

RESEARCH ARTICLE

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Frequency of Hepatitis B Markers in Systemic Lupus Erythematosus Patients in Iran

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Abstract

Background: Systemic lupus erythematosus (SLE) is a chronic autoimmune disease. Hepatitis B virus is the causative agent for chronic, acute, cirrhosis, and hepatocellular carcinoma. SLE patients with chronic or occult hepatitis B infection undergoing immunosuppressive drugs may become reactive and develop fatal hepatitis. Therefore, this study was conducted to determine HBV markers in SLE patients before the administration of immunosuppressive drugs in Ahvaz city, Iran. **Materials and methods:** The sera of 92 SLE patients were tested for HBs Ag and anti-HBc using ELISA, HBV DNA (by Nested PCR) testes. Real-time PCR was performed for the patients with positive anti-HBc and negative HBsAg. The positive HBV DNA samples were checked for HBV genotype and HBV subtypes. **Results:** Among the 92 SLE patients, three (3.3%) were males and 89 (96.7%) females. The patients' ages ranged from 14 to 70 years [mean age of 38.9±10.1]. Three of 92 (3.26%) subjects [2/3 males and 1/89 female] were positive for HBsAg, anti-HBc Ab, and HBV DNA detected with PCR (p=0.000003). Five of 89 (5.61%) subjects [1 male and 4/88 females] were only positive for anti-HBc and negative for HBsAg, HBV DNA(PCR) using Real-time PCR (p=0.05). The results of the nucleotide data and phylogenetic tree showed all three HBV patients were genotype D1. The results of amino acid sequencing revealed all three HBV patients were HBV subtype ayw2. **Conclusion:** This study proved that 3.26% of SLE patients were positive for overt HBV infection (positive for anti-HBc, HBsAg and HBV-DNA using PCR). All the three isolated HBV were genotype D1 and subtype ayw2. The fact that 5.61% of the patients were only positive for anti-HBc characterized the occult hepatitis B infection (OBI) although further investigation is needed. To prevent HBV or OBI reactivation for SLE patients before immunosuppression treatment, HBV markers including anti-HBc, HBsAg, HBV-DNA should be implemented using PCR and Real-time PCR.

Keywords: Chemotherapy- nested polymerase chain reaction- systemic lupus erythematosus

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Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease which causes inflammation and damage to tissues and organs including joints, skin, brain, lungs, kidneys, and blood vessels (Francis and Perl, 2010, Tsokos., 2011). Several factors may be implicated in SLE development including environmental, genetic, immunological alterations and infectious agents (Mu et al., 2015). Usually, the liver is not the target in SLE though 25-50% of lupus patients may have abnormal liver function throughout viral hepatitis (Bessone et al., 2014; Huang et al., 2012).

Hepatitis B virus (HBV) is a major global health problem. It is estimated that 230 million population are chronically infected worldwide (Weckerle and Niewold., 2011; Tordrup et al., 2019). The prevalence of hepatitis

B infection is low (<2%) among the population in Iran (Alavian et al., 2007). No data is available to validate the prevalence of HBV infection in the Khuzestan province. The persistence and replication of HBV in hepatocytes may result in wide spectrum of liver disease such as cirrhosis, fibrosis and hepatocellular carcinoma (Dandri and Petersen ,2016; Nour et al., 2022; Radwan et al., 2022). The frequency of HBV reactivation is estimated to be about 20% to 50% in HBV carriers undergoing immunosuppressive treatment and in some cases has led to fulminant or fatal hepatitis (Lok and McMahon, 2009; Huang et al., 2014; Chenet et al., 2015).

The HBV reactivation is described as levels of serum HBV DNA rising at least 10 times (compared to baseline) and (ALT level \geq 3-fold increases compared to baseline >100 U/L) (Patel et al., 2015; Paul et al., 2015). The reactivation of occult hepatitis B infection (OBI)

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in patients with autoimmune diseases who underwent immunosuppressive drugs resulted in fulminant hepatitis (Matsumoto et al., 2016; Chen et al., 2020). OBI is defined by the detection of HBV DNA liver tissue/or in serum of HBsAg negative subjects. The level of HBV-DNA in the serum is usually low (< 200 IU/mL/or 1,000 copy number/mL) (Kwak et al., 2014).

Two classes of OBI are described as seropositive and seronegative. Seropositive OBI refers to the presence of anti-HBc and/or anti-HBs in the serum but HBV DNA is detected in serum/liver. While seronegative OBI refers to when both anti-HBc and anti-HBs are negative in the serum but HBV DNA is detected in serum/liver. To determine OBI, the liver biopsy requires as a gold standard for detection of HBVDNA (Makvandi, 2016). The detection of anti-HBc alone in the blood is reported to be used as a surrogate marker to identify OBI (Raimondo et al., 2019). Similarly, the detection of anti-HBc alone was reported in some blood donors with OBI who had undetectable HBV DNA by NAT (nucleic acid testing) (Spreafico et al., 2015). HBV reactivation has been described in individuals negative HBsAg, positive anti-HBc who had undetectable HBV DNA in the blood (Cholongitas et al., 2018). The detection of OBI can be achieved by highly sensitive methods such as real-time PCR and nested-PCR (Raimondo et al., 2008; Sosa-Jurado et al., 2018). Therefore, this study was conducted to determine the frequency of HBV infection in SLE patients in Ahvaz city. Ahvaz is the capital of Khuzestan province and is located at Southwest region of Iran.

Materials and Methods

Patients: In this cross-sectional study, 92 (including 89 females and 3 males) consecutive patients with diagnosed SLE were selected in Golestan Hospital during January 2017 to December 2017. The rheumatology ward of Golestan hospital of Ahvaz city is a referral center for the Khozestan province (totally about 5 million population) and neighboring provinces for all treatment procedures. The patients' ages ranged from 14 to 70 with a mean age of 38.9 ± 10.1 years. 3/92 (3.3%) of patients were males and 89/92 (96.7%) were females. The SLE was diagnosed according to the American College of Rheumatology (ACR) revised criteria for the classification of systemic lupus erythematosus (Hochberg MC, 1997). The inclusion criterion required untreated diagnosed SLE patients. The exclusion criteria were rheumatoid arthritis, systemic sclerosis (scleroderma), vasculitis, dermatomyositis, and arthritis. Underlying malignancy, recognized chronic infection (e.g., human immunodeficiency virus, Endocarditis, osteomyelitis), or pregnancy. Their blood and sera were tested for the following criteria. The clinical and immunological criteria of SLE patients are presented in Table 1. ESR level was 47 ± 28 (mm/hour) and CRP level was 7.2 ± 5.1 (mg/dl).

Molecular Tests

The patients' sera were approved positive for HBsAg, anti-Hbc Ab and HBV DNA using Nested PCR. The SLE patients proved positive for anti-HBc Ab but negative for

HBsAg and HBV DNA using Nested PCR and also tested by Real time PCR.

DNA extraction

DNA was extracted from specimens using High Pure Viral Nucleic Acid Kit (Roche, Germany) according to manufacturer's instructions. The extracted DNA was stored at -20°C until used for further analysis.

Nested PCR

For the first round PCR, the following outer primers, FHBS1: 5'-GAGTCTAGACTCGTGGTGGACTTC-3' (nt. 246-269) and RHBS1: 5'-AAA TKGCACTAGTAACTGAGCCA-3' (nt. 670-693) were used (Sitnik et al., 2004). The PCR reaction mixture contained 7 μl 400 ng of isolated DNA sample as template, 2.5 μl 10x reaction buffer, 0.75 μl MgCl₂ (50 mM), 0.5 μl forward/reverse primer, 1 μl dNTP (10 mM), 0.2 Cinna Gen Taq DNA Polymerase (5 u/ μl), and double-distilled water up to 25 μl . The reaction mixture was subjected to thermal cycler (TC-512, Techne, Staffordshire, UK) with the following program: 1 cycle with initial denaturation at 94°C for 5 minutes, followed by 35 cycles, 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes, and final extension at 72°C for 10 minutes. For the second round, following inner primers, FHBS2: 5'-CGTGGTGGACTTCTCTCAATTTTC-3' (nt. 257-280) and RHBS2: 5'-GCCARGAGAAACGGRCTGAGGCC-3' (nt. 650-673) (Sitnik et al., 2004) was used. For the second round, 5 μl PCR product of first round was added to PCR reaction mixture containing the same amount of components as described in the first round and subjected to thermal cycler. The thermal cycler was programmed as follows: initial denaturation at 94°C for 5 minutes, 30 cycles at 94°C for 1 minute, 44°C for 1 minute, 72°C for 1.5 minutes, and final extension at 72°C for 10 minutes. All reactions were performed in the presence of negative and positive controls.

Sequencing and Phylogenic Analyses

The positive PCR products were sent to Bioneer Company (Korea) and sequenced in two directions to determine HBV genotyping. The results of nucleotide sequencing of partial "S" region of isolated HBV were aligned using NCBI HBV database (<https://blast.ncbi.nlm.nih.gov>) to determine the HBV genotyping. To determine HBV subtype, the results of "a" determinant of "S" gene of isolates HBV from Ahvaz were aligned with the consensus amino acid sequence of the reference HBV D genotype using SnapGene software (3.2.1). The phylogenic tree was constructed for each isolated partial S region by Neighbor Joining method under the Kimura 2-parameter distance model by 1000 bootstrap replicates (Studier and Keppler, 1988). The MEGA software version 6 was employed to implement the methods.

Real time PCR

To determine seropositive Occult Hepatitis B Infection (OBI). The quantitative Real Time PCR was performed for those sera positive for anti-HBc Ab but negative

for HBsAg and HBV DNA by the nested PCR. The quantitative Real Time PCR test kit (Primerdesign Ltd, UK) was used according to manufacturer's instruction.

Ethical consent: The project was approved with ethical code IR.AJUMS.REC.1395.396 by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. All of the subjects included in the study were orally informed about the purpose of the study and invited to participate voluntarily. Written informed consent was obtained from all the patients.

Statistical analysis: Data analyses were performed using the Statistical Package for the Social Sciences 22.0 (SPSS Inc., Chicago, IL, USA). Chi-Square test was used to calculate. $P < 0.05$ was accepted as significant.

Results

Three of 92 (3.26%) patients including 2/3 males and 1/89 female were positive for HBsAg, anti-HBc Ab and HBV DNA by nested PCR ($p = 0.000003$). The sera of these 3 (3.26%) patients had upper limited level of ALT (43 ± 4.0) and AST (42 ± 8.0). The sera of 5/89 (5.61%) samples of 1 male and 4/88 females were only positive for anti-HBc but negative for HBs Ag, HBV DNA using nested PCR and Real time PCR ($p = 0.05$) (Table 1). The sera of these 5 (5.61%) patients had upper limited level of ALT (41 ± 43.0) and AST (40 ± 40).

The results of partial sequences of S region of three HBV- DNA positive cases isolates from Ahvaz were recorded in GenBank with registration numbers MK886716, MK886717, and MK886718. The results of nucleotide data and amino acid sequencing and alignment of all three partial HBV DNA isolates revealed that all three samples belonged to genotype D1. The alignment of partial sequences of all three isolated HBV (MK886716, MK886717, and MK886718) showed the presence of amino acids at positions of 122R, 127P, 140T, 159G, 160K belonging to ayw2 subtype.

Determination of HBV subtypes

The analyses of partial amino acid sequences of surface (S) gene region of three isolated HBV (MK886716, MK886717, MK886718) were implemented with consensus reference (BAM75990.1). The results showed all three isolated HBV comprised of amino acids at positions 122R + 127P + 140T, 145G, 159G + 160K. All three isolates HBV were recognized as HBV subtype ayw2.

Table 1. shows the Clinical and Immunological Criteria of SLE Patients

	%
Clinical criteria	
Acute cutaneous lupus erythematosus	94
Chronic cutaneous lupus erythematosus	33
Oral ulcers	9
Non-scarring alopecia	89
Synovitis (≥ 2 joints)	38
morning stiffness	34
Serositis	28
Renal involvement (24-hour urine protein, >0.5 g)	62
Renal biopsy (nephritis)	62
Neurological manifestation	8
Hemolytic anemia	11
Leukopenia ($<4,000/\mu\text{L}$)	49
lymphopenia ($<1,000/\mu\text{L}$)	52
Thrombocytopenia ($<100,000/\mu\text{L}$)	84
Immunological criteria	
Anti-nuclear antibody	100
Anti-dsDNA antibodies	67
Anti-Sm antibodies	8
Antiphospholipid antibodies	33
Low complement (C3)	78
Low complement (C4)	71
Direct Coombs test	12

Results of Phylogenic trees

The results of the phylogenetic tree demonstrated that all the three HBV DNA isolations in Ahvaz (MK886716, MK886717, and MK886718) were clustered with genotype D1 isolated from Turkey (AB674418.1) (Figure 1). The results of qPCR was negative for all the 5 positive anti-HBc Ab.

Discussion

The chronic Systemic lupus erythematosus (SLE) may result in organ dysfunction over time (Francis and Perl, 2010; Tsokos, 2011). The SLE patients with HBV markers are at risk of HBV reactivation, which may lead to high morbidity and mortality rates since SLE patients need corticosteroid and immunosuppressive drugs treatment during the course of the disease (Lin et al., 2018; Kuo



Figure 1. Shows the Amino Acid Position at 122R + 127P + 140T, 145G, 159G and 160K of Surface (S) Gene of the Three Isolated HBV Genome in Ahvaz City which Compared with Consensus Reference (BAM75990.1). All three isolates recognized as HBV subtype ayw2.

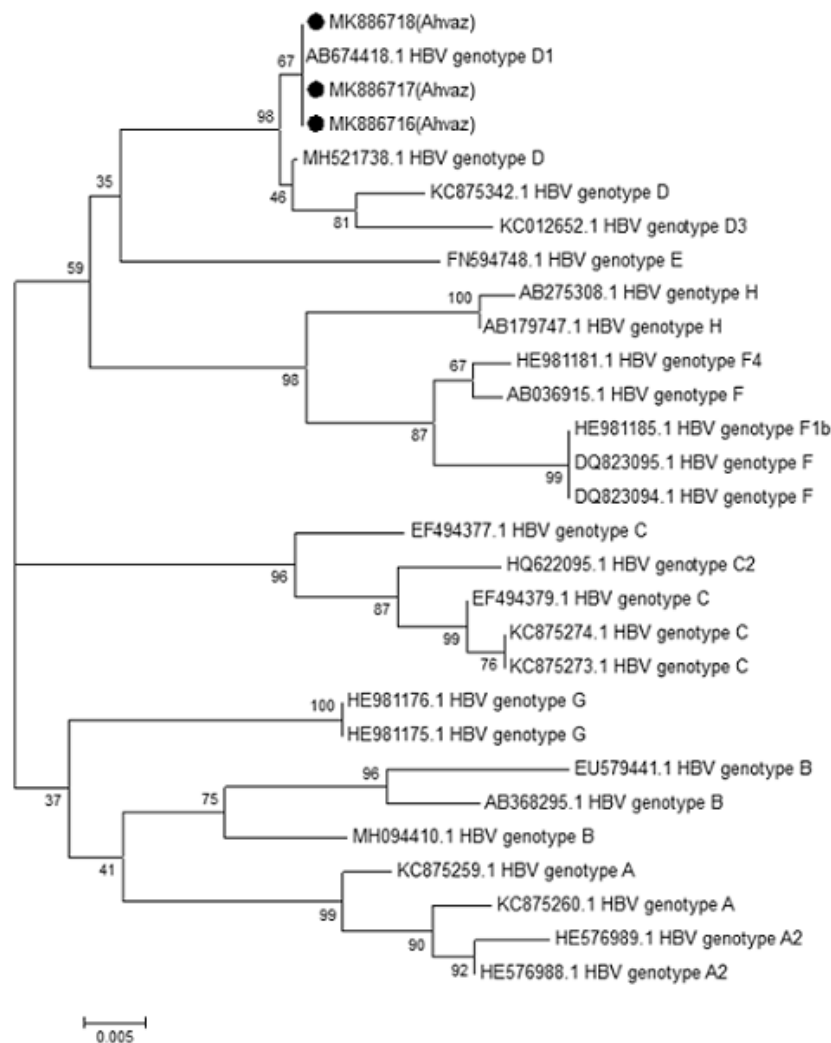


Figure 2. A Phylogenetic Tree was Constructed with Neighbor Joining Method for Partial Sequences of HBs Ag Gene Isolated from Ahvaz and Compared with 8 Different HBV Genotypes with Relevant accession Numbers Retrieved from GenBank Isolated from Different Regions of the World. The phylogenetic tree reveals all three HBV (MK886716, MK886717, and MK886718) isolates from Ahvaz city with black circles which are clustered with genotype D1 isolated from Turkey (AB674418.1). The accuracy of phylogenetic tree was assessed by 1000 bootstrap replicates. Scale bar=0.005

et al., 2020).

In our study, the low frequency of 3 (3.26%) samples including 2 males and one female were found positive for HBV markers, HBsAg, anti-HBc Ab and HBV DNA using nested PCR. Several studies have also reported low prevalence of HBV infection among SLE patients. Low prevalence of 2.7% HBV infection was observed in patients with SLE in China (Wang et al., 2017), 0.3% in Japan (Watanabe et al., 2014) and 1.5% (2/134) in Thailand (Sumethkul and Srivitidkul, 2017). Serological detection of “anti-HBc only” specifies exposure to HBV, lifelong persistence of cccDNA, and the risk for reactivation under strong immunosuppression (Gish et al., 2020). Presence of anti-HBc alone could be a surrogate marker for detection of OBI which indicates a significant clinical concern (Hourfar et al., 2009; Raimondo et al., 2008; Roman, 2018; Raimondo et al., 2019). The gold standard for OBI diagnosis is the detection of HBV DNA in the liver but a biopsy is not easily available (Raimondo et al., 2019). Thus, the detection of OBI is

very difficult due to very low virus load and mutations in HBV genome (Candotti et al., 2019; Spreafico et al., 2015). Nevertheless, recent reports on significance of anti-HBc detection show that it can play an alternative gold standard for OBI diagnosis. In agreement with OBI, it was found that donated samples may carry anti-HBc alone which are very infectious (Allain and Cox, 2011). Even individuals with certain PCR negative “anti-HBc alone” have been shown to be potentially infectious (Akcem and Demir., 2005). A recent study revealed that blood donors negative for both HBsAg and HBV DNA but positive for anti-HBc could be HBV carriers with viral loads below the detection limit (Oluyinka et al., 2005).

Thus, the Anti-HBc screening can potentially exclude the majority of OBIs which are undetectable by NAT (Taira et al., 2013; Vermeulen et al., 2014; Seed et al., 2015). The prevalence of OBI has been reported among the patients with SLE. In Our survey, 5/89 (5.61%) SLE patients including 1 male and 4/88 females were positive for anti-HBc but negative for HBs Ag, HBV DNA using

nested PCR and Real time PCR ($p=0.05$). It was reported that among 177 adults with active lupus nephritis, 68 cases were positive with OBI with undetectable HBV DNA (Fang et al., 2018). The results of phylogenetic analysis revealed that all three isolated HBV DNA (K886716, MK886717, and MK886718) were clustered with genotype D1 isolated from Turkey (AB674418.1). The alignment of partial sequences of all three isolated HBV (MK886716, MK886717, and MK886718) showed the presence of amino acids at positions of 122R, 127P, 140T, 159G, 160K and found to be of ayw2 subtype. (Rahman et al 2016). The predominant genotype D and HBV serotype ayw2 have been previously reported in Iran (Haghshenas et al., 2014). The reactivation of HBV in SLE patients is a great issue and can be life-threatening. The HBV reactivation has been investigated among the SLE receiving immunosuppressive therapy with consequences such as severe flare, decompensation and death (Lin et al., 2018). Recently, 3 antiviral therapies, entecavir, tenofovir disoproxil fumarate, and tenofovir alafenamide are recommended to be used in the prophylaxis or treatment of HBV reactivation and are recommended to be given concomitantly with anticancer therapy (Terrault et al., 2018; Huang et al., 2014)

SLE patients are generally under immunosuppression therapy and to halt HBV consequences such as reactivation, fulminant hepatitis, cirrhosis and HCC, preventive measures should be implemented. Therefore, the screening of HBV markers such as HBsAg, anti-HBc, AST/ALT, HBV DNA by nested-PCR or Real-time PCR should be performed for SLE patients prior to pretreatment and also during treatment periodically.

Author Contribution Statement

Study concept and design: Manoochehr Makvandi, Sedigheh Noormandi Pour, and Ali Teimoori; Analysis; Somayeh Shokriand interpretation of data: Shahab Mahmoudvand, Drafting of the manuscript: Somayeh Biparva Haghghi; Critical revision of the manuscript for important intellectual content: Elham Rajae Statistical analysis: Shahab Mahmoudvand. And Somayeh Shokri

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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