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E-Selectin Gene Polymorphisms in Iranian Chronic Hepatitis B Patients

Mehrdad Hajilooi [1], Amir-Houshang Mohammad Alizadeh [2], Mitra Ranjbar [3], Farahnaz Fallahian [4], Seyed-Moayed Alavian [4*]

[1] Department of Immunology, Hamedan University of Medical Sciences, Hamedan, Iran
[2] Department of Gastroenterology, Shaheed Beheshti University of Medical Sciences, Tehran, Iran
[3] Department of Infectious Diseases, Iran University of Medical Sciences, Tehran, Iran
[4] Baqiyatallah Research Center for Gastroenterology and Liver Disease (BRCGL), Baqiyatallah University of Medical Sciences, Tehran, Iran

Background and Aims: **The aim of this study was to detect the substitutions Ser128Arg (A128C) and Leu554Phe (T554C) which are responsible for E-selectin polymorphisms in patients with chronic hepatitis B and healthy controls. We investigated possible association of the Ser128Arg and Leu554Phe gene polymorphisms in the E-selectin gene with susceptibility to chronic hepatitis B.**

Methods: **Sixty-three patients with chronic hepatitis B virus infection and 150 healthy subjects were recruited sequentially as they presented to clinic. Classification of chronic hepatitis B virus (HBV)-infected patients was as asymptomatic carrier state (34 patients) and chronic hepatitis B (29 patients). Genomic DNA was isolated from anti-coagulated peripheral blood Buffy coat using Miller's salting-out method. The presence of the E-selectin gene polymorphisms was determined by using polymerase chain reaction amplification refractory mutation system (ARMS).**

Results: **Distribution of E-selectin 128 (A^+C^-, A^+C^+, A^-C^+) genotypes and E-selectin 554 (C^+T^-, T^+C^-, C^+T^+) genotypes were not statistically different in chronic hepatitis B patients and controls (P=0.41 and 0.96, respectively). Also, two groups had no significant difference in distribution of frequencies of allele 128A (P=0.41), 128C (P=0.15), allele 554C (P=0.85), and allele 554T (P=0.76). Carrying of allele 128A (OR=0.58, 95% CI=0.16-2.12), 128C (OR=1.52, 95% CI=0.84-2.74), 554C (OR=1.24, 95% CI=0.12-12.08), and allele 554T (OR=0.88, 95% CI=0.38-2.01) were not risk factors for susceptibility to chronic hepatitis B infection.**

Conclusions: **Carrying E-selectin gene polymorphisms of Ser128Arg and Leu554Phe is not considered risk factor for susceptibility to chronic hepatitis B infection.**

Keywords: **E-Selectin Gene, Chronic Hepatitis B, Gene Polymorphisms**

Introduction

Hepatitis B virus (HBV) is a serious health problem worldwide and major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). It was estimated that approximately 2 billion people have serological evidence of past or present HBV infection. More than 350 million are chronic carriers of HBV. It was reported that 15-40% of HBV infected patients would develop cirrhosis, liver failure, or HCC. Because of the high HBV-related morbidity and mortality, the global disease burden of HBV is substantial [(1)]. Carrying out the protective function, leukocytes migrate from the bloodstream into sites of infection. The initial interaction of neutrophils with the vascular endothelium is mediated by the binding of the selectin family of adhesion molecules to their glycoprotein ligands, and the firm adhesion

**** Correspondence:***
Seyed-Moayed Alavian, M.D., Professor of Gastroenterology and Hepatology, Baqiyatallah Research Center for Gastroenterology and Liver Disease (BRCGL), Baqiyatallah University of Medical Sciences, Mollasadra Avenue, Vanak Square, Tehran, Iran.

Tel/Fax: *+98 21 81264070*

E-mail: *alavian@thc.ir*

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and transmigration are mediated by the binding of integrins to their immunoglobin supergene family ligands, or as recently suggested through platelet endothelial cell adhesion molecule-1 (PECAM-1)-PECAM-1 interaction (CD31). This multistep paradigm also appears to hold true for T lymphocytes [2].

Selectins are a family of calcium-dependent adhesion molecules implicated in mediating the initial interaction of leukocytes with vascular endothelial cells. Three members, L-selectin, P-selectin and E-selectin have been identified [3]. E-selectin was originally described in 1987 as a 115 kD antigen that was induced on cultured human endothelial cells after stimulation with interleukin-1 (IL-1) and TNF-α, and was shown to be involved in the adhesion of neutrophils [4]. Disorders of the specific humoral and the specific cellular defense are the principle cause of inflammation. The recruitment and activation of inflammatory cells at the site of infection involve the expression of leukocyte and vascular adhesion molecules and the generation of pro-inflammatory cytokines, which contribute to the injury of endothelial barriers. An important role in the early transient adhesion phases of leukocytes is mediated by the selectin family, which includes three distinct carbohydrate receptors expressed by endothelial cells (E-selectin), leukocytes (L-selectin), or platelets and endothelial cells (P-selectin) [5]. ICAM-1 and VCAM-1 are membrane glycoproteins, belonging to the immunoglobulin superfamily; ICAM-1 is widely expressed on respiratory epithelial cells, monocytes, macrophages, dendritic cells, and, together with VCAM-1, on vascular endothelial cells. These adhesion molecules, after a proteolytic cleavage, are found as soluble forms in the circulation [6]. Patients with systemic inflammation present elevated levels of soluble adhesion molecules and the measurement of their levels is considered useful in the evaluation of inflammatory disorders [7]. In the complex cytokine network, IFN-γ, IL-12, IL-4, and IL-10 play an important role in responses to viral infections [8], regulate the Th-1/Th-2 balance [9]. However, little is known about the functional role of adhesion molecules in chronic liver diseases.

The aim of this study was to investigate possible associations of polymorphisms found in genes (Ser128Arg & Leu554Phe) encoding the adhesion molecule, E-selectin and chronic hepatitis B. We sought to evaluate the role of polymorphisms of this adhesion molecule to ascertain whether they produce anomalies in the immune response to hepatitis B virus infection and are associated with subsequent development of chronic hepatitis B.

Materials and Methods

Subjects

Sixty-three patients with chronic hepatitis B infection (mean age: 35.4±12.5 years) and 150 control subjects (mean age: 32.3±15.27 years) were recruited sequentially as they presented to the hepatitis clinic for regular follow-up examinations. Of chronic hepatitis B patients, 72.6% were male, 27.4% were female; of healthy control group 58.7% were male, and 41.3% were female. Two groups were matched for age and sex. None of the subjects of control group had clinical and serological evidences of hepatitis B infection. The study was carried out with the approval of the local hospital ethical committee. All participants signed the informed written consent.

Chronic hepatitis B was based upon biochemical [rise in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity for about 6 months], virological (positive for HBV markers), and liver histological activity if needed [histologic classification index (HAI) by Ishak score]. Chronic hepatitis B group included patients still on interferon or lamivudine treatment; those who finished treatment course, nonresponders to treatment (lack of virologic and/or histologic response by first treatment course in which sustained response was unlikely). Asymptomatic carrier state was defined as: chronically HBsAg positive patients who have anti-HBc in serum, anti-HBs is either undetected or detected at low titer against the opposite subtype specificity of the antigen regarded as inactive or asymptomatic carriers, HBV-DNA load less than 10^5 copies/ml, HBeAg (+/-), and serum liver transaminases of normal range. Inactive HBsAg carriers were monitored with periodic liver chemistries as liver disease may become active even after many years of quiescence.

Genotyping

Venous blood was collected from each subject into tubes containing 50 mmol of EDTA per liter, and genomic DNA was isolated from anti-coagulated peripheral blood Buffy coat using Miller's salting out method [10]. To detect the substitutions Ser128Arg (A128C) and Leu554Phe (T554C) which are responsible for E-selectin polymorphisms; we used a single-specific polymerase chain reaction method (ARMS-PCR). ARMS-PCR was performed in a total volume of 50 μl that contained 0.2 ng of genomic DNA, each primer pair consisting of 10 pmol of allele-specific

primer, 5 pmol of common primer, 200 mol/l each dNTP; 10 mol/l Tris-HCl (PH: 8.3); 50 nmol/l KCl, 1.5 mol/l $MgCl_2$ and 0.5 IU Taq DNA polymerase. PCR performed without DNA template represented the negative controls. The reaction was carried out as follow; initial denaturation was at 94 °C for 2 min, followed by 10 cycles of amplification at 96 °C for 20 s and annealing at 64 °C for 50 s, with extension for 40 s at 72 °C, followed by 20 cycles of denaturation at 96 °C for 20 s and annealing at 61 °C for 50 s, with extension for 40 s at 72 °C and then with final soak at 10 °C. The amplified PCR products were analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining and ultraviolet visualization.

Statistical Analysis

Each result was calculated as mean±SD. The Kolmogorov- Smirnov test of normality was used to test whether the distribution of variables had a normal pattern. The discrete variables were compared by Pearson's chi square test and Student's *t-test* compared the continuous variables. Non-parametric, Mann-Whitney *U* test, ANOVA, and Kruskal-Wallis tests were used when appropriate. All P values were two-tailed, and values less than 0.05 were considered to be statistically significant. Statistical analysis was performed with the SPSS version 13.

Results

Of chronic hepatitis B group, 34 cases (54%) were asymptomatic carriers, 18 (28.6%) cases on the first course of treatment, 5 (7.9%) cases were non-responders to the first course of treatment, and 6 (9.5%) cases were chronic hepatitis B. HBeAg was positive in 8 (12.7%), and negative in 55 (87.3%) of chronic hepatitis B patients. HBcAb was positive in 98.4%, HBeAb was positive in 88.9%, HBsAg was positive in 91.7%, and negative in 8.3%. Mean AST and ALT was 30.9±18.7 and 37.7±27 IU/L (normal range: 5-30 IU/L), liver biopsy was performed in 28 (44.4%) of patients and mean score, grade, and pathologic stage was 7.5±2.6, 5.6±1.9, and 1.9±1.1, respectively.

There were no statistically significant differences in age (33.14±11.88 vs. 35.70±12.74), mean ALT (53.71±27.36 vs. 35.67±26.62) and score (8±1.41 vs. 7.4±2.9) between HBeAg positive and negative chronic hepatitis B patients. Mean AST was 49.4±22.8 in HBeAg positive and 53.71±26.6 in HBeAg negative patients (P=0.005). Distribution of genotype frequency of E-selectin A128C & T554C polymorphisms in hepatitis B patients and controls is shown in Tables 1 & 2. Distribution of E-selectin 554 (T^+C^-, T^+C^-, T^+C^+) genotypes in chronic hepatitis B patients and controls is shown in Table 2.

Table 1. Distribution of E-selectin 128 (A^+C^-, A^+C^+, A^-C^+) genotypes in chronic hepatitis B patients and controls.

		Case (%)	Control (%)
	A^+C^-	38 (60.3)	104 (69.3)
A128C	A^+C^+	21 (33.3)	40 (26.7)
	A^-C^+	4 (6.3)	6 (4.0)
Total		63 (100)	150 (100)

P=0.41

Table 2. Distribution of E-selectin 554 (T^-C^+, T^+C^-, T^+C^+) genotypes in chronic hepatitis B patients and controls.

		Case (%)	Control (%)
	T^+C^-	1 (1.6)	3 (2.0)
T554C	T^+C^+	7 (11.1)	18 (12.0)
	T^-C^+	55 (87.3)	129 (86.0)
Total		63 (100)	150 (100)

P=0.96

Using Chi-square test, we did not find any statistically significant difference between frequencies of 128 (A^+C^-, A^+C^+, A^-C^+) genotypes and also 554 (T^+C^-, T^+C^-, T^+C^+) genotypes in chronic hepatitis B patients and healthy controls. So, these genotypes are not considered risk factors of susceptibility to chronic hepatitis B infection. Frequencies of alleles 128A, 128C, 554C, and 554T in chronic hepatitis B patients and healthy controls are shown in Table 3. Mean AST and ALT levels in every 6 genotype of E-selectin gene of chronic hepatitis B patients are shown in Table 4. Association of every 6 genotype with mean AST and ALT levels in chronic hepatitis B patients was assessed by Kruskal-Wallis test. There was no statistically significant difference between mean AST and ALT levels in 128 genotypes in chronic hepatitis B patients by ANOVA (P=0.26 & P=0.29, respectively). There was also no significant difference between mean AST and ALT levels in 554 genotypes in chronic hepatitis B patients by ANOVA (P=0.34 & P=0.29, respectively).

Table 3. Frequencies of allele 128A, 128C, 554C, 554T in chronic hepatitis B patients and healthy controls.

Allele frequency	Case	Control	Total	P value	OR (95% CI)
128A (+)	97	248	345		
128A (-)	4	6	10		
Total	101	254	355	0.41	0.58 (0.16-2.12)
128C (+)	29	52	81		
128C (-)	38	104	142		
Total	67	156	223	0.15	1.52 (0.84-2.74)
554C (+)	117	276	393		
554C (-)	1	3	4		
Total	118	279	397	0.85	1.24 (0.12-12.08)
554T (+)	9	24	33		
554T (-)	55	129	184		
Total	64	153	217	0.76	0.88 (0.38-2.01)

Discussion

Surveys for characterizing host immune state and endogenous cytoprotective pathways as potential avenues for therapeutic intervention are mandatory. Activation of endothelium is a critical component of inflammation, allowing the recruitment of leukocytes into the tissues and a shift towards a prothrombotic state [11]. While activation of proinflammatory cytokines leads to the expression of many proinflammatory genes, adhesion molecules and chemoattractants; this response is matched by a compensatory expression of anti-inflammatory response and to reduce endothelial injury [12]. In a study [13] on 30 chronic alcoholics without alcohol-related diseases and 30 controls, serum levels of endothelial adhesion molecules (ICAM-1, VCAM-1, and E-selectin) was measured. There was a significant correlation between daily alcohol intake and serum level of ICAM-1 and E-selectin. A significant positive correlation between E-selectin and total lifetime dose of ethanol was also observed. This article concluded these changes in serum levels of endothelial adhesion molecules of chronic alcoholics may reflect endothelial and or immune activation, and could interfere with the reactions between immune cells and the endothelium.

To investigate the effect of E-selectin A561C (S128R) polymorphism on blood pressure, in a study, 347 essential hypertensive patients and 315 normal controls were genotyped. According to this study, the frequencies of the AC and CC genotypes and C allele were significantly higher in hypertensive patients than normal controls and the study concluded that the E-selectin A561C (S128R) polymorphism might affect blood pressure in Chinese [14]. In another study [15], the authors believed that in all the studies they examined, the polymorphism G20210A in the prothrombin gene was associated with an increased risk of acute myocardial infarction (AMI) in young people, especially when other risk factors were present. Contradictory results have been found in the studies on factor V Leiden. An association was found between young AMI and polymorphism C-260T in the CD14 gene, between coronaries atherosclerosis and polymorphism A516C in the E Selectin gene or polymorphisms Leu125Val and Ser563Asn in the PECAM1 gene.

In a study [16] of 87 stable hemodialysis patients, predialysis serum soluble intercellular adhesion molecules-1 (sICAM-1), soluble vascular cellular adhesion molecules-1 (sVCAM-1), and soluble E-selectin were assayed. The sICAM-1, sVCAM-1 and E-selectin levels were higher in the anti-HCV-

Table 4. Mean AST, ALT in every 6 genotype of E-selectin gene in studied chronic hepatitis B patients.

A128C		AST	ALT	T554C		AST	ALT
A^+C^-	Mean±SD	31.97±18.45	39.51±22.84	T^+C^-	Mean±SD	20±0	15±0
	Range	5-90	10-93		Range	20-20	15-15
A^+C^+	Mean±SD	31.14±20.98	36.52±35.40	T^+C^+	Mean±SD	22.28±6.52	26.71±8.49
	Range	15-110	12-160		Range	12-30	13-38
A^-C^+	Mean±SD	20.75±2.50	27.25±10.50	T^-C^+	Mean±SD	32.29±19.68	39.55±28.41
	Range	18-24	12-36		Range	5-110	10-160
Total	Mean±SD	30.96±18.78	37.70±27.09	Total	Mean±SD	30.96±18.78	37.70±27.09
	Range	5-110	10-160		Range	5-110	10-160

positive group and concluded that HCV infection was determined as an independent determinant of sICAM-1 and sVCAM-1 by multiple linear regression analysis. In another study [17], whether the single nucleotide polymorphism (SNP) Ser128Arg in the E-selectin gene may affect cytokine-induced levels of soluble E-selectin, or be associated with proinflammatory or procoagulant properties was tested. 157 healthy male volunteers received a lipopolysaccharide (LPS) infusion and were genotyped for the S128R SNP, and outcome parameters were measured by enzyme immunoassays and real-time polymerase chain reaction. óAccording to this study, the S128R SNP had no pronounced effects on basal or inducible sE-selectin levels, or levels of tumor necrosis factor or interleukin-6. However, carriers of the S128R SNP had 20% higher monocytes counts at 24 hours after LPS infusion. The S128R E-selectin genotype is associated with procoagulant effects in a human model of endotoxin-induced, tissue factor (TF)-triggered coagulation. This could contribute to its linkage with various thrombotic cardiovascular disorders.

In an article [18], in order to investigate the clinical and pathogeneses role of P-selectin in chronic liver diseases, plasma P-selectin levels measured in 111 patients with chronic liver diseases. P-Selectin was significantly elevated in patients compared with controls. P-selectin correlated with platelet and white blood cell counts, but not with endothelial injury markers thrombomodulin and tissue factor or coagulation factors. Severe hepatic leukocyte infiltration in liver histology was associated with a tendency towards higher P-selectin levels. This study concluded that P-selectin elevation in chronic liver disease might suggest a possible pathogenesis role in the course of liver cirrhosis. In a study [19], the influence of chronic liver diseases on platelets morphologic parameters, their secretory activity and P-selectin expression was determined in 29 patients with chronic hepatitis and 27 with liver cirrhosis of post-inflammatory etiology (HBV & HCV). Liver biopsies were carried out in all patients. Thirty-two healthy individuals were the control group. According to this study, number, volume, and platelet count decreased with the advancement of a liver disease. Megathrombocyte fraction increased inversely with the severity of liver damage. The concentration of beta-thromboglobulin and platelet factor 4 α-granule contents in blood serum was higher 2- and 7-times, respectively than in healthy controls. P-selectin expression on resting platelets was considerably higher.

Thrombocytes in chronic liver diseases and liver cirrhosis are more activated.

The role of genetic susceptibility and polymorphisms influencing some biological functions such as coagulation and fibrinolysis, platelets, vascular function, lipid metabolism, atherosclerosis, inflammation, endothelial dysfunction, alteration of leukocyte-endothelial interactions, activation of proinflammatory cytokines that leads to the expression of many proinflammatory genes, adhesion molecules and chemoattractants from other studies mentioned.

The predominant routes of transmission vary according to the endemicity of the HBV infection. In areas with low HBV endemicity, perinatal transmission is the main route of transmission, whereas in areas with low HBV endemicity transmission is through sexual contact, intravenous drug use, acupuncture, and transfusion. HBeAg-negative chronic hepatitis B (e-CHB), characterized by HBV-DNA levels detectable by non-amplified assays and continued necroinflammation in the liver, has been reported worldwide, but is more common in Mediterranean countries and Asia [1]. Most patients with e-CHB, harbor HBV variants in the precore or core promoter region. The most common precore mutation, G1896A, creates a premature stop codon in the precore region thus abolishing production of HBeAg [20]. The most common core promoter mutations, A1762T+G1764A, decrease transcription of precore messenger RNA and production of HBeAg [21]. There are also clinical differences between HBeAg-positive and HBeAg-negative chronic hepatitis B.

In the present study, 55 (87.3%) of chronic hepatitis B patients were negative for HBeAg. Also, mean AST and ALT levels had no statistically significant difference in different 128 and 554 genotypes of E-selectin gene of patients with chronic hepatitis B patients. The clinical significance of mutant viruses and routes of transmission was not regarded in this study.

Conclusions

According to the present study, carrying E-selectin gene polymorphisms of Ser128Arg and Leu554Phe are not considered risk factors for susceptibility to chronic hepatitis B infection. Also, there was not any statistically significant association between serum AST & ALT levels and Ser128Arg and Leu554Phe gene polymorphism in chronic hepatitis B patients.

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