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No Detected Hepatitis B Virus-DNA in Thalassemic Patients Infected by Hepatitis C Virus in Kerman Province of Iran

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Abstract: This research was aimed to investigate the prevalence and clinical impact of occult HBV infection in thalassemic patients with chronic HCV infection. In this cross-sectional study we have totally examined 60 patients suffering HBV and HCV infections by PCR and RT-PCR methods, respectively, in Kerman province of Iran. ELISA technique (RADIM, Italy) was used to detect anti-HBc, anti-HBs and HBsAg. The serum level of liver enzymes (SGOT, SGPT, DB, TB and ALK) were analyzed in the HCV infected patients (MAN, IRAN). Statistical analyses performed using t-test and Chi-square. We found that 27 cases (out of 60) were infected by HCV but HBV-DNA was not seen in HCV infected patients. Present findings also showed that none of samples were HBsAg positive but 9 (33%) (out of 27) HCV-RNA positive patients were anti-HBc positive and 11 (40.7%) were positive for anti-HBs. We found that SGOT, SGPT, DB, TB and ALK are above normal in 27 (100%), 19 (70.3%), 12 (44.5%), 15 (55.5%) and 15 (55.5%) RNA-HCV positive patients, respectively. The prevalence of hepatitis C infection is very high in thalassemic patients and based on other studies our results showed that the prevalence of HCV infection in Kerman is more than other provinces of Iran. In contrast with other studies HBV-DNA in these patients could not be detected, hence, it seems that occult HBV infection isn't frequent in Iranian thalassemic patients who suffering from chronic HCV infection.

Key words: HBV-DNA, hepatitis C, hepatitis B and thalassemic patients

INTRODUCTION

Blood transmitted Hepatitis C (HCV) and B (HBV) infections are the most common causes of liver disease in thalassemia (Chakrabarti *et al*., 2006; Mirmomen *et al*., 2006). HBV infections in patients with undetectable HBsAg are called occult HBV infection (Jafarzadeh *et al*., 2008; Pourazar *et al*., 2005). It is possible that host immune mechanisms and viral interactions can maintain HBV infection in a latent state until more profound immunosuppression in these cases (Jafarzadeh *et al*., 2008). Occult HBV infection carries its own risks of disease transmission, acute exacerbations and development of hepatocellular carcinoma (Jafarzadeh *et al*., 2008; Pourazar *et al*., 2005; Hu, 2002). Although prevalence and clinical significance is unknown in chronic hepatitis C infected thalassemic patients, occult HBV infections are frequently reported in thalassemic patients with chronic hepatitis C liver disease (Carpenter *et al*., 2002; Kubo *et al*., 2001). Viral reactivation in patients undergoing immunosuppressive therapy is a well-known complication of occult HBV infection (Kubo *et al*., 2001). It is reported that there is a high prevalence of occult HBV infection in patients with chronic hepatitis C, HCC and hemodialysis patients, cryptogenic liver disease, drug injection users and HIV patients, multi-transfused individuals and blood donors (Hu, 2002; Marrero and Lok *et al*., 2004; Allain, 2004a, b; Toyoda *et al*., 2004; Oesterreicher *et al*., 1995; Jafarzadeh *et al*., 2008). Although it is well established that HCV infection prevalence is high in thalassemic patients (Mirmomen *et al*., 2006; Hu, 2002), the prevalence of occult HBV infection in thalassemic patients is yet to be identified. The aim of this study was to address epidemiological and clinical significance of occult HBV infection in multi-transfused thalassemic patients in Kerman province of Iran.

MATERIALS AND METHODS

This study was performed during June 2006 to March 2007 in the Department of hematology and Immunology, Rafsanjan University of Medical Sciences, Rafsanjan, Iran.

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Patients and informations: In this cross-sectional study a total of 60 thalassemic patients have been studied. The demographic data such as information sex, age and duration of blood and its components receiving was collected by questionnaire.

RNA extraction: 0.1 mL of samples were added to phenol and after vortexing, centrifuged for 5 min at 12000 rpm. Upper phase transferred to new tube and 0.2 mL chloroform was added and this was mixed vigorously for 15 sec. The sample was then incubated at room temperature for 5 min and centrifuged at 12000 g for 15 min at 4°C. The upper aqueous layer (containing RNA) was transferred to a fresh tube. 0.5 mL isopropanol was added to the isolated aqueous layer and this mixture was incubated at room temperature for 10 min. The RNA was precipitated by centrifugation at 12,000 g for 10 min at 4°C. The pellet was washed with 1 mL 75% (v/v) ethanol and centrifuged at 7500 g for 5 min at 4°C. The final pellet was air-dried for 10-15 min and was dissolved in 25-50 μL DEPC-treated water (volume added was dependent on the pellet size) at 60°C for 10 min.

Reverse Transcription Polymerase Chain Reaction (RT-PCR): To make complementary DNA (cDNA), reverse transcription reactions were performed using the following protocol: 4 μL 5x strand buffer [125 mM tris-HCl pH 8.3, 188 mM KCl, 7.5 mM $MgCl_2$ 25 mM DTT]; 1 μL of each dNTP [dATP, dCTP, dGTP, dTTP (stock concentration of 10 mM in DEPC-treated water)]; 4 μL oligo-dT (stock concentration of 125 μg mL^{-1}); 1 μL RNA (1 μg μL^{-1}); 4 μL DEPC–treated water; 1.5 μL M-MLV reverse transcriptase enzyme. After addition of M-MLV-reverse transcriptase and mixing, the tube was incubated for 1 h at 37°C. To amplify cDNA species, PCR reaction mixture was prepared by addition of the following reagents to a 0.2 mL micro-centrifuge tube in ice: 5 μL Taq polymerase buffer (10x); 1.5 μL $MgCl_2$ (stock concentration 1.5 mM); 1 μL of dNTPs [(dATP, dCTP, dGTP, dTTP) stock concentration of 10 mM]; 2 μL of each primer pair [(forward and reverse) stock concentration of 25 ng μL^{-1}]; 4 μL cDNA and sterile double distilled water to a final volume of 50 μL. Primers were design for polyprotein gene of HCV. The sequence of forward primer was 5'-ATCCCAGCTTCCGCTTAC-3' and the sequence of reverse primer was 5'-TGCAGTCCTGAACTGTC-3'. The PCR thermocycler was adjusted accordingly: 94°C for 5 min, 94°C for 40 sec, 40 sec at 63°C annealing temperature and 72°C for 45 sec. Denaturation, annealing and elongation procedures were repeated for 30 cycles. During the last 45 sec of first stage 1 mL Taq polymerase was added to the mixture. The presence of a 354 bp fragment indicated positive result. Positive samples, which were confirmed by ELISA and western blotting methods, were used as positive control and commercials negative control from Cinnagen Company was used as controls. All the HCV positive patients were followed up for at least 6 months before entering for this study.

Enzyme Linked Immunosorbent Assay (ELISA): HBsAg screening test were performed using HBsAg ELISA kit (RADIM, Italy). Anti-HBc and anti-HBs tests were also performed by a manual microplate enzyme immunoassay using anti-HBc and anti-HBs kits provided by RADIM (Italy).

DNA extraction: Viral DNA was purified from 100 μL of plasma samples. Briefly, each serum sample was incubated at 72°C for 10 min and then cooled at 4°C for 5 min in 100 μL proteinase K (200 μg mL^{-1}). After phenol/chloroform extraction (1:1), the viral DNA was precipitated with ethanol and the pellet was resolved in DNase free, deionized water and stored at -20°C for future use.

PCR and gel electrophoresis: PCR was carried out in a 50 μL mixture containing 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 5 units recombinant Taq DNA polymerase, 200 μM of each dNTPs, 0.6 μM of each primer and 5 μL of the DNA extracted from 100 μL of plasma. The sequence of forward primer was 5'-TCGTGGTGGACTTCTCTC-3' and the sequence of reverse primer was 5'-ACAGTGGGGGAAAGCCC-3'. These primers amplify a 500 bp of the gene S of HBV genome. A fast temperature cycling was performed. PCR amplification was done including one cycle of 93°C for 180 sec, 63°C for 30 sec and 72°C for 40 sec, then 35 cycles of 93°C for 40 sec, 63°C for 40 sec and 72°C for 40 sec. HBV genome provided by Cinnagen Company was used as positive control. For the analysis of PCR amplification, 10 μL of the amplified DNA were run on a 2% agarose gel after adding 4 μL loading dye. The presence of a 500 bp fragment indicated positive result. Ladder was also run in parallel with samples on the gels to estimate the molecular weights of DNA fragments in the gel. HBV genome provided by Cinnagen Company was used as positive control.

Liver enzyme evaluation: SGPT (Serum Glutamic-Pyruvic Transaminase), SGOT (Serum glutamic oxaloacetic Transaminase), direct and total Bilirubin (DB and TB respectively) and ALP (Alkaline phosphatase) from HCV chronic patients were done using MAN Ltd. kits, Iran.

RESULTS

Present results showed that HCV examination was positive in 27 (45%) patients and negative in 63 (55%) patients by RT-PCR method (Fig. 1). There were 16 male and 11 female subjects in the HCV positive group, with an average 14.5 years old and 10.13 in HCV negative patients. Therefore, the significant difference was seen in their age ($p<0.05$) (Table 1).

Present results also showed that 50% of males were HCV positive but 41% of women were HCV positive. These results don't show significant difference between two groups. The results from duration of receiving blood and their components are shown in Table 2. As it is clear there was a significant difference between two groups ($p<0.05$).

Table 1: Average age of HCV infected and none infected thalassemic patients

HCV	Plurality	Average age	SD	At least	Peak
Negative	33	10.13	5.18	1	23
Positive	27	14.50	6.07	2	25
Total	60	12.10	5.97	1	25

Table 2: Average of receiving blood of HCV infected and none infected thalassemic patients

HCV	Plurality	Average of receiving blood (month)	Mean±SD	At least (month)	Peak (month)
Negative	33	110.31	110.31±61/4	6	264
Positive	27	161.25	161.25±70	12	276
Total	60	133.27	133.27±69/312	6	276

Table 3: Average of serum level of liver enzymes of HCV infected thalassemic patients in a six month follow up study

Enzyme	Normal	Above normal
ALK	12 (44.5%)	15 (55.5%)
SGOT	0 (0.0%)	27 (100.0%)
SGPT	8 (29.7%)	19 (70.3%)
DB	15 (55.5%)	12 (44.5%)
TB	12 (44.5%)	15 (55.5%)

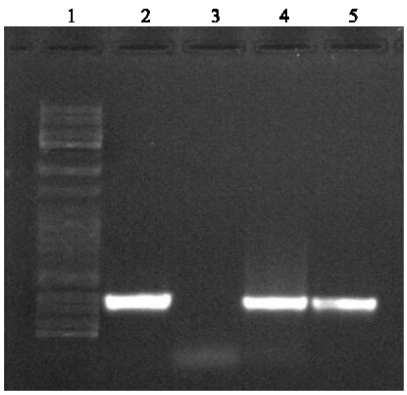

Fig. 1: The PCR amplification of HCV cDNA in thalassemic patients. Lane 1: 100 bp ladder. Lane 2: positive control. Lane 3: negative control. Lane 4 and 5 illustrated positive samples

Present results showed that none of samples were HBsAg positive but 9 (33%) out of 27 HCV-RNA positive were anti-HBc positive and 11 (40.7%) out of 27 patients were positive for anti-HBs. No HBV-DNA (0%) was detected through the PCR study in both patient groups with positive and negative HCV-RNA. Accordingly, there was no occult HBV infection in 27 patients in this study. Serum ALT, SGOT, SGPT, DB and TB levels of HCV infected patients in a 6 month following up study are shown in Table 3.

DISCUSSION

Occult HBV infection is defined as the presence of HBV infection with undetectable hepatitis B surface antigen (Jafarzadeh *et al*., 2008; Pourazar *et al*., 2005). Prevalence of occult HBV infection has been reported in several disease such as chronic hepatitis C, HCC and hemodialysis patients, in those with cryptogenic liver disease, drug users, HIV patients, blood and blood component receiving individuals (hemophilic patients, etc.) and blood donors (Hu, 2002; Marrero and Lok, 2004; Allain, 2004a, b; Toyoda *et al*., 2004; Oesterreicher *et al*., 1995; Jafarzadeh *et al*., 2008). The rate of co-infection of HBV and HCV in β-thalassemia patients is not identified yet. Some studies on Iranian thalassemic patients showed 19.3 to 55.9% of HCV infection but in Iranian patients HBV-DNA was not analysed yet (Kashef *et al*., 2008; Rezvan *et al*., 2007). In this study, HBV sequences were not found in all thalassemic patients with chronic HCV hepatitis. Among the 27 chronic HCV hepatitis, all of them were negative for HBsAg, 9 (33.3%) were anti-HBc positive and 11 (40.7%) were positive for anti-HBs. As they are transmitted in a similar manner, HBV and HCV co-infection are prevalent clinical signs. Anti-HBc prevalence which is reported in patients with chronic HCV infection is 81% (Matsuzaki *et al*., 1997) and 23% in Japanese and Italian respectively (Cacciola *et al*., 1999). These ratios are not a close value to present findings which is 33% (9/27). Therefore, this confirms present results and in the other hand probably shows that patients were less exposed to HBV than Japanese (which is an endemic area) and more contact than Italian HCV infected patients. A study from Zhihua at china showed that the prevalence of anti-HBc was higher among anti-HCV-positive individuals (Zhihua and Jinlin, 2006). Present results also are in agreement with their results because the rate of anti-HBc that was obtained by our previous study in blood donors is 5.18% in our province (Jafarzadeh *et al*., 2008). Most studies show that HBV-DNA genome existence is 22-87% (Fukuda *et al*., 1999; Cacciola *et al*., 1999) of patients with negative HBsAg and positive HCV-RNA. HBV-DNA is seen in 46% of anti-HBc positives and in 20% of anti-HBc negative (Cacciola *et al*., 1999). In contrast to the above

studies (Fukuda *et al*., 1999; Cacciola *et al*., 1999), there is no increased incidence of occult HBV infection in patients with thalassemia with chronic hepatitis C in our region while our results are comparable with Vedat *et al*. (2006) data which was performed in Turkey which failed to detect HBV-DNA in HCV infected patients. Therefore, present findings support Vedat *et al*. (2006) study that HBV infection in HCV infected patients in both regions (Turkey and Iran) is very low. It probably depends on several factors such as Intermediate prevalence of HBV in our region (High prevalence in South East Asia) (Mirmomen *et al*., 2006), safety of blood and its components and existence of HCV infection in young thalassemic patients. However, variable prevalence of occult HBV infection is seen in the permanence of HBV-DNA PCR (Vedat *et al*., 2006) and this makes us think that HBV viremia shows fluctuations during occult HBV infection. In conclusion, results showed that occult HBV infection is fluctuations among patients with HCV infected thalassemic patients. In the other hand, based on present findings it is suggested that due to the increased level of liver enzymes specially SGOT (100%) and SGPT (70.3%) in six months follow up of HCV positive thalassemic patients, the risk of shifting to liver malignancy is high in this group of patients. Therefore, we are going to monitor our patients in a longer period of time with complementary examinations to find if there are more complications such as cirrhosis and/or carcinomas.

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