg cells in vivo have reported an exacerbation of autoimmune disease [9,10]. Gene of CTLA-4 encodes a 233 amino acid protein [11]. Approximately 100 SNPs (single nucleotide polymorphism) have been reported in CTLA-4 gene [12,13]. The human CTLA-4 gene (CTLA-4) is known to contain polymorphisms in three regions: a cytosine-thymine single base substitution in the promoter at position −318 (C−318/T−318), and adenine-guanine dimorphism in the exon 1 leader sequence at position 49 (A49/G49), and a multiallelic dinucleotide repeat in the 3′ untranslated region (UTR) of exon 4. These polymorphisms have been investigated for linkage and association in a number of human certain diseases [14]. Previous studies have elucidated the effect of CTLA-4+49A/G with three kinds of diseases: autoimmune diseases [15,16], cancers like breast, lung and esophageal [17-19] and finally with few infectious diseases particularly tuberculosis, and hepatitis B infection [20].

This study aimed to assess the effect the vigor of the T-cell response to HBV infection, thus influencing viral persistence.

Methods

The current study was conducted in Baghdad City from September 2018 to April 2019. Ninety patients with CHB were enrolled in the present study, they were seeking treatment in the Gastrointestinal tract Hospital of Medical City. The CHB patients divided into two groups: the first group with complicated HBV infection 32 patients (fibrosis, cirrhosis, and hepatocellular carcinoma), and the second group with 58 uncomplicated patients. The diagnosis of each case was established by clinical examination done by a gastroenterologist and hepatologist in the Gastrointestinal Hospital along with the laboratory confirmatory testing for HBV infection. Data were collected through direct interviews with patients and examination of hospital records and previous medical reports. The data collected included subjects; name, age, sex, chronicity of disease, treatment and complication of disease. CHB patients with another type of viral hepatitis, alcohol abuse and patients with autoimmune disease were excluded.

Five ml of venous blood were obtained from all patients which divided into two parts: The first part (3 ml) was put in a plain tube from which serum was obtained for serological test to measure the HBeAg and anti-HBe by ELISA assay, the second part (2 ml) was placed in EDTA tube for each of DNA isolation for PCR, and for quantified the viral load by viral nucleic acid extraction Kit II (Geneaid-Tiwan), and then specific kit have been used to detect and quantify HBV- DNA by Bosphore® HBV Quantification Kit (Anatolia-Turkey).

Serological analysis

Serum preparation and storage

For complete clotting, the tubes had been left at room temperature (15-25 °C) for 20 min and centrifuged for 10 min at 1900 x g at 4 °C, then the serum was transferred to a new tube and centrifuged for 10 min at 16000 * g at 4 °C. Finally, the supernatant carefully transferred to a new tube and kept frozen in aliquots at -70 °C until use.

All the 90 samples were tested for HBeAg and Anti-HBe ELISA Kit (CTK Biotech / USA).

Hepatitis B envelop antigen (HBe Ag)

The RecombELISA HBe Ag ELISA test is a solid phase enzyme linked immunosorbent assay based on the principle of antibody sandwich technique for detection of HBe Ag in human serum or plasma.

The test is composed of two key components:

1- Solid microwells pre-coated with monoclonal anti-HBe Ag antibody.
2- Liquid conjugates composed of polyclonal anti-HBe Ag conjugates horseradish peroxidase (HRP-HBeAb conjugate).

Assay procedure

1- The desired number of strips was removed and secured them in the microwell.

2- Specimens was added according ELISA working sheet.
3- Fifty µl of HBe Ag positive, negative control was added to control well and 50 µl of samples were added to test well respectively.
4- Fifty µl of conjugate was added to each well.
5- The well was incubated at 37 °C for 30 min and washed with wash buffer for four times.
6- Fifty µl of TMB substrate A and B was added respectively, incubated in dark place for 10 minutes at 37 °C.
7- Finally, 50 µl stop solution was added rock gently.
8- The result was read at 450 nm within 15 minutes

Interpretation of Result

A. The cut-off value = N+2.1 N (Mean OD of the negative control).
B. Calculation of specimen OD ratio; Calculate an OD ratio for each specimen by dividing its OD value by the Cut-off value as follows: Specimen OD ratio = specimen OD /cut-off value.

Hepatitis B envelop antibody (HBe Ab)

Assay principle

HBe Ab ELISA test is a solid phase enzyme linked immunosorbent assay based on the principle of competitive technique for the detection of HBe Ab in human serum or plasma. The HBe Ab ELISA test is composed of two key components:

1- Solid microwells pre-coated with recombinant HBe Ag
2- Liquid conjugate composed of anti-HBe Ab conjugated with horseradish peroxidase (HRP-HBe Ab conjugate)

Assay procedure

Assay procedure same as HBe Ag test.

Interpretation of the result

A. Set up the cut-off value; the cut-off value = N*0.4+P*0.6 (N: mean OD of negative control, P: mean OD of positive control).
B. Calculation of specimen OD ratio: Specimen OD ratio = specimen OD /cut-off value.

Isolation of DNA and Polymerase Chain Reaction

Human DNA was isolated from whole blood using a ready kit (gSYNCTM DNA Geneaid / Korea) according to manufacturer's protocol. Tetra-Primer Amplification Refractory Mutation System (ARMS-PCR) method was used to amplify the fragment of CTLA-4 gene (+49A/G) rs231775 with four primers (Table 1).

Table 1. Sequences and resultant fragment lengths of primers used for CTLA-4 gene amplification with ARMS-PCR [21]

Polymorphism	Primers 5'→3'	Fragment length
+49 A/G (rs231775)	Outer pri. F: GTGGGTTCAAACACATTTCAAAGCTTCAGG	229 bp
	R: TCCATCTTCATGCTCCAAAAGTCTCACTC	
	Inner pri. F: ACAGGAGAGTGCAGGGCCAGGTCCTAGT	162 bp
	R: GCACAAGGCTCAGCTGAACCTGGATG	120 bp

The PCR conditions comprised of an initial denaturation for 10 minutes at 95 °C, followed by 35 cycles each with denaturation for 30 sec at 94 °C, annealing for 30 sec at 61 °C and an extension for 45 sec at 72 °C. The final steps were an elongation for 7 min at 72 °C [20]. The products of PCR were undergone gel electrophoresis and stained with ethidium bromide. The results were read under UV transilluminator with digital camera.

Quantitative Real time PCR (RT-PCR)

The viral DNA was isolated from whole blood using a ready kit (Geneaid/Korea) for Real time PCR according to manufacturer's protocol. Two hundred µl sample viral DNA was extracted via three main steps: lysis, nucleic acid binding and washing. The purified nucleic acid was eluted finally, the concentration and purity of the DNA were measured using the nucleic acid measuring instrument Nano Drop (England).

HBV was quantified by HBV Quantification kit (Real-time PCR/Bosphore/Anatolia/Turkey), the kit content in table (2) and table (3) shows the preparation PCR and table (4) shows the thermal cycler, and by the use of real-time PCR system software program calculates the baseline cycles and the threshold.

Table 2. Content of HBV quantitative kit

Component	REAGENT	100 Reactions	50 Reactions	25 Reactions
1	dH_2O	(1000 µl)	(500 µl)	(500 µl)
2	PCR Master Mix	(1650 µl)	(825 µl)	(413 µl)
3	Internal Control	(560 µl)	(280 µl)	(140 µl)
4	Positive Control	(44 µl)	(22 µl)	(15 µl)
5	Standard 1 (1×10^6) IU/ml	(880 µl)	(880 µl)	(440 µl)
6	Standard 2 (1×10^5) IU/ml	(880 µl)	(880 µl)	(440 µl)
7	Standard 3 (1×10^4) IU/ml	(880 µl)	(880 µl)	(440 µl)
8	Standard 4 (5×10^2) IU/ml	(880 µl)	(880 µl)	(440 µl)

Table 3. Preparation of PCR

PCR Master Mix	15 µl
Sample DNA (Standard, Negative/Positive Control)	10 µl
Total Volume	25 µl

Table 4. Instrument programming

Steps	Temperatures	Time
Initial denaturation	95 °C	14:30 min
Denaturation	97 °C	00:30 min
Annealing and Synthesis	54 °C	01:30 min
Hold	22 °C	05:00 min

Results

Clinical Characteristic of Patients

Table 5 shows that the rate of patients with HBeAg in both 174 complicated and uncomplicated CHB was very close (17.24% and 16.63%, respectively) (P>0.05). However, all patients in complicated group had anti-HBe compared to 87.93% in uncomplicated group with a significant difference. About 65.63% of patients in complicated group had active type of chronicity versus 58.62% of patients in uncomplicated group who had such type with a significant difference (P<0.05).

Table 5. Clinical characteristics of patients

Variables		Patients with CHB Uncomplicated (n=58)	Complicated (n=32)	P value
HBeAg	Negative	48(82.76%)	27 (84.38%)	0.844
	Positive	10 (17.24%)	5 (16.63%)	
Anti-HBe	Negative	7 (12.07%)	0 (0.0%)	0.041
	Positive	51 (87.93%)	32 (100%)	
Type of chronicity	Inactive	24 (41.38%)	11 (34.37%)	0.028
	Active	34 (58.62%)	21 (65.63%)	

Viral load

Data of viral load were subjected to normality test and were found to be non-normally distributed. As these data implies very large numbers, they were transformed in log formula which were found to be normally distributed. Accordingly, Student t-test was used to compared means between complicated and uncomplicated CHB. Uncomplicated CHB infections showed slightly higher Log10 viral load (5.0±1.86) than complicated CHB infection (4.4±1.4) without significant difference (Figure 1).

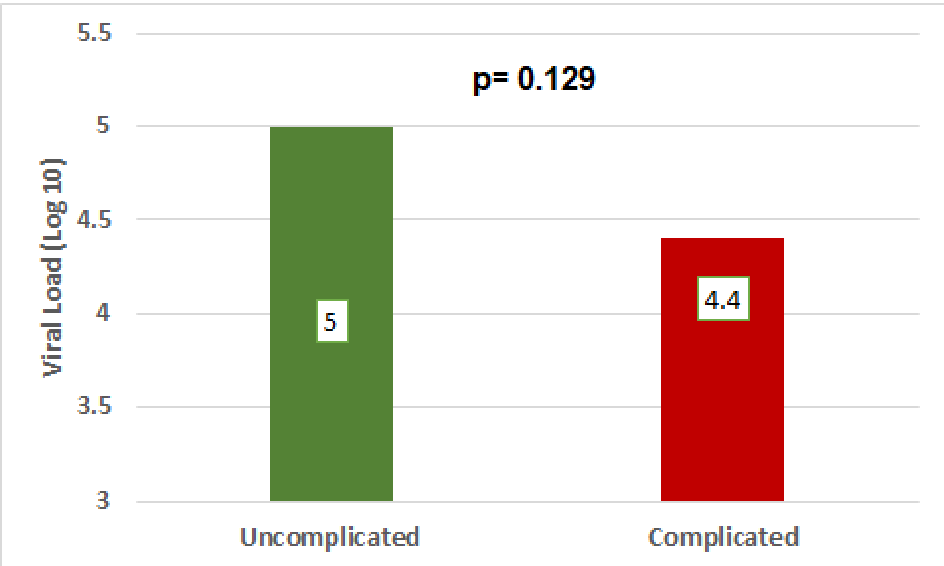

Figure 1. Mean Log10 hepatitis B viral load in complicated and uncomplicated CHB infection

CTLA-4 (+49G/A)

Allele specific PCR was used for gene amplification and genotyping of this SNP. Figure 2 shows the gel electrophoresis of PCR products which revealed that this SNP had three genotypes in complicated and uncomplicated patients. These were GG, GA and AA.

Figure 2. Genotype patterns of cytotoxic T-lymphocyte associated 208 antigen-4 +49A/G polymorphism using ARMS-PCR visualized under UV 209 transllluminator. *M: DNA marker, lanes 1,3 and 10: AG genotype, lanes 2, 4, 7, 8, and 9: AA genotype, 211 lanes 5 and 6: GG genotype

The frequency of either GG or GA did not differ significantly between the two groups. However, AA genotype was more frequent among uncomplicated than complicated CHB patients (44.83% versus 28.12%) with a significant difference (OR= 0.315, 95%, CI=1.0-0.99, P= 0.048) as shown in table 6. It seems that this SNP acts in recessive model more than in dominant model, despite the difference did not reach the acceptable significant level. Analysis of allele distribution revealed a higher frequency of A allele among uncomplicated than complicated group (63.79% versus 64.87%) with a significant difference (OR= 0.5, 95%CI= 0.27-0.93, P= 0.028)

Table 6. The frequency of different genotypes and allele of CTLA-4(+49G/A) polymorphism in complicated and uncomplicated HBV patients

CTLA-4(+49A/G)		Patients with CHB Un-complicated (58)	Complicated (32)	P-value	OR (95%CI)
Genotypes	GG	10 (19.23%)	11 (34.38%)	0.14	1.0 Reference
	GA	22 (37.93%)	12 (37.5%)	0.215	0.5 (0.16-1.5)
	AA	26 (44.83%)	9 (28.12%)	0.048	0.315 (1.0-0.99)
	HWE	0.173	0.162		
Dominant model	GG+GA	32(55.17%)	23(71.88%)	0.12	Reference
	AA	26(44.83%)	9(28.12%)		0.48 (0.19-1.22)
Recessive model	GG	10(19.23%)	11(34.38%)	0.066	Reference
	AA+GA	48(82.76%)	21(65.62%)		0.39 (0.15-1.08)
Alleles	G	42(36.21%)	34(53.13%)	0.028	1.0 Reference
	A	74(63.79%)	30(64.87%)		0.5 (0.27-0.93)

Discussion

In the current study, the rate of patients with HBeAg in both complicated and uncomplicated was very close. Traditionally, individuals who are HBeAg positive are seen during a phase with a high level of HBV replication and when the patient is highly infectious [22]. This is not completely accurate in view of new findings in this study, because patients with low levels of HBeAg can relatively easily achieve HBeAg loss or seroconversion to anti-HBe. Perhaps, a higher percentage of those patients, if treated with antivirals, will experience HBeAg loss [23]. According to the study conducted by Dienstag et al. [24] which suggested that patients with HBeAg-negative phenotype or precore mutants are unable to secrete HBeAg and tend to have severe liver disease.

In the current study, there was a significant difference in the complicated patients which have active type of chronicity compared with uncomplicated. The risk of developing complications (such as cirrhosis, liver failure, or liver cancer) depends on how rapidly the virus multiplies and how well the immune system controls the infection [25]. The specific virology factors which progress the chronic state to active complication in adult are: the type of genotype, the HBV DNA level and mutations, the external factors including co-infection with HCV or HDV [26]. Overweight or having diabetes increases the risk of having fatty liver in addition to drinking alcohol and other causes of liver injury can also influence the active type of chronicity [27]. The significant variation in the rate of progression of disease has led to the hypothesis that genes, may also determine the rate of disease progression [28].

The current study revealed significant protective role of AA genotype of CTLA-4 +49 rs231775 against progression of disease, which was more frequent among uncomplicated than complicated CHB (44.83% versus 28.12%) with significant differences (OR= 0.315, 95% CI=1.0-0.99, P=0.048). Analysis of allele distribution revealed a higher frequency of A allele among uncomplicated than complicated group (63.79% versus 64.87%). Previous study showed that CTLA-4 +49A/G polymorphism is assumed to confer a higher risk for persistent HBV infection in the Asian population [29]. In male Chinese population, A/A genotype and A allele of rs231775 increased the risk of developing HBV-related HCC according to study conducted by Gu et al. [30]. Both A/G heterozygosity and G/G homozygosity are significantly associated with chronic HBV infection in the study conducted by Xu et al. [31]. These disparities may be due to the apparent heterogeneity between different populations, and to the influence of environmental factors affecting different diseases.

The CTLA-4+49A/G polymorphism involves the substitution in CTLA-4 gene at the site 49 of adenine with guanine. Accordingly, the codon 17 (ACC) which encodes threonine is substituted by GCC which encodes alanine. The CTLA-4 receptor achieves essential regulatory function by controlling the strength of T-cell activation during immune response [32]. Two mechanisms have been postulated for this regulatory effect. The first one is interacting of CTLA-4 with its ligands B7.1 and B7.2 depriving the homologue receptor CD28 from their ligands, the second mechanism is the inhibition of T-cell activation through signal transduction pathway which down-regulates the T-cell receptor dependent signaling [33]. Substitution of threonine by alanine results in many phenotypic changes affecting one or both of these two mechanisms. It was postulated that alanine-containing CTLA-4 protein suffers from an altered spatial configuration which causes a fault in handling of this protein in the endoplasmic reticulum with less efficient N-glycosylation [34]. This glycosylation is very important in the dimerization and the triggering of inhibitory function of CTLA4 [35].

In conclusion, A allele of the SNP CTLA4+49 A/G appears to have a protective role against progression of CHB in Iraqi patients. Further studies with a larger sample and different ethnic population are required for more solid conclusion.

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Author contribution

Mahdi did the sampling and laboratory works; Dr. Kadhim supervised the study; Dr. Shemran did the statistics, helped in the laboratory works and prepared the manuscript.

Conflict of interest

There are no conflicts of interest.

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