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RANTES gene polymorphisms (-403G>A and -28C>G) associated with hepatitis B virus infection in a Saudi population

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ABSTRACT. Besides the host immune response, genetic and environmental factors play crucial roles in the manifestation of hepatitis B virus (HBV) infection. “Regulated on activation normal T-cell expressed and secreted”

factor (RANTES) plays a vital role in $CD4^+$, $CD8^+$ T-lymphocyte and dendritic cell activation and proliferation in inflammation. Single nucleotide polymorphisms (SNPs) in the RANTES gene are associated with several viral and non-viral diseases. Association studies have invariably indicated a lack of association between RANTES gene SNPs and HBV infection in ethnic populations, even though RANTES gene SNPs exhibit distinct ethnic distributions. Despite the high prevalence of HBV infections in Saudi Arabia, no studies have been made concerning a possible relationship between RANTES gene polymorphisms and susceptibility to and progression of HBV infection. We examined -403G>A and -28C>G RANTES gene variants in 473 healthy controls and 484 HBV patients in ethnic Saudi populations. Significant differences were found in the genotype and allele distributions of the SNPs between the controls and the HBV patients. Both SNPs were significantly linked to viral clearance in these subjects. Our data demonstrate for the first time in a Saudi population, a relationship between the RANTES gene polymorphisms and the clinical course of HBV infection and underscore the importance of evaluating the genetic background of the affected individual to determine how it may affect disease progression.

Key words: RANTES; Gene polymorphisms; Hepatitis B virus; Saudi Arabia

INTRODUCTION

Hepatitis B virus (HBV) infections are among the major health burdens with over 350 million people infected worldwide. The HBV infection that leads to hepatitis is hepatotropic with potentially fatal complications, including hepatocellular carcinoma (HCC) (Watson, 2002). The clinical course of HBV infection varies from spontaneous recovery after acute hepatitis to a chronic persistent infection that may progress to cirrhosis or HCC. 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 (Grakoui et al., 2003; Bowen and Walker, 2005; Park et al., 2006; Durantel and Zoulim, 2009).

RANTES (regulated on activation normal T-cell expressed and secreted) is a ligand for CC chemokine receptor 5 (CCR5) and produced principally by $CD8^+$ T-lymphocytes, platelets and epithelial cells (Cocchi et al., 1995; Gonzalez et al., 2002). Together with macrophage inflammatory protein 1 (MIP1)-α, MIP1-β and monocyte chemoattractant protein (MCP)-II, RANTES plays a vital role in $CD4^+$, $CD8^+$ T-lymphocyte and dendritic cell activation and proliferation (Ma et al., 2007). The effects of RANTES are mediated through the chemotaxis of monocytes and memory T-lymphocytes, the mechanism of which is involved in both acute and chronic phases of inflammation (Nelson et al., 1996). The regulatory region of the RANTES gene has two functional SNPs, namely -403G>A and -28C>G, which are associated with increased RANTES expression (Liu et al., 1999; Nickel et al., 2000). Both of these polymorphisms are linked to several viral and non-viral diseases including asthma, type 1 diabetes and HIV (Liu et al., 1999; An et al., 2002; Hizawa et al., 2002; Zhao et al., 2004; Zhernakova et al., 2006). Increased expression of RANTES in response to HBV infection or after exposure to viral antigens is reported in several experimental settings,

which is consistent with the anti-viral activity of this chemokine (Duan et al., 2005; Nam et al., 2006; Ma et al., 2007). Several studies have also verified the correlation of RANTES gene polymorphisms with HBV infection. These studies have invariably found a lack of association between the RANTES gene and HBV infection (Duan et al., 2005; Ahn et al., 2006; Park et al., 2006; Cheong et al., 2007; Thio et al., 2008). Despite the increased prevalence of HBV infections in Saudi Arabia, no information is available regarding RANTES gene polymorphisms and their relationships with viral susceptibility and disease progression. Thus, in this study we aimed to test this association for -403G>A and -28C>G RANTES gene polymorphisms in an ethnic Saudi population.

SUBJECTS AND METHODS

Subjects

The present study was carried out according to the guidelines set down by the Ethics Committee of the College of Science, King Saud University. Informed consents were obtained from all patients and normal healthy individuals. Blood samples from 484 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. Blood samples were also collected from 473 normal healthy subjects who volunteered to participate in the study. Control subjects were characterized by the absence of any known serological marker of HBV (HBsAg negative, anti-HBs negative, and anti-HBc negative) or the presence of anti-HBs antibodies. Among the patient subjects, chronically infected patients were determined by the presence of HBsAg, HBeAg and anti-HBc antibody, while 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 were obtained from all the participating subjects. A structured questionnaire was used to obtain the demographical, past and present medical data from the subjects. HBV infection in the patients was diagnosed by the method as detailed below. Blood samples were collected from the subjects and DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, USA) following the recommended procedures.

PCR diagnosis and genotyping of HBV

HBV was detected and genotyped using the INNO-LiPA HBV genotyping kit (Innogenetics, Gent, Belgium) according to manufacturer instructions. The assay is based on two-round PCR utilizing the HBV polymerase gene as a target for amplification. One of the second-round PCR primers is biotinylated, which allows the isolation of a biotinylated DNA strand for hybridization with an immobilized genotype-specific probe. Unhybridized DNA is washed away and color development is then performed based on alkaline phosphatase activity using BCIP/NBT as a substrate.

Amplification of target regions in the RANTES gene

In this study, we examined two functional SNPs, rs2280788 (-28C>G) and rs2107538 (-403G>A), in the promoter region of the RANTES gene. A 603-bp region that encompasses these polymorphic sites was amplified by PCR using the primers forward 5'-TGTGAAAAGGTTCCCAATGC-3' and reverse 5'-CATGGTACCTGTGGGAGAGG-3'.

DNA sequencing

Following PCR amplification, products were resolved on agarose gels and purified using 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 the BigDye® Terminator v3.1 Cycle Sequencing Kit, Applera, according to manufacturer instructions. Bidirectional sequencing was performed for all the samples to ensure the accuracy of the data. Sequencing analysis was performed using the DNA Sequencing Analysis Software v5.2.

Statistical analysis

Statistical analysis of genotype distributions and allele frequencies was performed by the chi-square analysis and the Fisher exact test. All analyses were carried out with the Statistical Product and Service Solutions software (Version 12.0; SPSS Inc., Chicago, IL, USA).

RESULTS

In this study, we evaluated the distribution of rs2280788 (-28C>G) and rs2107538 (-403G>A) SNPs in the promoter region of the RANTES gene in 484 HBV patients and 473 normal healthy subjects. PCR amplified a 603-bp RANTES gene-specific region, which was directly sequenced to determine the existence of SNPs. The clinical data of the studied patients are presented in Table 1. Both the genotypes were consistent with the Hardy-Weinberg equilibrium (Table 2; $P > 0.05$). The differences in the genotype (GG, GA, AA) and allele (G, A) distribution of SNP -403G>A are shown in Table 3. Genotype distribution among the HBV patients, irrespective of the disease severity, was found to be significantly different compared to controls ($\chi^2 = 21.7$; $P < 0.0002$). Likewise, compared to controls, genotype distributions in asymptomatic subjects, symptomatic subjects, and subjects with HCC were significantly different ($\chi^2 = 18.3$, $P < 0.0001$; $\chi^2 = 10.6$, $P < 0.005$, and $\chi^2 = 12.3$, $P < 0.002$, respectively). No significance in the genotype distribution was found between the control subjects and subjects with liver cirrhosis ($P > 0.05$). Comparisons of G and A allele carriers were also done between the controls and the HBV patients accounting for different disease severities. Subjects with HBV, regardless of disease course, had significantly different allelic distribution ($\chi^2 = 10.4$, $P < 0.001$) compared to controls. Similarly, allelic distribution in asymptomatic subjects and subjects with liver cirrhosis plus HCC was significantly different compared to controls ($\chi^2 = 4.7$, $P < 0.03$ and $\chi^2 = 12.0$, $P < 0.001$, respectively), while no significance was found between controls and symptomatic carriers and subjects with liver cirrhosis.

We also evaluated the SNP -28C>G of the RANTES gene in the controls and HBV patients. The differences in genotype (CC, CG and GG) and allele (C and G) distributions of -28C>G SNP among the controls and different disease states of HBV patients are presented in Table 4. Interestingly, no GG genotype carriers were found among the controls and patients studied. No significant difference in the genotype distribution was found between the controls and HBV patients. Contrarily, HBV patients with liver cirrhosis were significantly different compared to controls ($\chi^2 = 8.5$, $P < 0.003$) with respect to genotype distribution. Asymptomatic and symptomatic patients and patients with HCC had no statistically significant differences in genotype distribution. Comparisons of C and G allelic distributions revealed a significant difference between controls and HBV patients with HCC ($\chi^2 = 8.5$, $P < 0.004$), whereas, no significant differences were found in the allelic distributions

between controls and asymptomatic and symptomatic patients and patients with liver cirrhosis.

Table 1. Basic characteristics of the HBV patients.

Features	Average	Standard deviation
Age	46.60648148	14.6740636
Weight	71.08101852	16.2544208
Viral load	30442321.57	45169450.9
Hemoglobin	140.9953704	22.5492737
White blood cells	6.55212963	2.43954096
Platelets	238.0409259	83.6818789
Bilirubin	11.52314815	9.28411603
Alanine transaminase	81.8411215	82.5036234
Aspartate transaminase	60.49856459	48.5629691
Alkaline phosphatase	98.9342723	58.0901658
Gamma-glutamyltransferase	95.60465116	149.360462
Alpha-fetoprotein	7.659	12.3847215
Creatinine	140.6140791	239.611713
Urea	7.027692308	7.69660873
Albumin	40.08930233	5.94559658
Uric acid	345.8	131.50671
Cholesterol	4.790756303	4.93996143
Triglycerides	1.621769912	3.25127371
Thyroid-stimulating hormone	4.722090909	19.0400386
Na^+	139.8101852	8.57503613
K^+	4.086574074	0.54887227
Cl^+	103.6388889	2.99443929
Ca^+	2.317682927	0.16486692
Phosphate	1.160565217	0.41935732
Prothrombin time	13.1342723	2.50958522
Partial thromboplastin time	35.535	8.18382858
International normalized ratio	1.023004695	0.19973186

Table 2. Compliance of polymorphisms with Hardy-Weinberg.

SNP	(Chr. number) Position	Obs. HET[1]	Pred. HET	HW P value	% Genotype	MAF	Alleles
-28C>G	(17) 31231518	0.023	0.023	1	100	0.012	C:G
-403G>A	(17) 31231893	0.328	0.325	0.9893	100	0.204	G:A

[1]Genetic analysis of the SNPs studied. Obs. and Pred. HET = observed and predicted heterozygosities; HW = Hardy-Weinberg; MAF = minor allele frequency.

Table 3. Genotype and allele distribution of RANTES -403G>A polymorphism in controls and HBV patients.

	Control	Asymptomatic	Symptomatic	LC	HCC	P value
Genotype						
GG	299 (63.2)	156 (50)	58 (48.7)	23 (60.5)	3 (20)	0.002*
GA	155 (32.7)	149 (47.8)	58 (48.7)	13 (34.2)	10 (66.7)	0.0001**
AA	19 (4.1)	7 (2.2)	3 (2.6)	2 (5.3)	2 (13.3)	0.005^+
						0.002^{++}
Allele						
G	753 (79.6)	461 (73.9)	174 (73.1)	59 (77.6)	16 (53.3)	0.001*
A	193 (20.4)	163 (26.1)	64 (26.9)	17 (22.4)	14 (46.7)	0.03^+
						0.001^{++}

Data are reported as number with percent in parentheses. LC = liver cirrhosis; HCC = hepatocellular carcinoma. *Controls *vs* total HBV patients; **controls *vs* asymptomatics; $^+$controls *vs* LC; $^{++}$controls *vs* HCC. Significance was determined by the chi-square (χ^2) test.

Table 4. Genotype and allele distribution of RANTES -28C>G polymorphism in controls and HBV patients.

	Control	Asymptomatic	Symptomatic	LC	HCC	P value
Genotype						
CC	462 (97.7)	301 (96.5)	117 (98.4)	34 (89.2)	14 (93.3)	0.221*
CG	11 (2.3)	11 (3.5)	2 (1.6)	4 (10.8)	1 (6.7)	0.003**
GG	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Allele						
C	935 (98.8)	613 (98.3)	236 (99.2)	70 (94.6)	29 (96.7)	0.224*
G	11 (1.2)	11 (1.7)	2 (0.8)	4 (5.4)	1 (3.3)	0.004**

Data are reported as number with percent in parentheses. LC = liver cirrhosis; HCC = hepatocellular carcinoma. *Controls *vs* total HBV patients; **controls *vs* LC. Significance was determined by the chi-square (χ^2) test.

DISCUSSION

Our aim in this study was to check whether the polymorphisms in the RANTES gene affect susceptibility to HBV infection and the clinical course of the disease in an ethnic Saudi population. Here we examined the two most commonly studied RANTES gene polymorphisms, rs2280788 (-28C>G) and rs2107538 (-403G>A). In this study, we found a significant correlation between HBV infection and RANTES gene polymorphisms.

The majority of adults who are infected with HBV recover due to their ability to mount an efficient immune response. However, in about 5% of individuals, the HBV infection can lead to liver cirrhosis and HCC (Lee, 1997). A broad and strong T-cell immune response in the affected persons is the feature of recovery from HBV infection compared to those with a weak immune response (Rehermann et al., 1996). Differences in immune mediators at the genetic level may determine the host's ability to counter HBV infection. RANTES is a Th1 chemokine that is a ligand for receptor CCR5. RANTES promotes T-cell activation and proliferation. The binding of RANTES to its alternate receptor, CCR1, has been shown to upregulate the inflammatory response during sepsis (Ness et al., 2004) and to increase recruitment of natural killer cells to the liver in an autoimmune hepatitis mouse model (Ajuebor et al., 2007). Consistent with the pivotal role of RANTES in the adaptive immunity, the RANTES gene is considered to be the candidate gene in modulating the host response to HBV infection. Accordingly, several polymorphisms are reported in the RANTES gene. The -403G>A and -28C>G polymorphisms in the promoter region of the RANTES gene are widely studied and found to correlate with increased gene expression and are negatively linked to several diseases (Liu et al., 1999; An et al., 2000).

The SNP -403G>A was significantly linked to asymptomatic HBV subjects as well as to subjects with liver cirrhosis and HCC. Likewise, the SNP -28C>G was well correlated with asymptomatic HBV subjects and subjects with liver cirrhosis. Thus, our data clearly indicate the existence of significant association of the RANTES gene with susceptibility to HBV and subsequent progression of the disease. Our findings contrast with several other studies. The SNPs -403G>A, -28C>G and the intronic SNP, In 1.1T/C of the RANTES gene, were not associated with chronic HBV infection in a cohort of Chinese HBV patients; however, a significant correlation was found between plasma RANTES levels and HBV infection in the subjects (Duan et al., 2005). Similarly, no association was found in Korean HBV subjects in the clearance of virus with that of -403G>A and -28C>G RANTES SNPs (Park et al., 2006; Cheong et al., 2007; Ahn et al., 2009.). In a Caucasian population, the -403G>A SNP in combination with a CCR5Delta32 polymorphism in the CCR5 gene, but not by itself, is linked to

HBV clearance indicating at least in part the functional consequence of this variant (Thio et al., 2008). The findings in the present study, however, are consistent with the studies that have examined the association of -403G>A and -28C>G SNPs with viral and non-viral diseases including HIV, upper urinary tract infection, asthma, lymphoma, type 1 diabetes, and hepatitis C virus (Hizawa et al., 2002; Hellier et al., 2003; Zhao et al., 2004; Zhernakova et al., 2006; Bracci et al., 2010; Centi, et al., 2010; Zhang et al., 2010). Since we did not measure the RANTES levels in the studied subjects, it not clear whether -403G>A and -28C>G SNPs accounted for the increased RANTES gene expression. The differences in the observations made in the present study to those that found a lack of -403G>A and -28C>G SNP association with HBV could arise from the variations in the ethnicity, age, gender, number of samples analyzed, and the existence of different functional haplotypes.

The data in our study suggest that RANTES -403A and -28G would enhance recovery from an HBV infection. These findings could be explained by the characteristics of the cellular and cytokine profiles found in the conconavalin A (Con A)-induced hepatitis mouse model (Ness et al., 2004). In this model, natural killer (NK) cells did not infiltrate the liver in the wild-type mouse after Con A treatment, but accumulated in the liver of *CCR5* deficient mice upon Con A treatment, a condition that would apparently enhance an immune response and aid in the spontaneous recovery from HBV infection. The study showed that *CCR5* deficiency associates specifically with elevated RANTES expression in the mouse liver but not that of other CCR5 ligands. Thus, it is likely that the increase in liver RANTES subsequently leads to enhanced interactions between this chemokine and its alternative receptor, CCR1, an interaction that results in the recruitment of NK cells. Indeed, upon treatment of the mice with anti-RANTES antibody, there was a significantly reduced recruitment of NK cells into the liver after Con A administration.

In this study, we demonstrated for the first time the association of RANTES gene polymorphisms with HBV susceptibility and disease progression. The limitation of our study was that we analyzed only two of the SNPs of the RANTES gene. The possibility of the other unanalyzed SNPs contributing to these associations could not be ruled out.

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