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Single nucleotide polymorphisms in CXCR1 gene and its association with hepatitis B infected patients in Saudi Arabia

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ABSTRACT

Background/Aim. This study aims to investigate whether the SNPs of CXCR1 gene, could predict the likelihood of viral persistence and/or disease progression. **Material and methods.** We investigated the association of two different SNPs (rs2234671, and rs142978743) in 598 normal healthy controls and 662 HBV patients from a Saudi ethnic population. The HBV patients were categorized into inactive carriers (n = 428), active carriers (n = 162), cirrhosis (n = 54) and Cirrhosis-HCC (n = 18) sub-groups. Genetic variants in CXCR1 were determined by polymerase chain reaction (PCR)-based DNA direct sequencing. **Results.** The frequency of the risk allele 'C' for the SNP, rs2234671 was found to be insignificant when the patient group was compared to the uninfected control group, however, a significant distribution of the allele 'C' of rs2234671 was observed among active HBV carriers + cirrhosis + cirrhosis - HCC *vs.* inactive HBV carriers with an OR = 1.631 (95% C.I. 1.016-2.616) and p = 0.032. However, no significant association was observed for rs142978743 when the distribution of risk allele was analyzed among the different patient groups (i.e. inactive carriers, active carriers, cirrhosis and HCC). Furthermore, the most common haplotype, Haplo-1 (AG), was found to have an insignificant frequency distribution between HBV cases and controls, while the same haplotype was found to be significantly distributed when active carriers + cirrhosis + cirrhosis - HCC patients were compared to inactive HBV carriers with a frequency of 0.938 and p = 0.0315. Haplo-2 (AC) was also found to be significantly associated with a frequency of 0.058 and p = 0.0163. **Conclusion.** The CXCR1 polymorphism, rs2234671 was found to be associated with chronic HBV infection and may play a role in disease activity.

Key words. Hepatitis B Virus. CXCR1. Single nucleotide polymorphisms. Variations. Genetics. Haplotype. Saudi Arabia.

INTRODUCTION

Hepatitis B virus (HBV)-related diseases are among the major health burdens, especially in Asia, Africa, southern Europe and Latin America.[1,2] About 2 billion people are infected with HBV worldwide, and 400 million among them are suffering from chronic HBV infection.[1,2] HBV infection is hepatotropic that leads to hepatitis with potentially fatal complications including hepatocellular carcinoma (HCC).[3] The clinical course of HBV infection varies from spontaneous recovery after acute hepatitis to a chronic persistent infection that may progress to cirrhosis or hepatocellular carcinoma. The mechanisms underlying resolution of acute HBV infection or its progression to chronicity are not clearly understood but are suggested to depend on host immune response and genetic factors.[4-7]

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The CXCR1 receptor [chemokine (C-X-C motif) receptor 1] is a member of the superfamily of G-protein-coupled receptors that consists of seven transmembrane domains, three intracellular loops, and three extracellular loops.[8] CXCR1 is located at chromosome 2q34-35 and binds to a chemokine, known as interleukin-8 (IL-8).[9] IL-8 activates and attracts neutrophils, T cells, and basophils and is suggested to be a key mediator in inflammatory disorders.[10] CXCR1 and CXCR2 are important in mediating antimicrobial host defenses. CXCR1 functions in directing leukocyte recruitment and activation that leads to clearance of several infective agents.[11] In neutrophils, receptor activation also stimulates the release of granule enzymes as well as generation of superoxide in respiratory burst.[12] CXCR1 is expressed by several types of normal cells such as neutrophils, endothelial cells and various tumor cells.[11,13] IL-8 is known to be involved in tumor cell growth and metastasis in colon cancer.[14] Its receptors CXCR1 and CXCR2 have been demonstrated to be involved in tumor progression and angiogenesis.[10]

The variants of CXCR-1, CXCR-2 and IL-8, genes have been extensively investigated in different diseases such as HBV,[15] rapid disease progression of HIV-1+,[16] lung diseases, such as chronic obstructive pulmonary disease and asthma,[17] bronchiectasis,[18] systemic sclerosis[19] and lung cancer.[20] Some of these studies have reported positive associations between the diseases and single nucleotide polymorphisms (SNPs) in the CXCR1 gene.[16,17] Indeed, a significant association was demonstrated between the 860G > C (S276T) SNP in the CXCR1 gene with decreased lung cancer risk.[20] Also, it has also been shown that there is an association between CXCR1 polymorphism and survival of patients with advanced colorectal cancer.[21]

Little is known about the role of CXCR1 and its polymorphic forms in disease related to HBV and clinical outcome of patients with different stages of HBV-related diseases, including hepatocellular carcinoma (HCC). Therefore, the present study investigates the presence of human CXCR1 SNPs in HBV-infected Saudi Arabian patients and their possible role on the clinical outcome.

MATERIAL AND METHODS

Subject

Blood samples from 662 HBV patients attending the three major hospitals in Riyadh city, including King Faisal Specialist Hospital & Research Center, Riyadh Military Hospital, and King Khalid University Hospital. The study was approved by the institutional review board of the participating hospitals, in accordance with the Helsinki Declaration of 1975. Informed consents were obtained from all the patients and normal healthy individuals. The patient group was further sub-divided into case I-inactive HBV carriers, case II-active HBV carriers, case III-patients having cirrhosis and case IV-cirrhotic patients diagnosed with HCC.[22] Blood samples were also collected from 598 normal healthy subjects who volunteered to participate in this study. Control subjects were characterized by the absence of any known serological marker of HBV such as HBsAg negative, anti-HBs negative, and anti-HBc negative. All patients were infected chronically with HBV as they were all seropositive for HBsAg for at least six months. Patients with liver complications were identified by ultrasonography. Liver involvement was established based on the appearance of the liver and liver parenchymal texture. Anthropometric and clinical data was obtained from all the participating subjects.

PCR diagnosis and genotyping of HBV

Blood samples were collected from the subjects and DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, USA) following the recommended procedures. HBV was detected and genotyped using the INNO-LiPA HBV genotyping kit (Innogenetics, Gent, Belgium) according to the manufacturer's instructions.

Amplification of target regions in the CXCR1 gene

In this study we examined two SNPs for the CXCR1 gene, namely rs2234671 and rs142978743.

Figure 1. PCR of CXCR1 gene containing SNPs of interest.

A 276 bp region that encompasses these polymorphic sites was amplified by PCR using the primers forward 5'-CCATGAGGGTCATCTTTGCT-3' and Reverse 5'-GCCAAGAACTCCTTGCTGAC-3' (Figure 1).

DNA sequencing

Following PCR amplification, products were resolved on agarose gels and purified using the illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, UK) and subjected to sequencing for the detection of SNPs. DNA sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit, Applera, according to the manufacturer's instructions. Bidirectional sequencing was performed for all the samples to ensure the accuracy of the data. Sequencing analysis was performed using DNA Sequencing Analysis Software v5.2.

Statistical analysis

The SNPs were tested for Hardy-Weinberg equilibrium and their genotypic and allelic disease association analysis was performed using the DeFinetti program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). A cut-off p-value of 0.01 was set for HWE and SNPs with MAF < 1% were excluded from the study. Statistical analysis of genotype distribution and allele frequencies was performed by chi-square analysis and association was expressed as Odds Ratio (OR) with relative risk estimates of 95% confidence interval (CI). A probability value $P < 0.05$ was considered to be statistically significant. All analyses were recalculated using Statistical Product and Service Solutions software (Version 12.0; SPSS Inc, Chicago, Ill). Haplotype analysis and LD plots were generated using Haploview 4.2.[23]

RESULTS

Two SNPs were analyzed for the CXCR1 gene namely: rs2234671, which encodes a mis-sense mutation resulting in an amino acid change from Serine to Threonine at position 276 of the protein, and rs142978743. The frequency of the risk allele 'C' for the SNP, rs2234671 was found not to be significant when the patient group was compared to the uninfected control group with an OR of 0.821 (95% C.I. 0.602-1.120), χ^2-value of 1.55 and $p = 0.2135$ (Table 1). Also, under the dominant model, an insignificant association was observed with an OR of 0.829 (95% C.I. 0.595-1.154), χ^2-value of 1.24 and $p = 0.2655$. On the other hand, the allele showed a significant association when the active HBV carriers + cirrhosis + cirrhosis-HCC patients (case II + III + IV)

Table 1. Observed genotypic frequencies for the CXCR1 single nucleotide polymorphisms (SNPs), rs2234671 and rs142978743 between control and patient groups.

SNPs	Genotype/ allele distribution	Controls n = 598	Patients n = 662	OR (95% C.I.)	χ^2	P value
rs2234671	• Genotype			0.821 (0.602-1.120)	1.55	0.2135
	CC	6 (1.0%)	4 (0.6%)			
	GC	77 (12.9%)	74 (11.2%)			
	GG	515 (86.1%)	584 (88.2%)			
	• Allele					
	C*	89 (7.4%)	82 (6.2%)			
	G	1107 (92.6%)	1242 (94.0%)			
	CC+GC *vs.* GG			0.829 (0.595-1.154)	1.24	0.2655
rs142978743	• Genotype			9.975 (0.551-180.577)	4.53	0.0680
	TT	0 (0.0%)	0 (0.0%)			
	AT	0 (0.0%)	5 (0.8%)			
	AA	598 (100.0%)	657 (99.2%)			
	• Allele					
	T*	0 (0.0%)	5 (0.4%)			
	A	1196 (100.0%)	1319 (99.6%)			
	TT+AT *vs.* AA			10.013 (0.553-181.463)	4.53	0.0332

*Risk alleles marked in bold letters.

were compared to Inactive carriers HBV (case I), with an OR of 1.631 (95% C.I. 1.016-2.616), χ^2-value of 4.623 and a p-value of 0.032 (Table 2). However, the allele failed to show any significant association under the dominant model with an OR of 1.575 (95% C.I. 0.950-2.607), χ^2-value of 3.510 and a p-value of

Table 2. Observed genotypic frequencies for the CXCR1 single nucleotide polymorphisms (SNPs), rs2234671 and rs142978743 between Inactive HBV and active HBV + cirrhosis + cirrhosis - HCC patient groups.

SNPs	Genotype/ allele distribution	Inactive HBV patients (case I), n = 428	Active HBV + cirrhosis + cirrhosis - HCC patients (case II + III + IV), n = 234	OR (95% C.I.)	χ^2	*P* value
rs2234671	• Genotype			1.631 (1.016-2.616)	4.62	0.032
	CC	1 (0.2%)	3 (1.3%)			
	GC	42 (9.8%)	32 (13.7%)			
	GG	385 (90.0%)	199 (85.0%)			
	• Allele					
	C*	44 (5.1%)	38 (8.1%)			
	G	812 (94.9%)	430 (91.9%)			
	CC + GC *vs.* GG			1.575 (0.950-2.607)	3.51	0.061
rs142978743	• Genotype			0.456 (0.019-4.307)	0.52	0.4720
	TT	0 (0.0%)	0 (0.0%)			
	AT	4 (0.9%)	1 (0.4%)			
	AA	424 (99.1%)	233 (99.6%)			
	• Allele					
	T*	4 (0.5%)	1 (0.2%)			
	A	852 (99.5%)	467 (99.8%)			
	TT + AT *vs.* AA			0.455 (0.019-4.321)	0.52	0.4710

*Risk alleles marked in bold letters.

Table 3: Observed genotypic frequencies for the CXCR1 single nucleotide polymorphisms (SNPs), rs2234671 and rs142978743 between Active HBV carriers and Cirrhosis+Cirrhosis-HCC patient groups.

SNPs	Genotype/ allele distribution	Active HBV patients (case II), n = 162	Cirrhosis + cirrhosis -HCC patients (case III + IV), n = 72	OR (95% C.I.)	χ^2	*P* value
rs2234671	• Genotype			0.910 (0.410-1.982)	0.06	0.8
	CC	2 (1.2%)	1 (1.4%)			
	GC	23 (14.2%)	9 (12.5%)			
	GG	137 (84.6%)	62 (86.1%)			
	• Allele					
	C*	27 (8.3%)	11 (7.6%)			
	G	297 (91.7%)	133 (92.4%)			
	CC+GC *vs.* GG			0.884 (0.370-2.072)	0.09	0.76
rs142978743	• Genotype			-	0.45	0.505
	TT	0 (0.0%)	0 (0.0%)			
	AT	1 (0.6%)	0 (0.0%)			
	AA	161 (99.4%)	72 (100%)			
	• Allele					
	T*	1 (0.3%)	0 (0.0%)			
	A	323 (99.7%)	144 (100%)			
	TT+AT *vs.* AA	-	-	-	0.45	0.504

*Risk alleles marked in bold letters.

0.061. When this SNP was studied among patients suffering from cirrhosis + cirrhosis-HCC patients (case III + IV) in comparison to active carriers (case II), an OR of 0.910 (95% C.I. 0.410-1.982), χ^2-value of 0.06 and a p-value of 0.8000 was observed, suggesting an insignificant association of the risk allele with progression of the disease (Table 3). Under the dominant model, an OR of 0.884 (95% C.I. 0.370-2.072), χ^2-value of 0.093 and a p-value of 0.7600 was observed, which was also not significant enough to prove any association.

The risk allele 'T' for SNP, rs142978743 showed an insignificant association when the patient group was compared to the uninfected control group with an OR of 9.975 (95% C.I. 0.551-180.577), χ^2-value of 4.53 and a p-value of 0.0680 (Table 1). On the other hand, a significant association was observed under the dominant model with an OR of 10.013 (95% C.I. 0.553-181.463), χ^2-value of 4.53 and a p-value of 0.0332. However, the allele failed to show any significant association when case II + III + IV were compared to case I, with an OR of 0.456 (95% C.I. 0.019-4.307), χ^2-value of 0.52 and a p-value of 0.4720 (Table 2). Similarly, no significant association was observed under the dominant model with an OR of 0.455 (95% C.I. 0.019-4.321), χ^2-value of 0.52 and a p-value of 0.4710. Additionally, when this SNP was studied among cirrhosis + cirrhosis - HCC patients (case III + IV) in comparison to active carriers (case II), an insignificant association was observed with a χ^2-value of 0.445 and a p-value of 0.5050 (Table 3). Similarly, no significant association was observed under the dominant model with a χ^2-value of 0.446 and a p-value of 0.5040.

The heterozygosity and minor allele frequencies of CXCR1 SNPs are shown in table 4. Between patient and uninfected control group, genotypic data from 1,260 individuals was used to determine the LD pattern between rs2234671 and rs142978743. Two possible haplotype combinations were defined for these two SNPs, and their frequency of occurrence is represented in table 5.

In case of the comparison between case II + III + IV and case I, genotypic data from 662 individuals was used to determine the LD pattern between rs2234671 and rs142978743. Two possible haplotype combinations were defined for these two SNPs, and their frequency of occurrence is represented in table 6. Both of the blocks were found to be significant. With allele 'A' for rs142978743 and 'G' for rs2234671, the block was found to be significant with a frequency of 0.938, $\chi^2 = 4.623$ and a p-value of 0.0315. Also, with allele 'A' for rs142978743 and 'C' for rs2234671, the block was found to be significant with a frequency of 0.058, $\chi^2 = 5.774$ and a p-value of 0.0163.

Table 4. Heterozygosity and minor allele frequency of control subjects *vs.* patient.

Gene	Chr. No.	SNPs	Position	ObsHET	PredHET	HWpval	MAF	Alleles
CXCR1	2	rs142978743	219029088	0.004	0.004	1	0.002	A:T
		rs2234671	219029108	0.12	0.127	0.1111	0.068	G:C

Chr. No.: chromosome number. SNPs: single nucleotide polymorphisms. Position: chromosomal position. ObsHET: observed heterozygosity. PredHET: predicted heterozygosity. HWpval: hardy-weinberg P-value. MAF: minor allele frequency.

Table 5. Haplotype frequencies of the CXCR1 between control and patient groups.

Gene	Haplotype	Block rs142978743	rs2234671	Freq.	Case, control ratio counts	Case, control frequencies	χ^2	*P* value
CXCR1	Haplo 1	A	G	0.932	1242.0:82.0, 1107.0:89.0	0.938, 0.926	1.548	0.2135
	Haplo 2	A	C	0.066	77.0:1247.0, 89.0:1107.0	0.058, 0.074	2.699	0.1004

Table 6. Haplotype frequencies of the CXCR1 between inactive HBV and active HBV carriers + cirrhosis + cirrhosis - HCC patient groups.

Gene	Haplotype	Block rs142978743	rs2234671	Freq.	Case, control ratio counts	Case, control frequencies	χ^2	*P* value
CXCR1	Haplo 1	A	G	0.938	430.0:38.0, 812.0:44.0	0.919, 0.949	4.623	0.0315
	Haplo 2	A	C	0.058	37.0:431.0, 40.0:816.0	0.079, 0.047	5.774	0.0163

Table 7. Haplotype frequencies of the CXCR1 between active HBV carriers and cirrhosis + cirrhosis - HCC patient groups.

Gene	Haplotype	Block rs142978743	rs2234671	Freq.	Case, control ratio counts	Case, control frequencies	χ^2	*P* Value
CXCR1	Haplo 1	A	G	0.919	133.0:11.0, 297.0:27.0	0.924, 0.917	0.064	0.7996
	Haplo 2	A	C	0.079	11.0:133.0, 26.0:298.0	0.076, 0.080	0.02	0.8865

On comparing case III + IV against case II, genotypic data from 234 individuals was used to determine the LD pattern between rs2234671 and rs142978743. Two possible haplotype combinations were defined for these two SNPs, and their frequency of occurrence is represented in table 7. However, none of the two blocks were found to be significant.

DISCUSSION

To our knowledge, this is the first study of chemokine receptor polymorphisms and susceptibility to HBV infection and the clinical course of the disease in a Saudi ethnic population. In this study we examined two CXCR1 gene polymorphisms namely rs2234671 and rs142978743, and analyzed the association of these polymorphisms with different HBV patients' categories. According to NCBI HapMap database, the SNP rs2234671 has been studied and documented with frequencies for European (C = 0.06 and G = 0.94), Chinese Han (C = 0.12 and G = 0.88), Japanese (C = 0.1 and G = 0.9) and African Yoruba (C = 0.32 and G = 0.68) populations. The allele frequencies observed among the Europeans and the Asians were quite comparable to that observed among the Saudi Arabian population (C = 0.07 and G = 0.93).

Chemokines and their receptors have attracted attention in viral pathologies during recent years. It has been proved that chemokines participate in many pathological conditions like inflammation and are likely to be key regulators of immune responses.[24] However, there are very few reports about association between CXCR1 related SNPs and the outcome of HBV infection. A study conducted by Cheong, *et al.*, 2007,[15] suggested no association of CXCR1 rs2234671 polymorphism with the outcome of hepatitis B virus infection, a conclusion at variance with the present study which suggested a significant association between this SNP and disease progression. Such inconsistency in the results between the present study and those reported by Cheong, *et al.*[15] indicates that the effect of variation at this SNP is population dependent. Also, other factors that could account for the difference between the two studies might be related to the virus. Such factors include virus genotype/subgenotype, viral load and mutation in the viral genome. Furthermore, other host genetic variations could vary between both populations and could influence the outcome of the disease associated with HBV infection. Examples of such genetic variations, that were recently reported, include Ephrin receptor A3,[25] Estrogen receptor alpha,[26] TANK,[27] RANTES[28] and Toll-Like Receptor 3.[29]

The present study investigated the role of SNP rs2234671 in gene CXCR1 to determine the association of HBV infection and its progression to cirrhosis and HCC in Saudi Arabian patients. The risk allele 'C' for rs2234671 was insignificantly associated with the patient group when compared to uninfected control group. But quite interestingly, a significant association of the risk allele with viral persistence was observed (i.e. from inactive carriers to active carriers). This finding is relevant to our understanding of the disease whereby the persistence of HBV in an inactive form (defined as HBsAg-positive, low viremia and normal liver enzymes) is vastly recognized to be a benign state, marked by a general lack of significant fibrosis, development of cirrhosis or even HCC. Patients with the active disease (identified by high viremia, elevated liver enzymes and active histological disease) are recognized to be at greater risk for disease progression including development of cirrhosis and its sequelae. Hence, our findings suggest that the risk allele 'C' for rs2234671 could contribute to the active state of chronic HBV infection, although other risk factors (genetic or otherwise) may contribute to the eventual development of cirrhosis and HCC.

In the case of rs142978743, the risk allele 'T' showed no significant association when the patient group compared to the uninfected control group, but a highly significant dominant effect in relation to HBV infection. The comparison of risk allele 'T' between case II + III + IV *vs.* case I patient group, as well as in case III + IV *vs.* case II patient group, did not show any significant association, and hence no role in HBV viral persistence or with disease progression. An allele frequency of 0.0 was

observed for the risk allele 'T' and a frequency of 1.0 was observed for the wild allele 'A' within the Saudi Arabian population, which was consistent with the observations documented in the NHLBI Grand Opportunity Exome Sequencing Project (ESP) of T = 0.0 and A = 1.0 for European Americans and African Americans (https://esp.gs.washington.edu/drupal/).

Therefore, the present study suggests a significant association in the case of rs2234671 with regards to HBV viral activity but may lack an association in HCC occurrence among chronic or cirrhotic patients.

Haplotype analysis between patient and uninfected control group revealed 2 (Haplo 1 and Haplo 2) possible combinations. Both Haplo 1 (AG) (freq. = 0.93) and Haplo 2 (AC) (freq. = 0.07) was insignificantly distributed between patients and control subjects. While, haplotype analysis in case II + III + IV against case I revealed a significant association for both the haplotypes in relation to viral persistence. The most frequent haplotype, Haplo 1 (AG), was found to be significant with a frequency of 0.94. Haplo 2 (AC) (freq. = 0.06) was also found to be significant with a p-value of 0.016. Similarly, haplotype analysis was carried across case III + IV *vs.* case II group and none of the two observed haplotypes were found to be significant. Thus, the polymorphic variations related to this study might play a significant role in leading to different stages of HBV infection. However, resolving the precise functional consequences of these polymorphisms or SNPs need more detailed and careful *in vitro* as well as *in vivo* studies.

In conclusion, the studied polymorphisms in CXCR1 gene may have an important role in viral persistence but have no significant association with the disease progression. Further studies are needed to confirm the results observed in this study and to prove the existence of an association between the outcome of HBV infection and CXCR1 genetic polymorphisms. The authors speculate that, apart from chemokines and their receptors, some other host factors might be playing a prominent role in the progression of HBV infection.

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ABBREVIATIONS

- **HBV:** hepatitis B virus.
- **SNP:** single nucleotide polymorphism.
- **HCC:** hepatocellular carcinoma.
- **CXCR1:** chemokine (C-X-C motif) receptor 1.
- **HBsAg:** hepatitis B surface antigen.
- **Anti-HBs:** antibodies against hepatitis B surface antigen.
- **Anti-HBc:** antibodies against hepatitis B core antigen.
- **OR:** Odds Ratio.

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