



Association of HLA-G 3'UTR polymorphisms with hepatitis B virus infection in Tunisian population

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Received: 2 February 2024 / Accepted: 3 July 2024

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Abstract

Hepatitis B virus (HBV) infection is a major public health burden. The mechanisms of immune evasion during chronic HBV (CHB) infection are poorly understood. Human leukocyte antigen (HLA)-G, an immune checkpoint molecule, plays a crucial role in the tolerance mechanisms of various infectious diseases. The 3' untranslated region (3'UTR), including the HLA-G + 3142 C > G polymorphism (rs1063320) and the 14-pb Ins/Del (rs66554220) has been strongly suggested to influence HLA-G expression. This study conducted a case-control analysis to evaluate the potential correlation between the HLA-G + 3142 C > G polymorphism and HBV infection outcome in a Tunisian cohort. The HLA-G + 3142 C > G polymorphism was analysed by *PCR-RFLP* in 242 patients with chronic HBV infection (116 males and 126 females), 241 healthy controls (116 males and 125 females), and 100 spontaneously resolved subjects (52 males and 48 females). Patients with chronic HBV infection showed a higher frequency of the +3142G allele compared to healthy controls and spontaneously resolved subjects ($p=0.001$ and $p=0.002$, respectively). An association between the +3142G allele and high HBV DNA levels was observed when HBV patients were stratified based on their HBV DNA levels ($p=0.016$). Furthermore, the dominant model (GG + GC vs CC) was associated with liver function parameters, including AST, ALT, and high HBV DNA levels ($p=0.04$, $p<0.001$ and $p=0.002$, respectively). However, there was no significant association found between this polymorphism and the fibrosis stage ($p=0.32$). The haplotype analysis, using a subset of previously published data on the HLA-G 14-pb Ins/Del polymorphism, revealed an association between the Ins/G haplotype and chronic HBV infection (H1: InsG, $p<0.001$). Our findings suggest that the +3142G allele is a risk factor for the persistence and progression of HBV infection, while the +3142C allele serves as a protective allele associated with the spontaneous resolution of the infection. Additionally, the HLA-G 3'UTR haplotype Ins/G is associated with chronic HBV infection in the Tunisian population.

Keywords HLA-G · HLA-G + 3142C > G · HLA-G 14-pb Ins/del · Hepatitis B infection · Polymorphism

Introduction

Hepatitis B virus (HBV) is a major global public health concern. According to the latest data from the World Health Organization (WHO), approximately 296 million people were living with chronic hepatitis B (CHB) infection in 2019, and around of 820,000 deaths occurred annually due to HBV-related diseases, including cirrhosis, hepatocellular carcinoma (HCC), and liver failure [1]. Tunisia is a country with low HBV endemicity [2]. The prevalence of HBsAg ranges from 2.2 to 2.7% [3], with a predominance of genotype D [4]. To address this public health burden, the WHO has established a goal of eradicating the threat of viral hepatitis by 2030. The majority of immunocompetent adults infected with HBV can spontaneously resolve

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the virus during the acute phase. However, less than 5% develop chronic HBV infection, which can progress to severe stages of liver disease [5]. The host immune response has been shown to play a pivotal role in the natural history of chronic HBV infection. Experimental evidence shows that robust, polyclonal, and multispecific CD4⁺/CD8⁺ T-cell responses, combined with neutralizing antibody responses against all HBV antigens, are typically associated with viral clearance during acute HBV infection. However, persistent HBV infection is mostly characterized by dysfunctional or exhausted adaptive immune responses, and failure to activate the innate immune system such as dendritic cells (DCs), monocytes and NK cells [6, 7]. These investigations have clearly demonstrated the immunological exhaustion associated with the chronic phase of HBV infection. To achieve a functional cure of chronic HBV infection, a better understanding of the mechanisms leading to immune tolerance and affecting the effector function of immune cells is needed.

HLA-G is a non-classical (class Ib) major histocompatibility complex (MHC) molecule, characterized by a low level of polymorphism and restricted tissue expression. The immune checkpoint HLA-G molecule plays an important role in modulating the immune response [8]. The expression of HLA-G molecules induces immune tolerance function by binding to their specific inhibitory receptors expressed by immune cells, such as Ig-like transcript 2 (ILT-2) is expressed on DCs, B cells, NK cells and T cells, ILT-4 predominantly expressed in myeloid cells, whereas Killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4) is restricted to NK cells [8–10]. Given the importance of the HLA-G molecule, several studies have explored its involvement in susceptibility to different pathological conditions, including cancer [11], inflammatory [12], and infectious diseases [13].

The HLA-G (3' UTR) plays a crucial role in the control of transcriptional activity and mRNA stability. Variability in this region can effectively modulate the expression of the HLA-G gene [14]. Interestingly, several single nucleotide polymorphisms (SNPs) sites have been identified in the 3' UTR that could potentially affect alternative splicing and miRNA/mRNA binding. Among these, two variants have been reported so far in terms of functional studies. First, the 14-bp Insertion/Deletion (Ins/Del) variant (rs66554220) has been associated with alternative splicing, with the removal of 92 nucleotides between positions +2961 and +2974, and mRNA stability [15]. The Ins allele has been suggested to be associated with low HLA-G protein production, whereas the Del allele is associated with higher levels of HLA-G expression by providing more stability to the mRNA [16]. Furthermore, the +3142 C>G variant (rs1063320) has been identified as a target site for specific miRNAs, including miR-148a, miR-148b, and miR-152, which may potentially modulate the transcription of the HLA-G gene [15]. It has been reported that the +3142G allele enhances the binding

affinity of these miRNAs, leading to the degradation of HLA-G mRNA and a subsequent reduction in its expression. Conversely, the +3142C allele is associated with decreased production of soluble HLA-G [17]. It is noteworthy that the HLA-G +3142 C>G polymorphism has been associated with several diseases, including cancer [18], autoimmune diseases [19–21], and viral infections [22, 23].

In this retrospective study, we aimed to evaluate the potential association between the HLA-G +3142 C>G variant and the outcomes of HBV infection in a Tunisian population.

Subjects and methods

Study population

A total of 583 participants, including 242 patients who are chronically infected with HBV (CHB) [seropositive for hepatitis B surface antigen (HBsAg) for at least 6 months], attending the Department of Infectious Diseases at Farhat Hached University Hospital, 100 spontaneously resolved (SR) subjects of HBV who were seronegative for HBsAg and seropositive for HBV core antigen (anti-HBc), and 241 healthy blood donors included in the study as healthy controls (HC) who were seronegative for all known serological markers of HBV, hepatitis C virus (HCV), and human immunodeficiency virus (HIV) infection. CHB patients with co-morbidities, including liver disease (such as autoimmune and alcoholic liver disease) or other viral diseases (including HCV, hepatitis delta virus (HDV), and HIV) were excluded from the study.

CHB patients were divided into two subgroups according to their HBV replication status: Subgroup (1) consisted of 137 patients with low HBV replication (HBV DNA levels <2000 IU/mL) and subgroup (2) consisted of 105 patients with high HBV replication (HBV DNA levels ≥2000 IU/mL).

CHB patients, HC and SR were matched for sex, age and for geographical area. Informed written consent was obtained from all participants prior to blood collection in this study. Approval for the present study was obtained from the Ethical Review Board (ERB) of Farhat Hached University Hospital (ERB approval number: 35220228).

Laboratory assays

CHB patients were followed regularly with measurements of liver function tests including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBil), γ -glutamyltransferase (GGT) and alkaline phosphatase (ALP). Serological markers including HBsAg, HBeAg, anti-HBe and anti-HBc were detected using

Microparticle Enzyme Immunoassay technology (AxSYM; Abbott Laboratories, Abbott Park, IL, USA). Serum HBV-DNA levels were quantified by a commercial real-time polymerase chain reaction (RT-PCR) (COBAS AmpliPrep/COBAS TaqMan, Roche Diagnostics), with detection limits ranging from 20 IU/mL at the lower level to 110×10^6 IU/mL at the upper level. For statistical analyses, an arbitrary value of 20 and 110×10^6 IU/mL was assigned to samples with undetectable and uninterrupted HBV-DNA, respectively.

DNA extraction and genotyping assays

Genomic DNA was extracted from whole blood EDTA using the QIAamp DNA blood kit according to the manufacturer's instructions (Qiagen, Chatsworth, CA, USA). Genotyping assay for the HLA-G + 3142 C > G polymorphism was performed by restriction fragment length polymorphism (RFLP)-PCR with the following primers:

The forward primer (5'-CATGCTGAACTGCATTCC TTCC-3'), and the reverse primer (5'-CTGGTGGGACAA GGTTCTACTG-3') [22]. PCR was performed in a total volume of 25 μ L, including 100 ng genomic DNA, 1.5 mM MgCl₂, 0.2 mM concentrations of each dNTP, 1U of Taq DNA polymerase (Promega) and 0.4 μ M of each specific primer. The PCR conditions were 94 °C for 5 min, 32 cycles of 94 °C for 30 s, 30 s at 65.5 °C and 60 s at 72 °C, followed by a final extension step at 72 °C for 5 min. RFLP was performed by the incubation of PCR product with 3U of BaeGI restriction enzyme (Biolabs, New England, USA) at 37 °C for 3 h [24]. The restriction fragments (406 bp for allele C; 316 and 90 bp for allele G) were separated by electrophoresis on a 2% agarose gel with ethidium bromide, and observed under ultraviolet illumination using the Gel Doc XR Plus (Bio-Rad, USA) (Fig. 1). Additionally, the 14-pb Ins/Del was amplified by SSP-PCR, exclusively for the SR subjects, such as previously described [25] (Fig. 2).

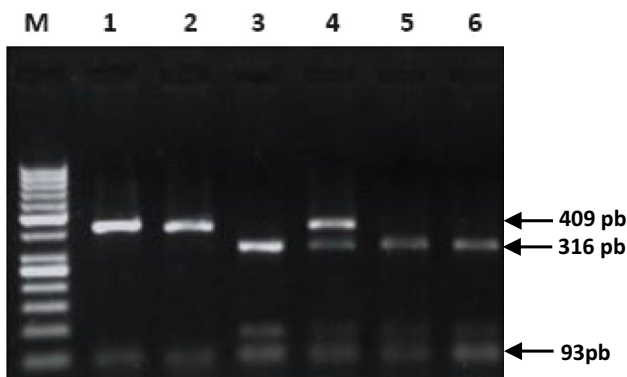


Fig. 1 HLA-G +3142 C <G genotyping by PCR-RFLP. M=DNA ladder of 50 pb; Lane 1=CC; Lane 2=CC; Lane 3=GG; Lane 4=CG; Lane 5=GG and Lane 6=GG genotype. Numbers on the right indicate the size of the amplicons. Agarose gel 2%

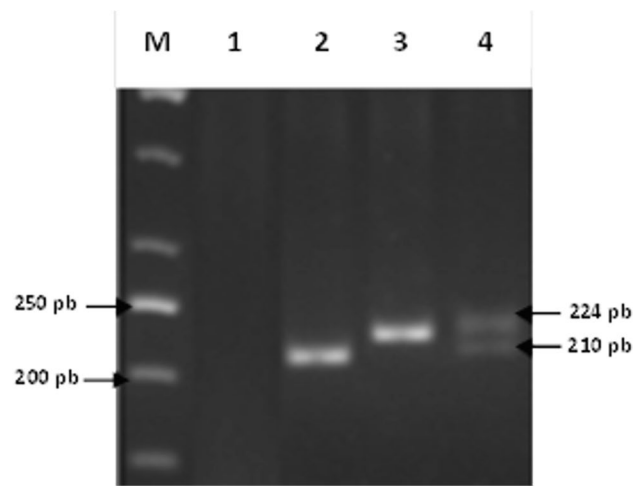


Fig. 2 HLA-G 14pb Ins/Del genotyping by PCR-SSP. M=DNA ladder of 50 pb; Lane 1=Negative control; Lane 2=Del/Del genotype and Lane 3=Ins/Ins genotype; Lane 4=Ins/Del genotype. Numbers on the right indicate the size of the amplicons. Agarose gel 2%

Statistical analysis

Statistical analysis was conducted using SPSS17.0 software (SPSS Inc., Chicago, IL, USA) and Graphpad prism 9. Allele and genotype frequencies were compared between groups using either the X^2 or Fisher's exact test. Odds ratios (OR) [95% confidence interval (CI)] were calculated to estimate the relative risk. The comparison of quantitative variables between groups was conducted using the Mann-Whitney test. The significance threshold for p -values was set at $p < 0.0125$ after applying the Bonferroni correction to address multiple comparisons. The Hardy-Weinberg equilibrium (HWE) and haplotype inference were analysed using Haploview software (version 4.2).

Results

Characteristics of the study population

Table 1 summarises the characteristics of the study population. The study enrolled 242 patients (116 males and 126 females), 241 HC (116 males and 125 females) and 100 SR (52 males and 48 females). The mean age [\pm standard error of the mean (SEM)] was 35.6 ± 0.7 for CHB patients, 34.4 ± 0.8 for HC and 37.5 ± 0.2 years for SR subjects. No significant differences in gender or age were reported between study groups. The clinical profiles of CHB patients with high and low HBV DNA levels are showed a significant difference in the HBV DNA levels between the two groups ($p < 0.001$). All patients included in our study exhibit HBV genotype D. In addition, high ALT and AST levels were

Table 1 Demographic characteristics and clinical profiles of study subjects

Characteristics	CHB patient (N=242)	Healthy controls (N=241)	Spontaneously resolved (N=100)
Gender			
Male	116 (48%)	116 (48%)	51 (51%)
Female	126 (52%)	125 (52%)	49 (49%)
Age*	35.6 ± 0.7	34.4 ± 0.8	37.5 ± 0.2
HBV status			
HBsAg +	242 (100%)	0 (0%)	0 (0%)
Ac anti-HBc IgG	242(100%)	0 (0%)	100 (100%)
Liver Functions Tests (LFT)	Low HBV DNA levels (N=137)	High HBV DNA levels (N=105)	
ALT (IU/L)* [¶]	23.97 ± 0.71	35.1 ± 2.8	
AST (IU/L)* [¶]	22.12 ± 0.65	29.94 ± 2.6	
GGT (IU/L)*	24.8 ± 1.63	26.73 ± 3.02	
ALP (IU/L)*	86.26 ± 4.67	83.89 ± 6.11	
T Bil (µmol/L)*	9.04 ± 0.23	9.83 ± 0.61	
HBV DNA Levels (IU/mL, log)* [¶]	2.09 ± 0.05	4.03 ± 0.09	
Liver biopsy specimen available during study period [‡]	5	55	
Stage of fibrosis			
F0-F1	5	40	
F2-F4	0	15	

*: Mean ± SEM

‡: Grading score, stage according to METAVIR classification

¶: *P*-value < 0.001

reported in patients with high HBV DNA levels ≥ 2000 UI/mL ($p < 0.0001$).

Association between the HLA-G rs1063320 and the different outcomes of HBV infection

To investigate the role of this polymorphism in the susceptibility to chronic HBV infection, we initially genotyped the rs1063320 in both CHB patients and HC. The corresponding genotype and allele frequencies are presented in Table 2. The genotype distribution of the HLA-G rs1063320 was in Hardy–Weinberg equilibrium in the HC ($p > 0.05$). The frequency of the **G** allele was significantly higher in CHB patients as compared to HC (62% vs 52%, $p = 0.001$), suggesting that CHB patients with the **G** allele have an increased risk of developing chronic HBV infection (OR = 1.49, 95% CI [1.16–1.93]). In addition, the **GG** and **GC** genotypes were overrepresented in CHB patients, conferring a susceptibility to persistent HBV infection (OR = 2.26, 95% CI [1.35–3.78], $p = 0.001$ and OR = 1.86, 95% CI [1.14–3.03], $p = 0.011$, respectively). Furthermore, to determine whether the rs1063320 was associated with spontaneously resolved HBV infection, the allele and genotype frequencies were

compared between CHB patients and SR subjects. Interestingly, the frequency of the wild-type **C** allele of the HLA-G rs1063320 was significantly overrepresented in SR subjects (51% vs. 38%, $p = 0.002$), and was associated with a protective effect that increased the spontaneous clearance of HBV infection (OR = 1.66, 95% CI [1.19–2.32]). Moreover, the frequency of the **CC** genotype was significantly higher in SR subjects than in CHB patients (30% vs. 15%, respectively; OR = 2.73, 95% CI [1.43–5.21], $p = 0.001$). However, there was no statistically significant difference in the comparison of SR subjects and HC. These results clearly show that the HLA-G +3142 C > G polymorphism has an impact on the outcome of HBV infection.

Association between the HLA-G rs1063320 and the HBV DNA level

To investigate the impact of the rs1063320 variant on the regulation of HBV replication, we classified patients into two subgroups according to their HBV DNA levels and compared the allele and genotype frequencies between those with low and high HBV DNA levels. As shown in Table 3, our finding revealed a higher prevalence of the **G** allele and

Table 2 Genotype and allele frequencies of HLA-G + 3142C > G polymorphism in the overall cohort

HLA-G	Alleles/genotypes distribution	CHB N = 242 (%)	HC N = 241 (%)	SR N = 100 (%)	P-value ^a	P-value ^b	P-value ^c	OR [95% CI] ^a	OR [95% CI] ^b	OR [95% CI] ^c
+ 3142C > G										
Allele	G	298 (62)	249 (52)	98 (49)	0.001	0.002	0.52	1.49 [1.16–1.93]	1.66 [1.19–2.32]	1.11 [0.79–1.54]
	C	186 (38)	233 (48)	102 (51)	Ref	Ref	Ref			
Genotype	GG	92 (38)	71 (29)	28 (28)	0.001	0.001	0.54	2.26 [1.35–3.78]	2.73 [1.43–5.21]	1.2 [0.65–2.23]
	GC	114 (47)	107 (45)	42 (42)	0.011	0.007	0.5	1.86 [1.14–3.03]	2.26 [1.24–4.12]	1.21 [0.69–2.1]
	CC	36 (15)	63 (26)	30 (30)	Ref	Ref	Ref	-	-	-
	GG+GC	206 (85)	178 (74)	70 (70)	0.002	0.001	0.46	2.02 [1.28–3.19]	2.45 [1.41–4.27]	1.21 [0.72–2.02]

Risk alleles marked in bold letters; Ref: reference

Significant P-values are shown in bold. ^a: CHB vs HC. ^b: CHB vs SR. ^c: HC vs SR

the **GG** genotype in patients with high HBV DNA levels compared with those with low HBV DNA levels [(68% vs. 57%, [OR = 1.57, 95% CI [1.08–2.29], **p = 0.016**) and (45% vs. 32%, [OR = 2.71, IC 95% [1.17–6.26], **p = 0.017**), respectively]. However, the significance did not survive the Bonferroni correction.

Moreover, the frequencies of the **G** allele and the **GG** genotype were significantly higher in CHB patients with high HBV DNA levels than in HC (68% vs. 52%, [OR = 1.95, 95% CI [1.39–2.74], **p < 0.001**), and similarly when compared with SR subjects (68% vs. 49%, [OR = 2.17, 95% CI [1.45–3.24], **p < 0.001**). This observation suggested that the presence of the HLA-G + 3142G allele in CHB patients increases the risk of active HBV replication.

Association between the HLA-G rs1063320 and the progression of CHB infection

To assess the influence of the rs1063320 on the progression of CHB infection, we stratified patients according to the stage of liver fibrosis using the METAVIR score. This score distinguishes between mild/moderate (F0–F1) and severe (F2–F4) fibrosis stages. However, we did not find any statistically significant association between the HLA-G rs1063320 polymorphism and the progression of liver disease in CHB patients in our cohort (Table 4).

Correlation of liver functions and virological features according to HLA-G + 3142 C > G genotypes

To evaluate the impact of the rs1063320 on variations of biochemical and virological features in CHB patients, we compared patients with dominant model (GG + GC) to those with CC genotype (Fig. 3). Notably, CHB patients with GG/GC genotypes had significantly elevated levels of transaminases (ALT and AST) and GGT compared to those with CC genotypes (**p = 0.04**, **p < 0.001** and **p = 0.002**, respectively) (Fig. 3A, B, C). Furthermore, the dominant model showed a correlation with higher HBV DNA levels (**p < 0.001**, Fig. 3F). In contrast, there was no significant correlation between HLA-G + 3142 C > G genotypes and ALP and T-Bil levels (Fig. 3D, E).

Haplotype analysis of HLA-G 3'UTR polymorphisms of HBV infection

To investigate the impact of HLA-G 3'UTR polymorphisms on the outcome of HBV infection, we constructed haplotypes for two polymorphisms. One polymorphism was derived from actual data of HLA-G + 3142 C < G and the other was based on our previously published study that investigated the HLA-G 14-bp Ins/Del in the same cohort [25]. Furthermore, we genotyped the HLA-G 14-bp Ins/Del specifically

Table 3 Genotype distribution and allele frequencies of HLA-G + 3'42C > G polymorphism between patients with low or high HBV DNA levels

Alleles/ genotypes distribution	High CHB DNA N = 105 (%)	Low CHB DNA N = 137 (%)	HC N = 241 (%)	SR N = 100 (%)	P-value ^a	P-value ^b	P-value ^c	P-value ^d	P-value ^e	OR [95% CI] ^a	OR [95% CI] ^b	OR [95% CI] ^c	OR [95% CI] ^d	OR [95% CI] ^e
+3'42C > G														
Allele G	142 (68)	156 (57)	249 (52)	98 (49)	0.016 *	< 0.001	< 0.001	0.16	0.087	1.57 [1.08– 2.29]	1.95 [1.39– 2.74]	2.17 [1.45– 3.24]	1.23 [0.91– 1.66]	1.37 [0.95–1.98]
C	68 (32)	118 (43)	233 (48)	102 (51)	-	-	-	-	-	-	-	-	-	-
Geno- type GG	47 (45)	45 (32)	71 (29)	28 (28)	0.017 *	< 0.001	< 0.001	0.15	0.085	2.71 [1.17– 6.26]	4.1 [1.94– 8.93]	5.03 [2.14– 11.8]	1.5 [0.85– 2.77]	1.8 [0.91–3.75]
GC	48 (46)	66 (47)	107 (45)	42 (42)	0.12	0.005	0.002	0.15	0.5	1.89 [0.83– 4.28]	2.82 [1.33– 8.97]	3.41 [1.46– 7.83]	0.66 [0.38– 1.16]	1.2 [0.69–2.12]
CC	10 (09)	26 (19)	63 (26)	30 (30)	-	-	-	-	-	-	-	-	-	-
GG+GC	95 (91)	111 (81)	178 (74)	70 (70)	0.04 *	< 0.001	< 0.001	0.11	0.04 *	2.22 [1.02– 4.84]	3.36 [1.64– 6.85]	4.07 [1.86– 8.87]	1.5 [0.9–2.52]	1.8 [0.99–3.34]

Risk alleles marked in bold letters; Ref: reference genotype

Significant P-values are shown in bold

*: Not significant after Bonferroni correction ($\alpha=0.0125$)^aHigh CHB DNA vs Low CHB DNA; ^bHigh CHB DNA vs HC; ^cHigh CHB DNA vs SR; ^dLow CHB DNA vs HC; ^eLow CHB DNA vs SR

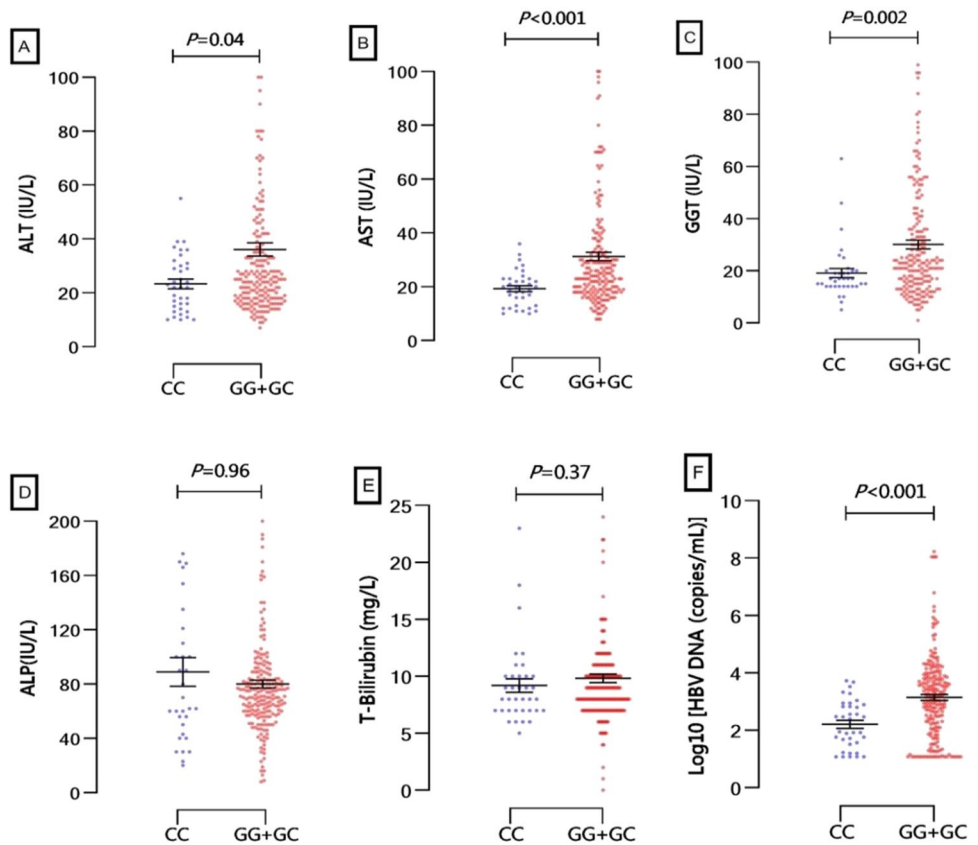
Table 4 Genotype and allele frequencies of HLA-G + 3142C>G polymorphism in patients stratified according to the liver fibrosis score

	Alleles/genotypes distribution	Fibrosis METAVIR score		X^2	P-value	OR [95% CI]
		F0-F1	F2-F4			
		(N=45) N (%)	(N=15) N (%)			
+ 3142C>G						
Allele	G	54 (60)	21 (70)	0.96	0.32	0.64 [0.26–1.5]
	C	36 (40)	9 (30)	Ref	-	
Genotype	GG	16 (36)	8 (54)	0.43	0.68*	0.57 [0.04–4.1]
	GC	22 (49)	5 (33)	0.57	1*	1.25 [0.09–10]
	CC	7 (15)	2 (13)	Ref	Ref	
	GG+GC	38 (85)	13 (87)	0.04	0.83	0.83 [0.15–4.5]

Risk alleles marked in bold letters; Ref: reference genotype

*: Fisher exact test

Fig. 3 Biochemical and virological features of CHB patients according to HLA-G + 3142C>G genotypes. Box plot illustrate Mean with SEM. P-value were calculated by Mann-Whitney test



for the SR subjects in this study. The comparison of allelic and genotypic frequencies of the 14-bp Ins/Del polymorphism between CHB and HC did not reveal any statistical significance (data not shown).

Four haplotypes were identified, denoted ('InsG' (**H1**), 'DelC' (**H2**), 'DelG' (**H3**) and 'InsC' (**H4**) (Table 5). The most prevalent haplotypes were **H1** and **H2**, with frequencies of 40% and 35%, respectively. The frequency of **H1** was significantly higher in the CHB patients compared to

HC and SR subjects (CHB vs. HC: $p < 0.001$ and CHB vs. SR: $p = 0.05$) indicating the association of 'InsG' haplotype with chronic HBV infection. However, the frequency of the **H4** haplotype was higher in the HC and SR subjects than in the CHB patients (CHB vs. HC: $p = 0.002$ and CHB vs. SR: $p = 0.041$), implying **H4** was suggested to be a protective haplotype against chronic HBV infection. These findings underscore the significant role of HLA-G in influencing disease outcomes.

Table 5 Haplotype frequencies of HLA-G 14-bp Ins/Del and +3142C>G polymorphisms between CHB patients and healthy controls

Haplotypes	CHB (%)	HC (%)	SR (%)	<i>P</i> -value ^a	<i>P</i> -value ^b	<i>P</i> -value ^c
H1: <i>InsG</i>	47	36	38	0.0003	0.05	0.43
H2: <i>DelC</i>	31	35	39	0.17	0.074	0.401
H3: <i>DelG</i>	15	16	11	0.68	0.202	0.107
H4: <i>InsC</i>	7	13	12	0.002	0.041	0.539

Significant *P*-values are shown in bold. ^aCHB vs HC. ^bCHB vs SR. ^cSR vs HC

Discussion

The clinical outcomes of HBV infection are heterogeneous. The host immune response emerges as a pivotal factor influencing the natural course of chronic HBV infection. In fact, efficient and robust immune responses are typically associated with viral clearance during acute HBV infection. However, chronic HBV infection is characterised by dysfunctional or weak immune responses [26]. The precise mechanisms that promote immune tolerance and the persistence of HBV infection are not fully understood. In this context, the HLA-G molecule continues to attract considerable interest as a molecule involved in immune tolerance in several diseases, such as cancer and infectious diseases [13, 27]. Previously, we have shown, as have several other studies, that the soluble HLA-G (sHLA-G) molecule is involved in the progression of HBV infection [28–31]. Given the substantial influence of the HLA-G +3142 C>G polymorphism on the regulation of sHLA-G expression [32, 33], this study aims to investigate the potential association of this variant with the outcome and the progression of HBV infection in the Tunisian population.

Our data showed that a higher frequency of the G allele of the HLA-G +3142 C>G polymorphism and the Ins/G haplotype were associated with an increased risk of developing chronic HBV infection. However, the C allele and the Ins/C haplotype appear to play a protective role during the acute phase and are associated with spontaneous resolution of HBV infection in our population. This finding seems to be in line with a recent study conducted by Okumura et al., showing a significant association of the G allele with susceptibility to chronic HBV infection in the Japanese population [34]. Interestingly, our results are supported by a previous study by Cordero et al. [22]. The study suggests that the presence of the G allele and the Ins/G haplotype are associated with an increased susceptibility to HCV infection in sickle cell disease patients of African descent. In addition, the G allele and the GG genotype of the HLA-G +3142 C>G polymorphism have been reported to be associated with several other viral infectious diseases. A recent study conducted by Medeiros et al. revealed that both the G allele, and the GG genotype were associated with a predisposition to HIV infection and the Ins/G haplotype

was overrepresented in African-derived patients HIV⁺ from Southern Brazil [35]. Moreover, Xu et al. showed that the G allele and the Ins/G haplotype were associated with a high risk of human papillomavirus (HPV) infection in Chinese Han women [36]. Likewise, Da Silva et al. also showed an increased prevalence of the Ins/G haplotype in HIV patients of African descent [37]. This consistent pattern emphasises the association between the +3142G allele and the insertion of 14-pb of HLA-G 3'UTR and the susceptibility to various viral infectious diseases, particularly in individuals with African ancestry. This not only strengthens our findings regarding the effect of these variants in the HBV infection, but also underscores the pivotal role of host genetic contributions in determining HBV outcomes.

Furthermore, to determine the correlation of the HLA-G +3142 C>G polymorphism with the progression of chronic HBV infection, we stratified CHB patients according to their viral load (patients with low HBV DNA levels <2000 IU/mL and patients with high HBV DNA levels ≥2000 IU/mL). This threshold is clinically important because it differentiates between active and inactive viral replication. It also serves as a critical indicator to assess the progression of inflammation during HBV infection, as recognised by healthcare professionals [38]. Interestingly, our results showed a significant association between the G allele and the GG genotype with elevated levels of HBV DNA in patients with chronic hepatitis B (CHB). We also observed an increased frequency of the GG +GC model, which correlated with elevated levels of inflammatory liver markers, including AST, ALT and GGT. To the best of our knowledge, the present study is the first to assess the association between HLA-G +3142 C>G polymorphism and the progression of chronic HBV infection. Previous studies have investigated the level of sHLA-G with different phases of chronic HBV infection. Han et al. showed that serum of sHLA-G levels correlated with ALT levels in CHB patients [29]. Subsequently, increased expression of sHLA-G was correlated with HBV DNA levels [28, 39]. However, our cohort did not show a consistent association between the HLA-G +3142 C>G polymorphism and liver fibrosis. Notably, our findings have revealed for the first time a potential involvement of the G allele in activating HBV replication and triggering an inflammatory response that may contribute

to the progression of HBV infection to liver failure. It's worth noting that we could not conclusively demonstrate this association in the progression of liver fibrosis within our cohort, possibly due to the limited sample size. Similarly, Okumura et al. also reported a lack of correlation between HLA-G + 3142 C > G polymorphism and progression to HCC [34].

Overall, several lines of evidence suggest that the G allele and/or GG genotype of the HLA-G + 3142 C > G polymorphism was strongly associated with the susceptibility to several infectious diseases [35–37]. In the context of HBV infection, both the recent study by Okumura et al. [34] and our current research have confirmed the following findings. In addition, elevated levels of sHLA-G expression have been consistently reported in CHB patients across different cohorts, underscoring the important role of the HLA-G molecule in influencing the outcome of this disease [28–31]. On the other hand, the HLA-G + 3142 C > G polymorphism is suggested to influence HLA-G expression through post-transcriptional mechanisms by affecting the binding of specific miRNAs, in particular the miR-148/152 family (miR-148a, miR-148b, and miR-152) [17, 33, 40]. Tan et al. showed that the presence of guanine at position + 3142 reduces the expression of soluble HLA-G through mRNA degradation following a high affinity for miR148/152 [33]. Furthermore, Rousseau et al. suggest that the deletion of 14-bp in the 3'UTR of HLA-G correlates with high sHLA-G expression by stabilising the mRNA [41].

Several hypotheses may explain this discrepancy observed between functional studies and genetic and molecular studies carried out on HBV infection. To address this paradox, we hypothesise that the persistence of HBV infection and the elevated levels of sHLA-G, despite the presence of the + 3142G allele, may be due to strategies used by the virus to evade the immune response. Notably, Bian et al. showed that the expression of sHLA-G protein is upregulated by HBV, but not the mRNA, through the downregulation of miR148/152, which serves to attenuate NK cell cytotoxicity [42]. Likewise, Sartorius et al. showed that the HBx protein of HBV downregulates the miR-148/152 to suppress the immune response against HBV infection [43]. Recently, the miR-152 has been reported to be associated with dendritic cell dysfunction during acute and chronic hepatitis B virus infection [44].

A further hypothesis can be proposed in relation to the effect of the HLA-G + 3142 C > G variant on the expression of the HLA-G molecule. Veit et al. suggest that the HLA-G + 3142 C > G polymorphism alone may not be the sole determinant that controls the expression of HLA-G and the 14-pb polymorphism is not the main element on the regulation of gene expression at the RNA level [45]. Furthermore, a previous study investigated the impact of the most frequent variation sites described in the HLA-G

3'UTR on plasma sHLA-G levels. The study showed that the UTR-2 haplotype ((InsTCCCGAG), which contains the 14-pb insertion and + 3142G allele, is correlated with medium production of sHLA-G [46]. It is noteworthy that UTR-2 is among the most frequent haplotypes in the African population [47], which may explain the increased susceptibility of individuals of African origin to various types of infectious diseases [20, 35, 48]. Furthermore, Hviid et al. showed a significant correlation between the 14-pb ins/ins genotype and increased IL-10 levels [49]. IL-10 has recently been identified as a crucial mediator of T-cell dysfunction during chronic HBV infection and HIV-1/HCV coinfection diseases [50, 51]. Altogether, these observations highlight the important role of the 14-pb Ins/Del and + 3142 C > G polymorphisms of HLA-G 3'UTR in the susceptibility to various infectious diseases including the HBV infection. Hence, it is crucial to take into account other polymorphisms in the 3'UTR and 5'UTR to fully understand the complexity of genetic factors that regulate the HLA-G molecule and its involvement in susceptibility to infectious diseases, including HBV infection.

Several studies have demonstrated elevated expression of the HLA-G molecule in chronic HBV patients [28–31]. On the other hand, it has been reported that chronic HBV patients are characterized by lower levels and dysfunction of NK cells [52] and a depletion of HBV specific CD4 + and CD8 + T cells [53]. Furthermore, a number of studies have indicated that immune system cells in patients with CHB, including NK cells, T lymphocytes, and dendritic cells, express inhibitory receptors of the HLA-G molecule (ILT2, ILT4 and KIR2DL4) during chronic HBV infection [54, 55]. These findings suggest that the interaction between the HLA-G molecule and its inhibitory receptors is implicated as a mechanism of immune evasion by HBV. This occurs through the protection of infected cells, achieved by modulating the expression of specific cytokines associated with HBV infection, such as IL-1 β , IL-10, IL-12, INF- γ , and TNF- α [56]. Moreover, research conducted by our team and other investigators on genetic polymorphisms influencing HLA-G expression, such as the 14-bp Ins/Del and + 3142 C/G in the context of HBV infection could provide valuable insights into the genetic mechanisms regulating HLA-G expression during chronic HBV infection [25, 39]. This underscores the significant role of host genetics as a key factor controlling the expression of immune system genes.

In conclusion, this study is the first to demonstrate an association between the HLA-G + 3142 C > G polymorphism and the outcome and progression of HBV infection in the Tunisian population. The + 3142G allele and GG genotype were found to be linked to susceptibility to chronic HBV infection and elevated levels of HBV DNA. Conversely, the C allele and CC genotype exhibited a protective effect, correlating with the spontaneous resolution

of HBV infection. Furthermore, the Ins/G haplotype located within the HLA-G 3'UTR was found to be linked with persistent HBV infection. Our study has certain limitations, including a small number of patients with fibrosis and HCC, as well as a limited number of individuals who spontaneously resolved the infection. Considering the crucial role of the HLA-G molecule in the immune response to HBV infection outcomes, it would be interesting to investigate the association of the entire HLA-G gene, including the 5'UTR, coding region, and 3'UTR, with susceptibility to HBV infection in future studies.

Author contributions **A.B Laaribi:** Conceptualization, Formal analysis, Investigation and Writing—original draft. **A. Letaif:** Resources. **N. Hannachi and J. Boukadida:** Resources, Validation, Supervision and Writing—review & editing. **A. Mehri, H. Ben Yahia, H. Chaouch, W. Babay:** Formal analysis, Investigation and Data curation. **H.I Ouzari:** Supervision, Writing—review & editing and Funding acquisition. **I. Zidi:** Conceptualization, Supervision, Writing—review & editing, Project administration.

Funding This research was supported by the Ministry of Higher Education and Scientific Research of Tunisia. Programme d'Encouragement à l'Excellence Scientifique (P2ES), Grant Number: P2ES2020-D5P2.

Declarations

Ethics approval This research was approved by the ethical review board (ERB) of Farhat Hached University Hospital. ERB approval number: 35220228.

Consent to participate Informed written consent was obtained from all participants.

Consent for publication All authors reviewed and approved this manuscript for publication.

Conflict of interest The authors declare no competing interests.

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