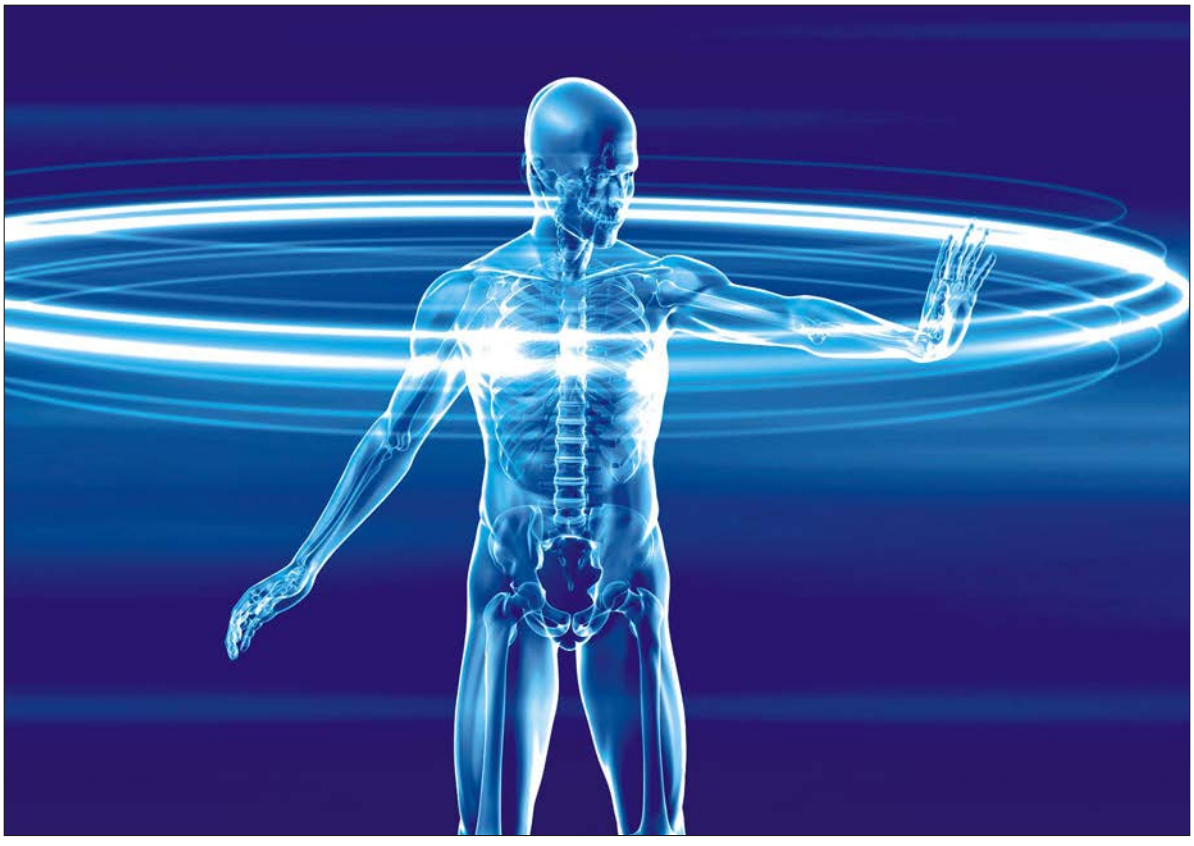

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Research Article
Interleukins-10 and 18 Genes Polymorphisms in Hepatitis B Virus Infected Saudi Patients

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Abstract

Background and Objective: Interleukin-10 (IL-10) and interleukin-18 (IL-18) are important immune regulators that may have a role in HBV infection outcome. Gene polymorphisms of these cytokines can affect their expression and serum level and may be associated with liver disease severity in patients with viral hepatitis. This study aimed to investigate the polymorphisms of IL-10 and IL-18 and to explore whether such polymorphisms are associated with the natural course of HBV infection in Saudi population. **Materials and Methods:** The study included 327 Saudi patients with different HBV clinical outcomes. Single Nucleotide Polymorphisms (SNPs) were detected by restriction fragment length polymorphism after polymerase chain reaction (PCR-RFLP) for both IL-10 (1082G/A and 592A/C) and IL-18 (607C/A and137G/C). **Results:** The IL-10-1082GG genotype among cirrhotics was significantly lower than controls ((3.7 and 12.7%, respectively). While, IL-10-592AA genotype was significantly less prevalent among cirrhotics (6.4%) compared to recovered and control groups. IL-18 (607C>A) SNP analysis showed no significant difference between the studied groups. The IL-18-137CC genotype was less prevalent among cirrhotic patients (1.8%) compared to recovered group (9.7%). Also, GG genotype was statistically more prevalent among HBV cirrhotics. The polymorphisms of the promoter region of IL-10 and IL-18 genes are closely associated with susceptibility to chronic active hepatitis B and development of hepatic cirrhosis. **Conclusion:** The patients with AA at position IL-10-592AA may be protected against HBV infection. Moreover, CC genotype at position IL-18-137CC may be closely linked to HBV-DNA recovery.

Key words: IL-10, IL-18, SNP, PCR-RFLP, HBV, hepatitis, cirrhosis, Saudi Arabia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatitis B Virus (HBV) is one of the most common causes of liver disease; about 350 million persons are currently asymptomatic carriers or suffering from Chronic Hepatitis B (CHB) infection worldwide[1]. The CHB mostly ends by serious complications as hepatic cirrhosis and hepatocellular carcinoma (HCC)[2]. In Saudi Arabia, HBV is hyper-endemic infections may occur either by horizontal transmission early in life (most common) or by vertical transmission[3]. A prevalence of more than 70% of hepatitis B surface antigen (HBsAg) was detected in the first large-scale surveillance done on Saudi children[4]. The WHO reported that the prevalence of HBV in Saudi Arabia[5] was about 8%. Prevalence was low in young ages health college's students in Saudi Arabia. A prevalence of 0.17 and 0.78% was found in 18-21 years old males and females, respectively and 0.39 and 0.90% in the 22-30 year old[6]. Saudi government introduced program in 1990 to vaccinate all Saudi children at school entry, vaccination of healthcare workers, patients under hemodialysis and all children born[3] after October, 1989. These efforts helped in decreasing HBV prevalence. Despite significant decline of HBV prevalence in KSA, it still causes significant morbidity, mortality and represents a challenge to the country's healthcare system[7]. The HBV serological screening must be done before used to decrease HBV transmission risk in blood transfusion. However, low levels of HBV-DNA were detected in some HBsAg negative individuals with the risk of HBV transmission HBV[8].

There are eight genotypes of HBV named from A-H and classified according to the distance of nucleotide sequence from the viral genome of 8% or greater[9,10]. In Saudi Arabia, genotypes B and C were the most common (75 and 16%, respectively) with minimal proportion of other genotypes (3.9 genotype A, 2.2 genotypes E and 1.7% for other genotypes)[11]. In chronic HBV infection dynamic interactions between the virus and immune response of the host occur resulting in a great variation of the natural course of the disease in different patients. While, some patients have a very rapid progression of liver disease with frequent hepatitis flares and others are inactive carriers with more benign prognosis. Natural course of CHB is influenced by many factors related to both virus and host[12]. Virus factors include genotypes, basal core promoter mutations and viral loads[13,14]. Host factors include age, sex, immune status and metabolic factors[15].

The host immune response plays an important role in pathogenesis and the clinical outcome of HBV infection. However, there are individual variations in this immune response as indicated by presence of better response to interferon (IFN) therapy in patients with higher baseline alanine transaminase (ALT) levels[16]. Interleukin-10 (IL-10) is secreted from T-lymphocytes and has many functions as inhibition of inflammatory and immune-proliferative responses. It also inhibits secretion of many cytokines from T-cells and monocytes/macrophages. It stimulates differentiation and proliferation of B-lymphocytes producing IgM, IgG and IgA[17]. Polymorphism of IL-10 at -1082 region which increases production of G allele is associated with HBV clearance during intrauterine infection. Also, G/G genotype at -1082 may decrease HBV viral load at the immune inflammatory phase in chronic HBV infected children. Generally, increased IL-10 production plays an important protective role against HBV infection[18]. However, effect of IL-10 gene polymorphism on HBV infection is still conflicting, IL-10 polymorphism was found to be related to the increased risk of HCC in Korean, Taiwanese and Chinese patients[19]. Also, Zhang *et al.*[20] reported an association between the IL-10 polymorphism at -1082GA and persistent HBV infection susceptibility.

The IL-18, which is secreted from active macrophages is a potent pro-inflammatory cytokine and an immune activator. It also increases induction of IFN-γ and TNF-α and cytotoxicity of natural killer cells[21]. The IL-18 can promote hepatitis B virus clearance as indicated by the positive relationship between serum IL-18 level and the severity of HBV infection in many clinical studies[22]. The human IL-18 gene is located on chromosome 11 q22.2-q22.3 and is composed of six exons and five introns[23]. Genetic analysis showed that two SNPs of the IL-18 gene at position -607 and -137 were suggested to have an impact on IL-18 gene activity[24]. There is no study carried out to examine the association between polymorphisms of the IL-10 or IL-18 gene and CHB infection in Saudi Arabia. This study aimed to investigate the possible role of IL-10 and IL-18 gene polymorphisms in the natural course of CHB infection.

MATERIALS AND METHODS

The study included 327 Saudi patients categorized into three groups; group I (asymptomatic carriers): 115 inactive HBV carriers, group II (cirrhotics): 109 HBV infected patients complicated with cirrhosis, group III (recovered): 103 patients completely recovered from previous HBV infection and group IV (controls): 110 healthy for age and sex matched. Patients suffering from any hepatic decease or double HBV and HCV infection were excluded. Asymptomatic HBV carriers were characterized by being HBsAg positive for a period more than 6 months but with normal levels of transaminases and were both HBeAg and anti-HBe negative, in addition to

Table 1: Primer sets, restriction endonuclease and RFLP fragments of IL-10 and IL-18 SNP

SNP	Primer set (Macrogen, Korea)	Base change	RE enzyme (Fermentas, Lithuania)	PCR-RFLP fragments
IL-10 (1082G/A)	F-CCAGGTAGAGCAACACTCCT R-CTCTTACCTATCCCTACTTCCGC	G>A	*Ear*I	AA: 128 and 27 bp GA: 155, 128 and 27 bp GG: 155 bp
IL-10 (592A/C)	F-GTGGAAACATGTGCCTGAGA R-ATGAGGGGGTGGGCTAAATA	A>C	*Rsa*I	CC: 79 and 75 bp AC: 154, 79 and 75 bp AA: 154 bp
IL-18 (607C/A)	F-TCAGTGGAACAGGAGTCCAT R-GCAGAAAGTGTAAAAATTTTT	C>A	*Dra*I	AA: 109 and 41 bp CA: 150,109 and 41 bp CC: 150 bp
IL-18 (137G/C)	F-AGGTGCTTTCTTAAAGTCAGA R-AATATCACRATTTTCATGGAA	G>C	*Hin*fI	CC: 107 and 42 bp CG: 149, 107 and 42 bp GG: 149 bp

absence of clinical symptoms of liver disease and radiological evidence of cirrhosis. The HBV infection was confirmed by detection of HBV-DNA by real time PCR. Liver cirrhosis was diagnosed by abdominal ultrasonic examination. While, complete recovery form HBV was confirmed when both HBsAg and HBV-DNA by PCR turned negative and anti-HBs become positive.

After a written consent, peripheral venous blood sample (4 mL) was drawn from each individual. One milliliter of whole blood was collected in heparinized tube for DNA extraction and determination of IL-10 and IL-18 single nucleotide polymorphisms (SNP). Sera from the other 3 mL were separated and tested for liver function tests and hepatitis B viral markers. Liver enzymes, serum bilirubin and albumin were measured by enzymatic methods (Spinreact, Girona Spain). Serum AFP and HBV markers were measured by commercially available ELISA kits (Bio-Rad, CA, USA). Genomic DNA was extracted according to the manufacturer's instructions using DNA Blood Mini Kit (Qiagen, Hilden, Germany). The SNPs were detected by restriction fragment length polymorphism after polymerase chain reaction (PCR-RFLP) for both IL-10 (1082G/A and 592A/C) and IL-18 (607C/A and137G/C) as formerly described[25,26]. Primer sets, restriction enzymes and RFLP fragment products are shown in Table 1. The PCR reactions were set-up to amplify the target genes using Amplitaq Gold PCR master Mix (Applied Biosystems, Foster City, USA) with a 25 µL reaction. The PCR protocols were as follows: for IL-10; initial denaturation at 94°C for 2 min followed by of 35 cycles at 94°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec. For IL-18; initial denaturation at 94°C for 2 min followed by 40 cycles at 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec. Then, a final extension step at 72°C for 2 min was added after the last PCR cycle. The PCR products were digested with 10 U of each of the restriction enzymes (Table 1) for 1 h. The digested PCR products were visualized on 3% agarose stained with ethidium bromide (Fig. 1).

Statistical analysis: Data were analyzed using SPSS version 16 (SPSS Inc., Chicago, IL, USA). Normality of distribution was assessed using kolmogorov-Smirnov z-test. Quantitative variables were expressed in Means±SD. Qualitative variables were expressed in frequencies and percentages. Univariate analysis was performed by Kruskal-Wallis test for quantitative data and by chi-square test (χ^2) or Fisher exact test (where appropriate) for qualitative data. Statistical significance was set at 0.05 level. All data were evaluated using SPSS software version 16.0.

RESULTS

Age, gender and biochemical parameters of the studied groups are shown in Table 2. The majority of the studied subjects were males (57.4%). There was no significant difference in the age and gender between the studied groups. Laboratory biochemical parameters show significant higher level of serum bilirubin (both total and direct), ALT and lower level of albumin ($p<0.001$) in cirrhotic patients. Also, serum AFP level was higher among cirrhotic patients than controls. Gel electrophoresis of PCR products and restriction fragments showed the different genotypes of both IL-10 and IL-18 (Fig. 1). The IL-10 (1082A>G) Single Nucleotide Polymorphisms (SNPs) was significantly higher in recovered group compared to HBV carriers and cirrhotics (17.5, 6.9 and 3.7%, respectively). Also, the prevalence of GG genotype among cirrhotics (3.7%) was significantly ($p<0.05$) lower than controls (12.7%). About 1082-A allele was more prevalent among the cirrhotics (73.9%) and 1082-G allele was more prevalent among the recovered group (36.4%) however, the differences between the studied groups were non-significant statistically. On the other hand, IL-10 genotype-592AA was significantly less prevalent among cirrhotics (6.4%) compared to recovered and control groups (19.4 and 16.4%, respectively). Also, allele distribution showed no significant differences between studied (Table 3). The IL-18

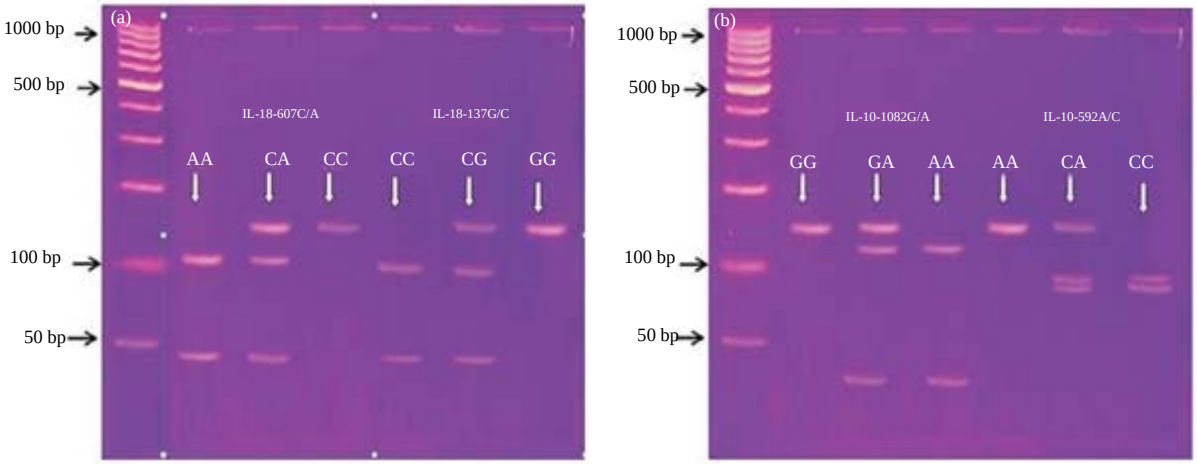

Fig. 1(a-b): Gel electrophoresis of PCR products and restriction fragments, (a) Genotypes of IL-18-607C/A in lanes 2, 3 and 4, IL-18-137G/C in lanes 5, 6 and 7 and (b) Genotypes of IL-10-1082G/A in lanes 2, 3 and 4, IL-10-592A/C in lanes 5, 6 and 7

Table 2: Demographic and biochemical laboratory parameters of the studied groups

Parameters	Group I (Carriers) n = 115	Group II (Cirrhotics) n = 109	Group III (Recovered) n = 103	Group IV (Controls) n = 110
Age (Mean±SD)	42.5±2.5	49.7±4.5	48.2±4.5	45.2±2.4
Gender				
Male	66 (57.4%)	62 (56.9%)	58 (56.3%)	62 (56.4%)
Female	49 (42.6%)	47 (43.1%)	45 (43.7%)	48 (43.6%)
AFP (ng mL^{-1})	4.5±0.2	5.5±1.2[a]	3.8±1.1	2.9±0.8
ALT (IU L^{-1})	31±8	41±21[b]	38±18	27±2
AST (IU L^{-1})	39.5±23	43.5±12	29.3±2[a]	23±4
GGT (IU L^{-1})	18±4	19±6	13±6	14±5.2
Albumin (g dL^{-1})	3.5±0.8	2.1±0.5[c]	3.9±0.8[a,b]	4±1.4
Total bilirubin (mg dL^{-1})	0.9±1.1	4.8±1.5[d]	0.8±0.4[a,b]	0.8±0.2
Direct bilirubin (mg dL^{-1})	0.1±0.02	0.6±0.8	0.1±0.2[a,b]	0.1±0.03

[a]Significant difference from carriers (t-value = 23.36, p-value<0.0001) and control group (t-value = 33.4052, p-value<0.0001) and [b,c,d]Significant difference versus control, carriers and recovered groups

Table 3: Genotype and allele distributions of IL-10 genes among the different studied groups

SNP	Group I (Carriers) n = 115	Group II (Cirrhotics) n = 109	Group III (Recovered) n = 103	Group IV (Controls) n = 110
IL-10 (1082A>G)				
AA	60 (52.2%)	56 (51.4%)	46 (44.7%)	53 (48.2%)
AG	47 (40.9%)	49 (44.9%)	39 (37.9%)	43 (39.1%)
GG	8 (6.9%)	4 (3.7%)[b]	18 (17.5%)[a]	14 (12.7%)
A allele	167 (72.6%)	161 (73.9%)	131 (63.6%)	149 (67.7%)
G allele	63 (27.4%)	57 (26.1%)	75 (36.4%)	71 (32.3%)
IL-10 (592C>A)				
AA	16 (13.9%)	7 (6.4%)[c]	20 (19.4%)	18 (16.4%)
CA	49 (42.6%)	49 (45.0%)	36 (35.0%)	49 (44.5%)
CC	50 (43.5%)	53 (48.6%)	47 (45.6%)	43 (39.1%)
A allele	81 (35.2%)	63 (28.9%)	76 (36.9%)	85 (38.8%)
C allele	149 (64.8%)	155 (71.1%)	130 (63.1%)	135 (61.4%)

[a]Significant difference versus HBV carriers χ^2 = 4.766, p-value = 0.029, OR: 0.6, CI 95%: 0.38-0.93 and cirrhosis groups χ^2 = 9.420, p-value = 0.002, OR = 2.13, CI 95%: 1.41-3.13, [b]Significant difference versus control group χ^2 = 4.814, p-value = 0.028, OR: 0.59, CI 95%: 0.32-0.91, [c]Significant difference versus control group χ^2 = 4.413, p-value = 0.035, OR: 0.85, CI 95%: 0.71-0.98 and recovered group χ^2 = 6.920, p-value = 0.008, OR: 1.57, CI 95%: 1.12-2.12

(607C>A) SNP analysis showed no significant difference between the studied groups. On the other hand, IL-18 -137CC genotype was less prevalent among cirrhotics (1.8%) compared to recovered group (9.7%). Also, the GG genotype was statistically more prevalent among HBV cirrhotics (74.3%) group compared to the recovered group (56.3%), HBV carriers group (60.9%) and control group (59.1%), 607-A allele was more prevalent among the cirrhotics followed by carriers

Table 4: Genotype and allele distributions of IL-18 genes among the different studied groups

SNP	Group I (Carriers) n = 115	Group II (Cirrhotics) n = 109	Group III (Recovered) n = 103	Group IV (Controls) n = 110
IL-18 (607C>A)				
AA	26 (22.6%)	17 (15.6%)	25 (24.3%)	22 (20.0%)
CA	55 (47.8%)	55 (50.5%)	42 (40.7%)[a]	47 (42.7%)
CC	34 (29.6%)	37 (33.9%)	36 (35.0%)[b]	41 (373.0%)
A allele	107 (46.5%)	89 (40.8%)	92 (44.7%)	91 (41.4%)
C allele	123 (53.5%)	129 (59.2%)	114 (55.3%)	129 (58.6%)
IL-18 (137G>C)				
CC	6 (5.2%)	2 (1.8%)[a]	10 (9.7%)	8 (7.3%)
CG	39 (33.9%)	26 (23.9%)	35 (34.0%)	37 (33.6%)
GG	70 (60.9%)	81 (74.3%)[b]	58 (56.3%)	65 (59.1%)
C allele	51 (22.2%)	30 (13.8%)	55 (26.7%)	53 (24.1%)
G allele	179 (77.8%)	188 (86.2%)	151 (73.3.%)	167 (75.9%)

[a]Significant difference versus recovered group χ^2 = 4.762, p-value = 0.029, OR: 0.6, CI 95%: 0.38-0.93, [b]Significant difference versus recovered group χ^2 = 4.011, p-value = 0.045, OR: 1.8, CI 95%: 1.13-2.21, HBV carriers χ^2 = 6.825, p-value = 0.009, OR: 1.59, CI 95%: 1.32-2.93 and control groups χ^2 = 5.043, p-value = 0.025, OR: 1.2, CI 95%: 1.42-2.93

(46.5 and 44.7%, respectively) however, the differences between the studied groups were non-significant statistically (Table 4).

DISCUSSION

The clinical outcome of Hepatitis B Virus (HBV) infection is diverse clinically ranging from asymptomatic carrier state to a progressive Chronic Hepatitis B (CHB) infection, which may be complicated by liver cirrhosis and HCC[27,28]. It is established that age and immune status are the classic risk factors of HBV persistence[29]. Recently, certain immuno-modulatory cytokines including IL-10 and IL-18 are reported to be risk factors which are involved in determination of natural history of persistent HBV infection[30]. Since HBV is a non-cytolytic virus, HBV-induced liver injury is attributed to the host immune response against the virally infected cells[31]. These cytokines play a fundamental role in the immune-pathogenesis of HBV so, they influence the outcome of HBV infection[32]. Therefore, effects of these cytokine polymorphisms on the disease outcome and response to vaccination and treatment have been studied[33].

The IL-10 is an immune-regulatory cytokine produced by Th2 cells, T regulatory lymphocytes and activated macrophages. It inhibits secretion of IFN-γ, IL-2 and TNF-α by Th1 cells[34]. Polymorphisms in IL-10 gene were suggested to have an effect on the transcription, translation and the IL-10 serum levels[35]. In the present study, the relation between polymorphisms of IL-10 promoter regions and HBV clearance and disease progression were investigated. The results showed significant differences in frequencies of IL-10 genotypes at positions -1082 and -592 among the studied groups. The IL-10-1082GG genotype was more prevalent in the recovered group compared to HBV carriers and cirrhotics. The prevalence of GG genotype among cirrhotics was significantly lower than controls. Also, a low prevalence of IL-1082GG was reported in cirrhotics and a decreased risk of cirrhosis and HCC in association with IL-10-592AA[30]. Moreover, it was found that a more prevalence of G/G genotype at position-1082 in recovered HBV individuals. Also, IL-10 -592AA genotype was significantly less prevalent among cirrhotics compared to the recovered and control groups[18]. These results are in agreement with the findings of many previous studies[36-39] that reported lower IL-10 levels with favorable HBV disease outcome in people with IL-10-592 A/A genotype. In contrary to results, a previous study reported more prevalence of IL-10-1082GG genotype and IL-10-592AA genotype in chronic active hepatitis B patients compared to asymptomatic HBV carriers[40]. Also, it was found that IL-10-592CC genotype carriers had a better capacity of spontaneous recovery from HBV versus individuals with IL-10-592AA[32]. On the other hand, some studies[35,41] showed no significant difference at positions-1082G/A and -592A/C of IL-10 gene promoter region among normal controls, recovered HBV individuals and chronic HBV patients. These conflicting results may be explained by racial differences, association with other genetic markers and the influences of other genes on HBV progression than just a direct link between IL-10 expression and HBV outcome[40].

Interleukin-18 (IL-18), a cytokine which is mainly produced by activated macrophages is able to induce IFN-γ and TNF-α and enhances cytotoxicity of Natural Killer (NK) cells. An association between serum levels of IL-18 and disease severity in patients with HBV was previously reported[32]. The SNPs in promoter of IL-18 gene at positions -607 and -137 were suggested to have a critical impact on IL-18 gene activity, which results in variable levels of IL-18 production[42]. In the present study, the association of IL-18 gene promoter

SNPs-137 C/G and -607 A/C with HBV infection was investigated. The results showed that IL-18 -137CC genotype was significantly less prevalent among cirrhotic patients compared to the recovered group. Also, GG genotype was statistically more prevalent among cirrhotics compared to the recovered patients, HBV carriers and control groups. On the other hand, no significant difference in position -607 was observed. These results are in agreement with a previous study that showed no significant difference in position -607 A/C SNP between patients with CHB infection and controls[20]. Also, this study showed a significant higher frequency of genotype IL-18-137 GG in cirrhotic patients compared to the controls. Also, a higher frequency of genotype -137 GG was observed in patients with chronic hepatitis B compared to healthy controls[43]. On the other hand, other studies[44,45] reported that the -607A/A genotype was significantly higher in the patients with chronic hepatitis B than in the controls. They suggested that -607A/A genotype likely results in inhibition of transcription and production of IL-18 from hepatic macrophage, leading to less inhibitory effects on HBV replication. This hypothesis was confirmed by Pavlovna *et al*.[46] who reported that -607A/A genotype was associated with lower level of IL-18 compared to C/A and C/C genotypes. The anti-viral effect of IL-18 is thought to be mediated by its ability to activate hepatic NK cells and T cells to produce IFN-γ[22]. The IL-18-137 SNPs may contribute to differences in IL-18 gene activity resulting in variable levels of IL-18 production. Furthermore, the carriage of allele C at position -137 in the promoter of IL-18 gene was suggested to play a protective role against development of chronic HBV infection. These results suggest that IL-18 may have a therapeutic value for patients with chronic hepatitis due to its potential role in control of HBV replication during self-limited infection[43]. Conflicting results on the role of these polymorphisms may be due to various ethnic groups studied[47]. Moreover, these can be related to epidemiological and geographical factors and study circumstances (such as the characteristics and numbers of patients and HBV genotype variations).

CONCLUSION

It speculated that host genetic factors play an important role in determining the outcome of HBV infection. Cytokine gene polymorphisms have a major contribution in predicting disease susceptibility and clinical outcome. However, due to the limited number of patients in the current study, the roles of IL-10 and IL-18 gene promoter polymorphisms in HBV infection outcome need further clinical studies within large population from different ethnic groups.

SIGNIFICANT STATEMENT

Hepatitis B virus is one of the most common causes of liver disease in Saudi Arabia, which is considered hyper-endemic region. IL-10 and IL-18 are important immune regulators that may have a role in HBV infection outcome. Gene polymorphisms of these cytokines may be associated with liver disease severity in patients with viral hepatitis. This study aimed to investigate the role of IL-10 and IL-18 polymorphisms in the natural course of HBV infection in Saudi population.

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