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Single nucleotide polymorphisms of interleukin-35 subunit genes predict host susceptibility to chronic hepatitis B virus infection among Iraqi patients

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ABSTRACT

Chronic hepatitis B virus (HBV) infection represents a complication of a major concern in public health. Interleukin-35 (IL-35) has been suggested to play a role in maintaining the viral persistence. It consists of two subunits (IL-12p35 and EBI3) that are coded by two genes located on different chromosomes; *IL12A* (3q25.33) and *EBI3* (19p13.3), respectively. Single nucleotide polymorphisms (SNPs) of both genes have been associated with susceptibility to different diseases. Therefore, this study examined two intronic SNPs of *IL12A* (rs582054 and rs583911 in intron 2) and *EBI3* (rs428253 and rs7254021 in intron 1) genes in 80 chronic HBV patients and 96 control with the aim to understand their susceptibility role in the disease. The method of SNP detection was allele-specific PCR using specific primers. Frequencies of rs582054 *A* allele and AT genotype were significantly increased in patients compared to control. *C* allele and CC genotype frequencies of rs428253 SNP were also significantly increased in patients. For rs583911 and rs7254021 SNPs, allele and genotype frequencies demonstrated no significant variation between patients and control. Haplotype analysis revealed that A-A haplotype of *IL12A* SNPs (rs582054 and rs583911) was associated with a significantly increased HBV risk. In contrary, G-C haplotype of *EBI3* SNPs (rs428253 and rs7254021) was associated with a significant decreased risk. In conclusion, IL-35 subunit gene SNPs (*IL12A* rs582054 and *EBI3* rs428253) are associated with susceptibility to develop chronic HBV infection among Iraqi patients.

1. Introduction

Chronic hepatitis B virus (HBV) infection represents a complication of a major concern in public health. In 2015, the World Health Organization (WHO) estimated that 257 million people were living with chronic HBV infection. The infection resulted in an estimated 887,000 deaths, mostly due to liver cirrhosis and hepatocellular carcinoma (WHO, 2017a). Clearance or persistence of HBV infection is mostly determined by host immune responses. A complex interaction between HBV and an inadequate immune response is required to establish the chronicity of HBV infection (Li et al., 2015). Upon infection, effective antiviral immune responses mediated by CD8+ and CD4+ T cells, as well as natural killer (NK) cells and monocytes can consequence in partial or complete eradication of HBV (Peeridogaheh et al., 2018). It has been demonstrated that acute HBV infection induces CD4+ and CD8+ T responses together with elevated production of interferon-gamma (IFN-γ). IFN-γ is prominent cytokine in clearing the virus and controlling HBV infection (Sandhu et al., 2017). In contrast, increased production of inhibitory cytokines; for instance, interleukin 10 (IL-10) and transforming growth factor-beta (TGF-β), has been associated with persistent HBV infection and inability to eradicate the virus due to a down-regulation of T cells (Maini and Pallett, 2018). Recently, a further cytokine (IL-35) has been suggested to play a role in maintaining viral persistence. It suppresses anti-HBV immune responses and reduces inflammatory responses in chronic HBV infection (Shao et al., 2017).

IL-35 is the latest member of IL-12 family, which is recognized to have anti-inflammatory and immunosuppressive properties (Su et al., 2018). It is heterodiemeric cytokine consisted of two subunits; IL-12α chain p35 (IL-12p35) and IL-27β chain Epstein-Barr virus-induced 3 (EBI3) (Song and Ma, 2016). Several cells have been reported to produce IL-35 including stimulated natural T regulatory (Treg) cells, IL35-producing regulatory B cell (i35-Breg) and stimulated pan T cells (Xue et al., 2019). Two distinct immunological functions are linked to IL-35; suppression of T cells proliferation and transformation of these cells into Treg cells (Collison et al., 2010). Due to these functions, abnormal regulation of IL-35 has been suggested to have a prominent role in immune-associated diseases; for instance autoimmune diseases, cancer

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Table 1
Forward and reverse primers for *IL12A* and *EPI3* gene SNPs.

Gene	SNP	Primer	Position	Tm (°C)	Molecular Size (bp)
IL12A	rs582054 A/C/T	FA: 5'-TGGGTGTCCCAACTCTAGGAA-3' FC: 5'-TGGGTGTCCCATCTCTAGGAA-3' FT: 5'-TGGGTGTCCCACCTCTAGGAA-3′ R: 5'-CCCAAATGACCAGGAGGCAT-3′	Chr3:159992203 + 159,992,308	57	106
	rs583911 G/A	FG: 5'-ATGCCATTCAAAAACCAAACATTT-3' FA: 5'-ATGCCATTCAAAAACCAAACGTTT-3′ R: 5'-CAGACAGAGGTAACAGCATGAG-3′	Chr3:159991422 + 159,992,160	59	739
EBI3	rs428253 G/C	FG: 5'-AAAACCAAAAAGAAACCAAAGGAATG-3' FC: 5'-AAAACCAAAAAGAAACGAAAGGAATG-3′ R: 5'-CCTCTCAAGTCTCCCACGA-3'	Chr19:4229493–4,229,932	61	440
	rs7254021 T/C	FT: 5'-CAGGGTTTTACCATGTTGGCC-3' FC: 5'-TAGGGTTTTACCATGTTGGCC-3′ R: 5'-TACAGAGAGGAACTCGGGTAAG-3'	Chr19:4230112 + 4,230,948	61	837

F: Forward; R: Reverse; Tm: Melting temperature; bp: Base pair.

and viral and bacterial infections (Zhang et al., 2019). In HBV, recent studies suggest a critical immunopathogenesis role for IL-35 in potentiating a chronic infection (Shi et al., 2015; Shao et al., 2017; Cheng et al., 2018; Tao et al., 2018).

The two subunits of IL-35 (IL-12p35 and EBI3) are coded by two genes located on different chromosomes; *IL12A* (3q25.33) and *EBI3* (19p13.3), respectively (Song and Ma, 2016). Single nucleotide polymorphisms (SNPs) of both genes have been associated with susceptibility to different diseases (Guan et al., 2019). With respect to HBV, three studies have so far investigated *IL12A* SNPs in infected patients. In the first, *IL12A* and *IL12B* SNPs were suggested to play a role in determining the response to HBV vaccination (Pan et al., 2012). It has also been demonstrated that development of anti-HBV surface antigen (anti-HBs) antibodies in hemodialysis patients are associated with T helper 1 cytokine SNPs including *IL12A* gene (Grzegorzewska et al., 2012). In the third investigation, a variant of *IL12A* was considered as a marker for risk of HBV clearance (Tan et al., 2016). In the case of *EBI3* gene, searching the PubMed revealed that SNPs of this gene have not been investigated in HBV.

This study examined two intronic SNPs of *IL12A* (rs582054 and rs583911 in intron 2) and *EBI3* (rs428253 and rs7254021 in intron 1) genes in HBV patients with the aim to understand their susceptibility role in the disease.

2. Materials and methods

2.1. Patients

During June–October 2018, a case-control study was conducted in Baghdad to determine SNPs of IL-35 subunit genes (*IL12A* and *EBI3*) in 80 cases with chronic HBV infection (age mean ± SD: 41.2 ± 13.7 years; 50 males and 30 females). The patients were recruited from the outpatient clinic at the Specialized Center for Gastroenterology and Hepatology in Baghdad. HBV testing guidelines established by the WHO and EASL (European Association for the Study of the Live) were followed in the diagnosis (Lampertico et al., 2017; WHO, 2017b). Serological diagnostic tests included anti-hepatitis B core antigen (HBc) IgM, anti-HBc IgG and anti-hepatitis surface antigen (HbsAg) antibodies. Molecular diagnosis included detecting pre-S1 through S genes of HBV. Patients with HCV infection, other chronic hepatic diseases (autoimmune liver disease and drug-induced hepatic diseases) and autoimmune diseases were excluded from the study. A control sample of 96 healthy blood donors matched patients for age (43.2 ± 11.9 years), gender (58 males and 38 females) and ethnicity was also included in the study. The study protocol was approved by the Ethics Committee at the Department of Biology (University of Baghdad) and the Iraqi Ministry of Health (Reference: BEC/1019/002). All participants gave a written informed consent to participate in the study.

2.2. Serological diagnostic tests

Anti-HBc IgM, -HBc IgG and -HbsAg antibodies were qualitatively screened using ELISA kits (MyBioSource, USA). Standard procedures recommended by the manufacturers were followed.

2.3. Molecular detection of HBV

DNA was isolated from whole EDTA blood using a rapid blood genomic DNA extraction kit (Bio Basic, Canada) employing standard procedure recommended by the manufacturer. The isolated DNA was subjected to PCR to amplify 1063 bp region of pre-S1 through S genes using sense (5'-TCACCATATTCTTGGGAACAAGA-3′) and antisense (5'-CGAACCACTGAACAAATGGC-3′) primers. The PCR reaction mixture consisted of 5 μL DNA, 2 μL of sense primer, 2 μL of antisense primer, 2 μL of master mix (AccuPower PCR Premix, Bioneer, Korea), and the volume was made up to 20 μL with free-nuclease water. PCR cycling conditions consisted of initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (20 s) and elongation at 72 °C (1.5 min). This was followed by a final elongation step at 72 °C (5 min). The PCR products were electrophoresed on 2% agarose gel at 5 V/cm^2 for 60 min and stained with diamond dye. The migrating PCR products alongside a pattern of 100 bp DNA ladder were then visualized using gel documentation system (Naito et al., 2001).

2.4. IL-35 subunit gene SNPs

Four SNPs of IL-35 encoding genes; *IL12A* (rs582054 and rs583911 in intron 2) and *EBI3* (rs428253 and rs7254021 in intron 1), were selected on the basis of their minor allele frequency (MAF ≥ 10%) (https://www.ncbi.nlm.nih.gov/snp/). An allele-specific PCR method was employed to detect the SNPs using specific primers (Table 1). Geneious software (version 10.2.2) was used to design the primers (Kearse et al., 2012). After DNA isolation, PCR amplification was carried out. The PCR mixture (20 μL) consisted of 1.5 μL DNA, 1 μL of each primer, 5 μL Master Mix (Integrated DNA Technologies, Inc., USA), and 11.5 μL nuclease free water. The PCR conditions included initial denaturation at 94 °C (5 min), followed by 40 cycles of denaturation at 94 °C (35 s), annealing for 35 s (the temperature is given in Table 1) and extension at 72 °C (35 s). This was followed by a final extension at 72 °C for 10 min. The PCR products were electrophoresed on 1.5% agarose gel at 5 V/cm^2 for 60 min and stained with diamond dye. The migrating PCR products alongside a pattern of 100 bp DNA ladder were then visualized using gel documentation system (Figs. 1-4).

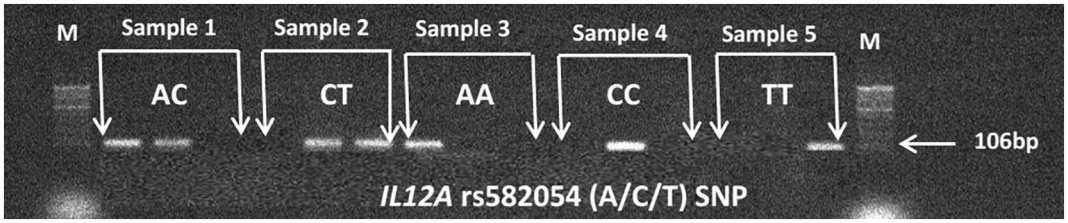

Fig. 1. Gel electrophoresis of DNA-PCR amplified products (106 bp) for the SNP rs582054 (A/C/T) on 3% agarose (5 V/cm^2 for 60 min) showing genotypes for five samples. Sample 1: AC, Sample 2: CT, Sample 3: AA, Sample 4: CC, Sample 5: TT. M: DNA Ladder (100 bp).

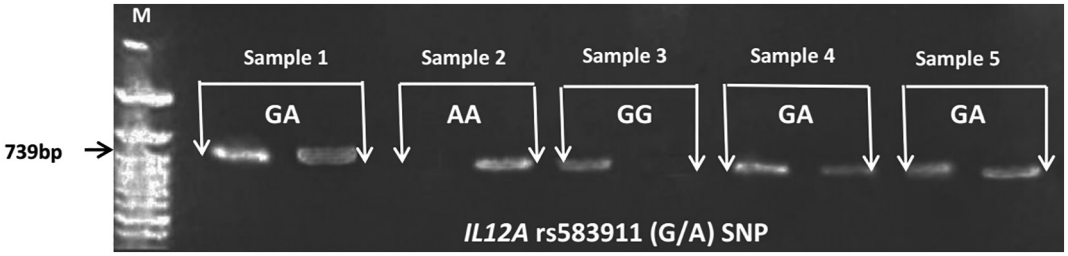

Fig. 2. Gel electrophoresis of DNA-PCR amplified products (739 bp) for the SNP rs583911 (G/A) on 1.5% agarose (5 V/cm^2 for 60 min) showing genotypes for five samples. Sample 1: GA, Sample 2: AA, Sample 3: GG, Sample 4: GA, Sample 5: GA. M: DNA Ladder (100 bp).

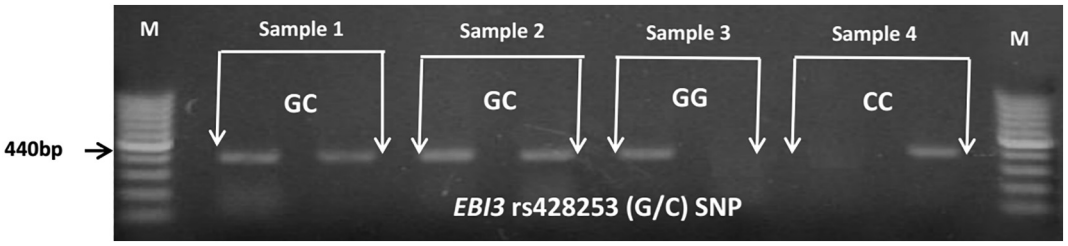

Fig. 3. Gel electrophoresis of DNA-PCR amplified products (400 bp) for the SNP rs428253 (G/C) on 1.5% agarose (5 V/cm^2 for 60 min) showing genotypes for four samples. Sample 1: GC, Sample 2: GC, Sample 3: GG, Sample 4: CC. M: DNA Ladder (100 bp).

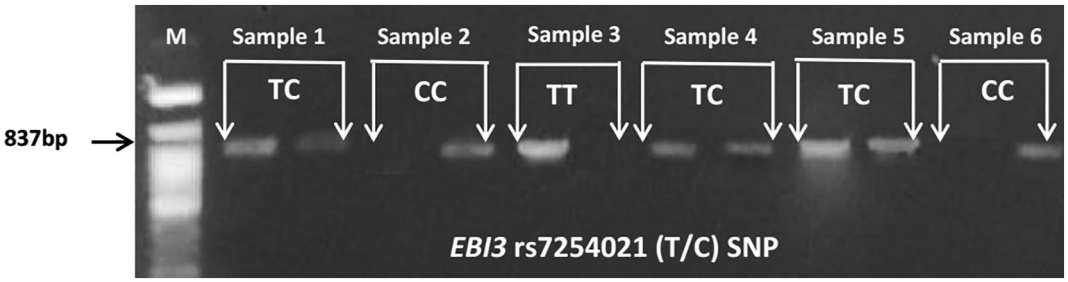

Fig. 4. Gel electrophoresis of DNA-PCR amplified products (400 bp) for the SNP rs7254021 (T/C) on 1.5% agarose (5 V/cm^2 for 60 min) showing genotypes for six samples. Sample 1: TC, Sample 2: CC, Sample 3: TT, Sample 4: TC, Sample 5: TC, Sample 6: CC. M: DNA Ladder (100 bp).

2.5. Statistical analysis

The sample size was estimated using G*Power 3.1.9.4 (Faul et al., 2007). Hardy-Weinberg equilibrium (HWE) evaluation of SNP genotype frequencies was made by Pearson's Chi-square test. Odds ratio (OR) and its 95% confidence interval (CI) were used to assess SNP-disease association, and significant differences were determined by two-tailed Fisher exact probability (p). Bonferroni correction was applied to correct the p-value due to multiple comparisons. A corrected p (pc) ≤ 0.05 was considered significant. The statistical package SPSS (version 19.0) and Winpepi software (version 11.65) were employed to carry out these analyses. SHEsis software was used to estimate haplotype frequencies and linkage disequilibrium (LD) between SNPs. The LD was expressed as LD coefficient (D') (Yong and He, 2005).

3. Results

3.1. Sample size

The power of present sample size of HBV patients was calculated by G*Power 3.1.9.4 software. At $\alpha = 0.05$and effect size of 0.3, a sample of 80 patients gave a power of 0.79, which was close the accepted minimum power (0.80). However, a higher power will certainly more preferable.

3.2. HBV diagnosis

Serum assessments of anti-HBV antibodies (anti-HBc IgM, -HBc IgG and -HbsAg) revealed that all patients were seropositive for anti-HBc IgG and -HbsAg antibodies, while they were seronegative for anti-HBc

Table 2
Allele and genotype frequencies of *IL12A* and *EBI3* gene SNPs in hepatitis B virus infection patients and control.

Gene	SNP	Allele/genotype	N (%)		OR (95% CI)	*p* (*pc*)
			Patients (*N* = 80)	Control (*N* = 96)		
IL12A	rs582054	*A*	66 (41.2)	37 (19.3)	2.94 (1.83–4.73)	< 0.001 (S)
		C	43 (26.9)	89 (46.3)	0.43 (0.27–0.67)	< 0.001 (S)
		T	51 (31.9)	66 (34.4)	0.89 (0.57–1.39)	0.651 (NS)
		AA	12 (15.0)	6 (6.2)	2.65 (0.95–7.37)	0.079 (NS)
		AC	14 (17.5)	14 (14.6)	1.24 (0.56–2.78)	0.681 (NS)
		AT	28 (35.0)	11 (11.5)	3.97 (1.83–8.61)	< 0.001 (S)
		CC	8 (10.0)	18 (18.8)	0.46 (0.19–1.11)	0.091 (NS)
		CT	13 (16.2)	39 (40.6)	0.26 (0.13–0.54)	< 0.001 (S)
		TT	5 (6.3)	8 (8.3)	0.70 (0.22–2.22)	0.579 (NS)
HWE *p*			0.146 (NS)	0.107 (NS)		
	rs583911	*G*	96 (60.0)	112 (58.3)	1.07 (0.70–1.64)	0.828 (NS)
		A	64 (40.0)	80 (41.7)	0.93 (0.61–1.43)	0.828 (NS)
		GG	26 (32.5)	29 (30.2)	1.11 (0.59–2.10)	0.747 (NS)
		GA	44 (55.0)	54 (56.3)	0.95 (0.52–1.72)	0.880 (NS)
		AA	10 (12.5)	13 (13.5)	0.91 (0.38–2.20)	1.000 (NS)
HWE *p*			0.192 (NS)	0.124 (NS)		
EBI3	rs428253	*G*	70 (43.8)	120 (62.5)	0.47 (0.30–0.71)	0.001 (S)
		C	90 (56.2)	72 (37.5)	2.14 (1.40–3.28)	0.001 (S)
		GG	14 (17.5)	34 (35.4)	0.39 (0.19–0.79)	0.010 (S)
		GC	42 (52.5)	52 (54.2)	0.94 (0.52–1.69)	0.880 (NS)
		CC	24 (30.0)	10 (10.4)	3.69 (1.65–8.25)	0.002 (S)
HWE *p*			0.551 (NS)	0.128 (NS)		
	rs7254021	*T*	90 (56.3)	85 (44.3)	1.62 (1.06–2.47)	0.032 (NS)
		C	70 (43.7)	107 (55.7)	0.62 (0.41–0.94)	0.032 (NS)
		TT	22 (27.5)	15 (15.6)	2.05 (0.98–4.27)	0.064 (NS)
		TC	46 (57.5)	55 (57.3)	1.01 (0.56–1.83)	1.000 (NS)
		CC	12 (15.0)	26 (27.1)	0.41 (0.20–0.88)	0.028 (NS)
HWE *p*			0.132 (NS)	0.115 (NS)		

IL12A: Interleukin-12 alpha; *EB13*: Epstein–Barr virus induced 3; HWE: Hardy-Weinberg equilibrium; OR: Odds ratio; CI: Confidence interval; *p*: Two-tailed Fisher's exact probability; *pc*: Corrected *p*; S: Significant ($pc \leq 0.05$); NS: Not significant ($p > .05$).

IgM antibody. Such pattern of reactions suggests the chronicity HBV infection (Loh and Kew, 2007). Molecular diagnosis also demonstrated that all patients showed a band of 1063 bp after PCR amplification and gel electrophoresis. In contrary, all control subjects were seronegative for anti-HBV antibodies, and no virus DNA band was detected after gel electrophoresis.

3.3. HWE

The four investigated SNPs showed no significant difference from HWE in patients and control (Table 2).

3.4. IL12A gene SNPs

Frequencies of rs582054 *A* allele and AT genotype were significantly increased in patients compared to control, while *C* allele and CT genotype frequencies were significantly decreased. For rs583911 SNP, allele and genotype frequencies demonstrated no significant variation between patients and control (Table 2).

3.5. EBI3 gene SNPs

C allele and CC genotype frequencies of rs428253 SNP were significantly increased in patients compared to control. In contrary, frequencies of *G* allele and GG genotype were significantly decreased. With respect to the second SNP (rs7254021), allele and genotype frequencies showed no significant differences between patients and control (Table 2).

3.6. LD and haplotype frequencies

There was a weak LD between SNPs of *IL12A* or *EBI3* gene in patients and control (Fig. 5). However, haplotype estimation revealed that A-A haplotype of *IL12A* SNPs (rs582054 and rs583911) was associated with a significantly increased HBV risk. In contrary, G-C haplotype of *EBI3* SNPs (rs428253 and rs7254021) was associated with a significant decreased risk (Table 3).

4. Discussion

Preliminary findings of present study suggest a role for *IL12A* rs582054 (A/C/T) and *EBI3* rs428253 (G/C) SNPs in susceptibility to develop chronic HBV infection. A range of 2.14–3.97 fold higher risk was associated with alleles or genotypes of the two SNPs. Moreover, a haplotype analysis depicted that A-A haplotype of *IL12A* SNPs (rs582054 and rs583911) recorded an estimated OR of 3, and its association with HBV was suggested. Equally important, the *EBI3* G-C (rs428253 and rs7254021) might be considered as a protective haplotype. These findings could provide a significant evidence for the association between haplotypes of IL-35 subunit gene SNPs and the risk of HBV. Therefore, employing haplotype-based analyses in disease-association studies are suggested to be potentially superior to single marker association studies, because these analyses utilize information from individual markers as well as determine the LD structure between the markers (Shi et al., 2007). To our knowledge, the present study is the first examination of the four polymorphisms in chronic HBV patients. However, the association between *IL12A* gene SNPs and HBV appeared in only three publications. In the first, the influence of *IL12A* rs2243115 SNP on response to HBV vaccination in a Chinese Han population was investigated, but allele and genotype frequencies showed no significant variations between low and high responders. However, it was implicated that SNPs in *IL12A* (rs2243115) and *IL12B* (rs17860508) genes might have a combined effect in determining the response to HBV vaccination (Pan et al., 2012). In a further investigation, hemodialysis patients bearing *IL12A* rs568408 AA genotype had a 10.9-fold lower chance to develop anti-HBs antibodies (anti-HBV) compared with those

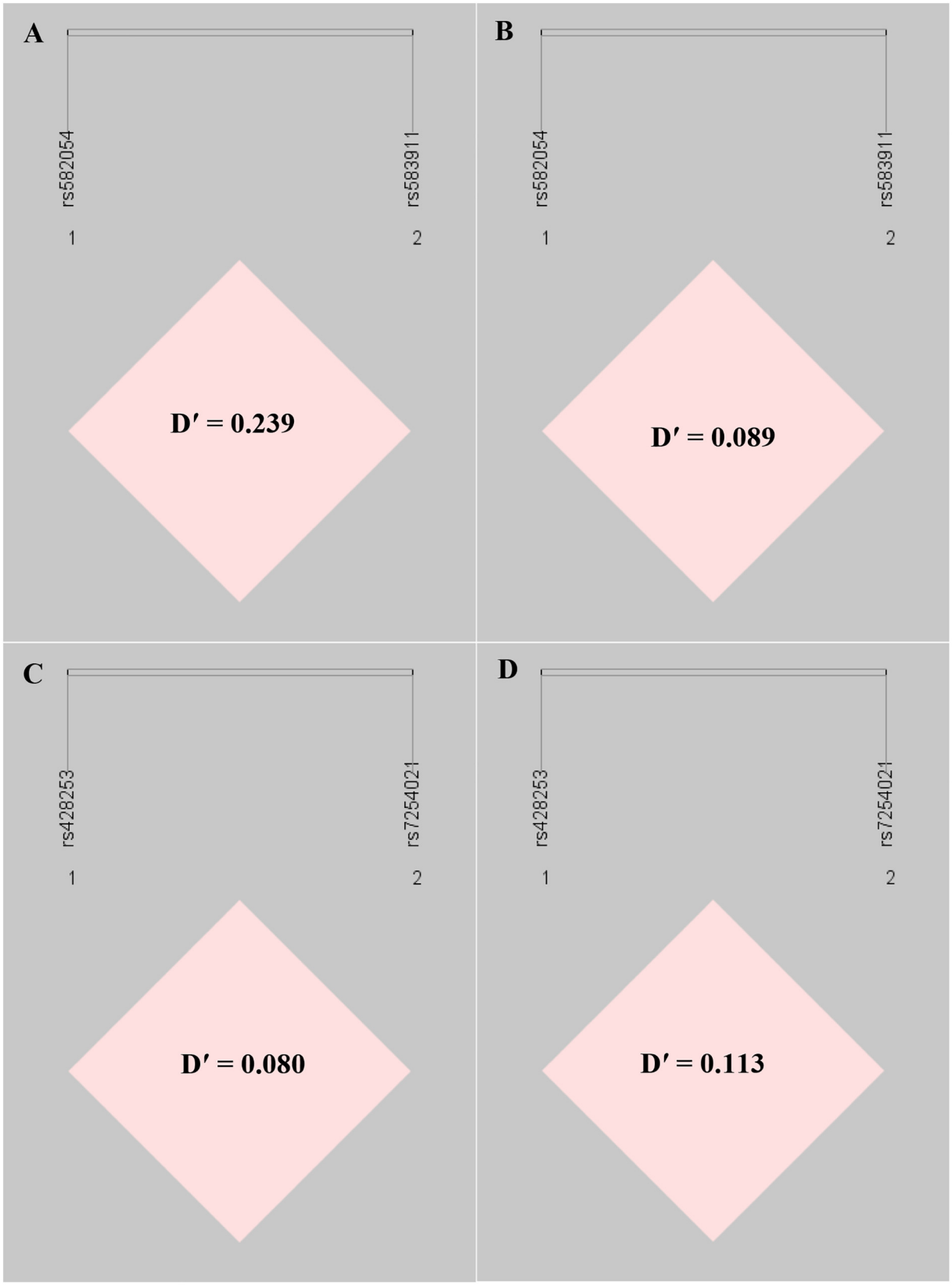

Fig. 5. Pairwise analysis depicting linkage disequilibrium coefficient (D′) between SNPs of *IL12A* (rs582054 and rs583911) and *EBI3* (rs428253 and rs7254021) genes in hepatitis B virus infection patients (A and C, respectively) and control (B and D, respectively).

Table 3
Estimated two-locus haplotype frequencies between SNPs of *IL12A* (rs582054 and rs583911) and *EBI3* (rs428253 and rs7254021) genes in hepatitis B virus infection patients and control.

Gene	Haplotype	%		OR (95% CI)	*p* (*pc*)
		Patients (N = 80)	Control (N = 96)		
IL12A	A-A	22.1	8.6	3.00 (1.60–5.62)	< 0.001 (S)
	A-G	19.1	10.6	1.99 (1.09–3.6)	0.024 (NS)
	C-A	7.8	17.1	0.41 (0.21–0.82)	0.009 (NS)
	C-G	19.1	29.2	0.57 (0.34–0.94)	0.028 (NS)
	T-A	10.1	15.9	0.59 (0.31–1.13)	0.109 (NS)
	T-G	21.8	18.5	1.23 (0.73–2.07)	0.439 (NS)
EBI3	C-C	26.1	18.5	1.56 (0.94–2.58)	0.086 (NS)
	C-T	30.1	19.0	1.84 (1.12–3.02)	0.015 (NS)
	G-C	17.6	37.2	0.36 (0.22–0.60)	< 0.001 (S)
	G-T	26.1	25.3	1.05 (0.65–1.70)	0.8572 (NS)

IL12A: Interleukin-12 alpha; *EB13*: Epstein–Barr virus induced 3; OR: Odds ratio; CI: Confidence interval; *p*: Two-tailed Fisher's exact probability; *pc*: Corrected *p*; S: Significant ($pc \leq 0.05$); NS: Not significant ($p > .05$).

carrying any other genotypes (Grzegorzewska et al., 2012). The third investigation suggested an association of *IL12A* rs568408 mutant *A* allele with an increased risk of persistent HBV infection (Tan et al., 2016). For *EBI3* SNPs, they have not been investigated in HBV infection. Accordingly, the available evidence supporting or refuting the present findings has not been well-elaborated. Apart from HBV, three SNPs of present study (rs582054, rs583911 and rs428253) have shown associations with some diseases especially those of inflammatory and autoimmune in nature; for instance systemic lupus erythematosus (Su et al., 2018; Guan et al., 2019), while rs7254021 has not been investigated in any human disease. Therefore, further functional and genetic analyses of IL-35 represent a critical theme in the understanding of HBV development, progression and control. However, serum level of IL-35 was not determined in the present sample of chronic HBV patients and therefore no correlation was made with the IL-33 subunit gene SNPs in order to understand the SNP's impact on IL-33 level. In future studies, it is necessary to determine IL-35 level and correlate it with SNP genotypes of *IL12A* and *EBI3* genes. The study was further limited by the low sample size because the estimated power of present sample size was at the minimum accepted level.

5. Conclusions

IL-35 gene SNPs (*IL12A* rs582054 and *EBI3* rs428253) are associated with susceptibility to develop chronic HBV infection among Iraqi patients.

CRediT author statement

Rana Mohsen: Conceptualization, Visualization, Methodology, Investigation, Validation, Writing.

Raghad Al-azzawi: Conceptualization, Visualization, Investigation, Supervision, Reviewing.

Ali Ad'hiah: Conceptualization, Methodology, Visualization, Investigation, Supervision, Software, Validation, Writing-Reviewing and Editing.

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Declaration of Competing Interest

None.

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