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Interleukin16 and Interleukin 28B Genes Polymorphism in HBV Infected Saudi patients.

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Abstract: *The course of hepatitis B virus (HBV) infection is variable depending on many factors. In this study, we investigated single nucleotide polymorphisms of interleukin-28B and interleukin-16 as possible host factors which may determine the occurrence of hepatocellular carcinoma (HCC) in Saudi patients. Chronic hepatitis B (CHB) patients (75), HCC patients (42), and healthy controls (70) were analyzed for polymorphisms of the IL16 and IL28B genes using PCR and restriction fragment length polymorphism (RFLP). Results showed that HCC and chronic HBV patients had higher prevalence of rs11556218TG genotype than controls. The rs11556218GG genotype was higher among HCC (14.4%) compared to chronic HBV (2.7%) patients. The IL-16 genotype rs4072111CT was higher among HCC (47.6%) and chronic HBV (46.7%) patients than controls (28.6%). The rs4072111TT genotype was higher among HCC patients compared to the other two groups. The T allele frequency was higher among HCC patients than controls. The CT and TT of the IL-28B rs12979860 genotype were significantly less frequent in chronic HBV and HCC patients. The IL-28B rs8099917 TG genotype was more frequent among HCC (19%) compared to chronic HBV (8%) patients. However, no significant difference was detected in the allele distribution.*

Keywords*: Hepatitis B virus, Interleukin 28B, Interleukin-16.*

I. Introduction:

Hepatitis B virus (HBV) infection is an important cause of morbidity and mortality worldwide [1]. Patients with chronic HBV infections are at an increased risk of developing cirrhosis and hepatocellular carcinoma (HCC) [2]. HBV is considered hyper-endemic in Saudi Arabia, where infections are acquired mainly through horizontal and less commonly by vertical transmission [3]. Data about prevalence of HBV infection in Saudi Arabia varies, WHO reports mentioned that it is about 8%. On the other hand, Al-Thaqafy et al., [4] reported a lower rate of about 4%. This prevalence diminishes greatly in lower age groups [5]. This fall in the prevalence was attributed to initiation of vaccination program in 1990 that aimed for vaccinating all Saudi children at school entry. Mandatory vaccination of healthcare workers and hemodialysis patients was also introduced around this time [3]. Despite significant decline in the prevalence of HBV infection in Saudi Arabia, it still causes significant morbidity and mortality and imposes a great burden on the country's healthcare system [6].

Chronic HBV infection is a result of the dynamic interactions between virus and host immune response and the natural course varies greatly among different individuals. Some patients showed more rapid progression of liver disease, while others remain at inactive carrier state with a relative benign prognosis. Several host and viral factors have been reported to be associated with the natural course of chronic HBV infection [7]. Genotypes, basal core promoter mutations and viral loads of HBV may influence the progression of HBV-related liver disease [8,9]. Similarly, age, sex, host immune status and metabolic factors of the host affect disease progression [10].

Currently, the genetic determinants of host immune responses to HBV infection remain unclear. Studies have shown that single nucleotide polymorphisms (SNPs) at or near the interleukin 28B gene (IL28B) region on chromosome 19, which encodes interferon lamda 3 (IFN-λ3), are associated with spontaneous hepatitis C virus (HCV) clearance and sustained virological response (SVR) in patients with chronic hepatitis C (CHC) treated with pegylated interferon (PEG-IFN) and ribavirin [11]. Furthermore, a recent study showed that patients with lower serum levels of interferon gamma-inducible protein 10 (IP-10) in combination with favorable IL28B genotypes had higher chance of spontaneous HCV clearance [12]. The influence of IL28B polymorphisms on host immune response to HBV infection is not fully understood. It is possible that genetic

variations at IL28B region determine host susceptibility to HBV infection and influence the progression of liver disease [13].

IL16 is one of the potent pro-inflammatory cytokines and has a wide array of biological functions, initially identified as lymphocyte chemo-attractant factor [14]. It activates T-cells, monocytes, macrophages and dendritic cells through binding to the CD4 molecule. In addition, IL16 can stimulate the production of different pro-inflammatory cytokines such as IL1b, IL6, IL15 and tumor necrosis factor (TNF) by monocytes [15]. Recently, genetic polymorphisms of IL16 have been reported to be associated with susceptibility to a range of cancers, including nasopharyngeal, colorectal, gastric and prostatic cancer [16]. To our knowledge, there is no study examined the association between single-nucleotide polymorphisms (SNPs) of the IL16 and IL28B genes and HBV-related HCC in Saudi Arabia. Therefore, the aim of this study was to investigate the relationship between IL16 and IL28B gene polymorphisms in patients with chronic HBV infection and HBV related HCC.

II. Material and methods

2.1 Subjects: The study included 187 Saudi individuals who were categorized into three groups; group I: 70 healthy controls, group II: 75 patients with chronic HBV infection and group III: 42 HBV infected patients complicated with HCC. Demographic, clinical and laboratory data were collected, through a structured data collection sheets. Chronic hepatitis B patients were diagnosed by being HBs Ag positive for at least six months and hepatitis B virus core antibody (Hbc Ab) positive. The infection was confirmed by detection of HBV-DNA by PCR. HBV-related HCC was diagnosed based on pathological findings and/or radiological results (computed tomography and magnetic resonance imaging) combined with elevated alpha-fetoprotein (AFP) level (>400 ng/ml).Patients with HCV or HIV co-infection and those having other chronic liver disease were excluded.

2.2. Collection of Blood Samples: after a written consent, 4 ml of venous blood were drawn from each individual after an overnight fasting. One ml of blood was collected in heparinized tube for DNA extraction and determination of IL28B and IL16 genotypes. The other 3 ml of blood were collected in plain tubes for separation of sera to be tested for liver functions, viral markers and AFP.

2.3 Biochemical measurements: Alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyle transferase (GGT), albumin and serum bilirubin (total and direct) were measured by routine enzymatic methods (Spinreact, Girona Spain). Serum AFP concentration was measured by ELISA according to the Manufacturer's recommendation using Elecsys 2010 (Roche Diagnostics, Madison USA). HBV markers were measured by commercially available ELISA kits (Bio-Rad, CA, USA).

2.4. DNA extraction and genotyping: Genomic DNA of the studied individuals were extracted according to the Manufacturer's instructions from 200 µl of whole blood using DNA Blood Mini Kit (Qiagen, Hilden, Germany) [14]. The IL28B (rs12979860 and rs8099917) and IL16 (rs11556218 and rs4072111) SNPs were studied by the PCR-RFLP method as formerly described (18 and 14 respectively). Primer set, restriction endonuclease and RFLP fragment products are shown in table 1. PCR reactions were set up to amplify the IL28B gene DNA using Amplitaq Gold PCR master Mix (Applied Biosystems, Foster City, USA) with a 20 µl reaction. The temperature profile, which was used for the PCR amplification of both SNPs consisted of a 94°C for 5 minutes, followed by 35 cycles of 94°C for 20 seconds, 66°C for 20 seconds and 72°C for 20 seconds, followed by a 72°C for 5 minutes. PCR reactions were set up to amplify the IL16 gene DNA using Amplitaq Gold PCR master Mix with a 20 µl reaction tube type. The reaction conditions of both SNPs consisted of a 94°C for 5 minutes, followed by 35 cycles of 94°C for 20 seconds followed by annealing phase (61°C in case of rs11556218 and 67°C in case of rs4072111) for 20 seconds and 72°C for 20 seconds, followed by a 72°C for 5 minutes. For the RFLP assay, the PCR amplicon containing the SNP was digested with 10 units of each of the restriction endonucleases (table 1) for at least one hour. The digested PCR products were visualized after separation on 3% agarose gel containing 0.5 µg of ethidium bromide per ml [13,17].

2.5. Statistical analysis: The results for continuous variables are expressed as Means ± SD. The means of the groups were compared using one-way analysis of variance (AVOVA). The statistical significances of differences in frequencies of variants were tested using chi-square (x^2) test. A difference was considered significant at $P<0.05$. All data were evaluated using SPSS software version 16.0.

III. Results

3.1 Demographic and laboratory biochemical parameters: The age and gender of the studied 3 groups are shown in table (2). The majority of the studied subjects were males (65.4%). There was no significant difference in the age and gender between the 3 groups. The laboratory biochemical parameters of them are shown in table (2). HBV patients with HCC had statistically significant differences in their serum bilirubin (total and direct), AST, ALT, ALB and GGT, ($P<0.001$). Serum AFP level was higher in HCC group (881 ± 158 ng/ml) than chronic HBV (881 ± 158 ng/ml).

3.2 IL16 genotypes and alleles distributions: The IL16 genotype rs11556218 polymorphism was significantly different among the studied groups. HCC patients and chronic HBV patients had higher prevalence of rs11556218TG genotype (33.2% and 33.3% respectively) than controls (15.7%). On the other hand, rs11556218GG genotype was more commonly found among HCC patients (14.4%) compared to chronic HBV (2.7%). The IL16 genotype rs4072111CT polymorphism had higher prevalence among HCC patients and chronic HBV patients (47.6% and 46.7% respectively) than controls (28.6%). While, rs4072111TT genotype had a higher prevalence among HCC patients (14.3%) compared to the other two groups (2.7% and 2.9%). Regarding allele distributions of rs4072111, HCC subjects had significantly higher T allele frequency than controls (38.1% and 17.1% respectively) (table 3).

3.3 IL28B genotypes and alleles distributions: IL28B rs12979860 genotype was characterized in all subjects. Overall, CT and TT genotypes were significantly less frequent in patients with chronic HBV and HCC patients (CT, 18.7% and 16.7% and TT, 2.7% and 7.1% respectively) compared to controls (CT, 35.7% and TT, 10.0%) while on the opposite, IL28B rs8099917 TG genotypes were more frequently found among HBV with HCC (19%) compared to Chronic HBV (8%). Regarding allele distributions, no significant (>0.05) difference was detected between the 3 groups (table 4).

Table (1) The primer sequences, restriction endonuclease and RFLP fragments of IL28B and IL16 genes

SNP	The primer set (Macrogen, Korea)	Base change	RE enzyme (Fermentas, Lithuania)	PCR-RFLP fragments
IL28B rs12979860	Sense, 5'-GCGGAAGGAGCAGTTGCGCT-3'	C>T	BstUI	CC: 196 & 45 bp
	Antisense, 5'-GGGGCTTTGCTGGGGGAGTG-3'.			CT: 241, 196& 45 bp
				TT: 241 bp
IL28B rs8099917	Sense, 5'-CCCACTTCTGGAACAAATCGTCCC-3'	T> G	BsrDI	TT: 552 bp
	Antisense, 5'-TCTCCTCCCCAAGTCAGGCAACC-3'.			GT: 552, 322 & 230 bp
				GG: 322 & 230 bp
IL16 rs11556218	Sense5'-GCTCAGGTTCACAGAGTGTTTCCATA-3'	T>G	Nde I	TT:171bp
	Antisense, 5'-TGTGACAATCACAGCTTGCCTG-3'.			GG:147 & 24bp
				TG:171, 147 & 24bp
IL16 rs4072111	Sense, 5'-CACTGTGATCCCGGTCCAGTC-3'	C> T	BsmA I	CC:164bp
	Antisense, 5'-TTCAGGTACAAACCCAGCCAGC-3'.			TT:140 &24bp
				CT:164, 140 & 24 bp

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

	Control group (n = 70)	**Chronic HBV (n = 75)**	**HBV with HCC (n = 42)**
Age (Mean± SD)	51.2 ± 5.4	49.7 ± 2.5	57.2 ± 6.5
Gender - Male - Female	43 (61.4%) 27 (38.6%)	44(58.7%) 20 (41.3%)	26 (61.9%) 16 (38.1%)
AFP (ng/ml)	2.9 ± 1.3	7.5 ± 5.2	881 ± 158[a,b]
ALT (IU/L)	26 ± 2	121 ± 38 [a]	72.2 ± 29.2[a]
AST(IU/L)	22 ± 5	45.5 ± 27	83.3 ± 23.3[a]
GGT (IU/L)	14 ± 5.2	29 ± 5.3 [a]	180 ± 51.2[a]
Albumin (g/dl)	4±1.1	3.5±0.4	1.9±0.8[a,b]
T-Bil. (mg/dl)	0.8 ± 0.2	2.8 ± 1.5	16 ± 8.4[a,b]
D-Bil. (mg/dl)	0.1 ± 0.03	0.6 ± 0.2	5.7 ± 1.3[a,b]

[a] Significant difference from control group

[b] Significant difference from chronic HBV group.

Table (3): Genotype frequencies of IL16 genes among the different studied groups

SNP	**Control group (n = 70)**	**Chronic HBV (n = 75)**	**HBV with HCC (n = 42)**
rs11556218			
TT	48(68.6%)	48 (64.0%)	22(52.4%)
TG	11 (15.7%)	25 (33.3%) [1]	14(33.2%) [2]
GG	11 (15.7%)	2 (2.7%)	6(14.4%) [3]
T allele	107(76.4%)	121(80.6%)	58(69.0%)
G allele	33(23.6%)	29(19.4%)	22(31.0%)
rs4072111			
CC	48(68.6%)	38 (50.7%)	16(38.1%)
CT	20 (28.6%)	35 (46.7%) [4]	20(47.6%) [5]
TT	2 (2.9%)	2 (2.7%)	6(14.3%) [6]
C allele	116(82.9%)	111(74.0%)	52(61.9%)
T allele	24(17.1%)	39(26.0%)	32(38.1%) [7]

[1] Significant difference from control group (P=0.0141), [2] Significant difference from control group (P= 0.030)
[3] Significant difference from Chronic HBV group (P = 0.016), [4] Significant difference from control group (P = 0.025),
[5] Significant difference from control group (p=0.042), [6] Significant difference from Chronic HBV group and control (P = 0.023, 0.018) [7] Significant difference from control group (P = 0.0004).

Table (4): Genotype frequencies of IL28B genes among the different studied groups

SNP	**Control group (n = 70)**	**Chronic HBV (n = 75)**	**HBV with HCC (n = 42)**
rs12979860			
CC	38 (54.3%)	59 (78.7%)	32 (71.2%)
CT	25 (35.7%)	14 (18.7%) [1]	7 (16.7%) [2]
TT	7 (10.0%)	2 (2.7%)	3 (7.1%)
C allele	101(72.1%)	132(88%)	71(84.5%)
T allele	39(27.9%)	18(12%)	13(15.5%)
rs8099917			
TT	55 (78.6%)	69 (92.0%)	34(81.0%)
TG	15 (21.4%)	6 (8.0%) [3]	8(19.0%)
GG	0 (0%)	0 (0%)	0(0%)
T allele	125(89.3%)	144(96%)	76(90.5%)
G allele	15(10.7%)	6(4%)	8(9.5%)

[1] Significant difference from control group (P = 0.021) [2] Significant difference from control group (P = 0.030) [3] Significant difference between chronic HBV versus control group (P=0.022)

IV. Discussion

HBV infection is a serious and common infectious disease with high morbidity and mortality in many areas of the world. It has been associated with chronic hepatitis, cirrhosis, and HCC [18]. The genetic background of the host and host-pathogen interactions may affect the outcome of infection [19]. Single nucleotide polymorphism (SNP) may change the structure and biological function of genes encoding cytokines or regulatory molecules that may affect immunopathogenesis of HBV infection [20]. Detection of the effect of these polymorphisms may help to develop new strategies for prevention and treatment of HBV infection [19].

In this study, we investigated the effect IL16 gene polymorphisms on the occurrence of HCC in Saudi population. Our results revealed that IL16 rs11556218 TG and GG polymorphisms were significantly higher among patients with HCC. IL16 rs4072111CT polymorphism was significantly higher among chronic HBV and HCC patients compared to the control group. Moreover, IL16 rs4072111TT polymorphism was significantly higher among HCC patients when compared with either chronic HBV or the control group. These results are consistent with that reported by Shan et al., [13] who indicated that IL16 SNPs were associated with chronic HBV infection and HCC. Previous studies suggested that IL16 rs4072111 and rs11556218 polymorphisms were related to the course of hepatitis B infection and IL16 gene polymorphisms were suggested as considerable host genetic factors which affect the susceptibility to chronic HBV infection [21]. Recently, rs11556218` and rs4072111 genetic polymorphisms of IL16 gene have been reported to be associated with susceptibility to a range of cancers [13]. The rs11556218 TG genotype was reported to be associated with a significantly higher risk of nasopharyngeal carcinoma (NPC) compared to the TT genotype and patients carrying the G allele were more labile to develop NPC [15]. Moreover, TG and GG genotypes were associated with a significantly higher risk of gastric cancer compared to patients with the TT genotype. The role of IL16 in the development of HCC may be attributed to its role in inflammation and tumor growth and progression. IL16 can augment inflammation in Th1-mediated hypersensitivity and the link between inflammation and cancer is well established. The

inflammatory microenvironment can increase mutation rates and enhance the proliferation of mutated cells [22]. Although, IL16 may help in killing virus-infected cells and minimizing cellular damage during HBV infection, the host immune response may be fairly weak and fail to completely clear the infection. This results in chronic stimulation of the antigen-specific immune response in persistently infected patients and leads to continued expression of cytokines and recruitment of activated lymclear cells to the liver leading to liver damage, hepatic fibrosis and ultimately HCC [13,23].

In the present study, IL28B polymorphisms (rs12979860 and rs8099917) were investigated to determine the potential influence of the IL28B polymorphism on HBV infection outcome. Our results revealed that IL28B rs12979860 CT polymorphisms were significantly lower among both chronic HBV and HCC patients and IL28B rs8099917 TG polymorphism was significantly lower among chronic HBV patients when compared with that of the controls. These results are consistent with a recent study [24] that reported an association between IL28B polymorphisms (rs12979860, rs12980275, and rs8099917) and the outcome of HBV infection. IL-28B was found to inhibit HBV replication in hepatocyte cell lines. It has been suggested as a potential treatment for viral hepatitis [25]. IL28B triggers a cascade through the JAK-STAT pathway that up-regulates the IFN-stimulated genes (ISGs). The effects of IL28B are similar to those of IFN-α and β; however, it binds to a distinct receptor that triggers a different set of ISGs [14]. Recently, IL28B was identified as a key factor of the immune response to HCV that strongly determines the outcome of HCV infection [26,27]. It is reported that polymorphisms near IL-28B gene are strongly associated with sustained viral response and spontaneous viral clearance in patients with chronic HBV infection. Genetic variation of IL-28B may prevent progression of HBV infection by reducing viral load and liver inflammation [28].

Our results showed higher prevalence of the major alleles rs12979860 CC (78.7% and 71.2%), and rs8099917 TT (92.0% and 81.0%) in subjects with chronic HBV and HCC patients respectively as compared to the controls. However these alleles were demonstrated as favorable predictors in terms of spontaneous clearance of HCV infection. These results may indicate that IL28B polymorphisms have a distinct effect on the immune response to HCV in spite of the same signal from both type I IFNs and IL28B through the JAK-STAT pathway.

ISGs were found to play a major role in non-cytolytic inhibition of HBV replication in a transgenic mouse model and IFN-l2 (IL28A) was demonstrated to inhibit HBV replication through up-regulated ISGs in HCC cell lines. Moreover, there are differences between HBV and HCV infection regarding their replication strategies, mechanism of viral persistence and host response [25,29]. Guo et al., [30] reported that patients with the rs8099917 TG and GG genotypes had an increased risk of null virological response in HCV/HBV dually-infected patients. However, IL28B was reported to have no role in the development of chronic HBV infection among HIV-infected patients. Moreover, on comparing patients with persistent infection with individuals recovered from HBV infection, IL28B polymorphism was found to have no role in clearance of HBV and the outcome of HBV infection [31,32]. In another study, which investigated the effect of rs12979860 polymorphism on the INF-α responsive HBV, the rs12979860 CC genotype was not associated with spontaneous HBV recovery [33]. Furthermore, no significant association was found between IL28B rs12979860 genotypes and the risk of developing HCC in Turkish patients [34].

V. conclusion

In conclusion, SNP of IL16 was significantly higher among chronic HBV and HCC patients. The SNP of IL28B that was proposed to have an association with HCV recovery does not have the same effect on HBV recovery and the effects of this SNP cannot be generalized to chronic viral infections. Additional studies are needed to understand the mechanisms underlying the effects of this SNP in HBV infection and to clarify the possible role of IL28B polymorphism on the risk of developing HCC in larger patient groups.

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