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Prevalence of Occult Hepatitis C Virus Infection in Iranian Patients With Lymphoproliferative Disorders

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Occult HCV infection is a form of chronic HCV infection characterized by absence of detectable anti-HCV antibodies or plasma HCV-RNA but presence of HCV-RNA in liver biopsy and/or peripheral blood mononuclear cells (PBMCs). The aim of this study was to determine the presence of HCV-RNA in PBMCs of patients with lymphoproliferative disorders. One hundred and four consecutive patients with lymphoproliferative disorders admitted to Firouzgar Hospital from January 2010 to March 2011 were recruited in this cross-sectional study. A 6-ml sample of whole blood was taken from the patients, the total RNA was extracted from the samples after the separation of plasma and PBMCs. The HCV-RNA of the samples was amplified by reverse transcriptase-nested polymerase chain reaction (RT-nested PCR). The HCV genotypes of the positive samples were tested using the INNO-LiPA^{TM} HCV II kit, and the HCV genotypes were then confirmed by sequencing of the 5′-UTR fragments after the PCR products were cloned into a pJET1.2/blunt cloning vector. The mean age of the patients was 48.3 $\pm$ 1.76 years (range: 16–83). HCV-RNA was found in PBMCs from 2 (1.9%) of the 104 patients. Genotyping showed that the patients were infected with HCV subtype 1a. One patient suffered non-Hodgkin's lymphoma and the other suffered chronic lymphocytic leukemia. Patients with lymphoproliferative disorders with negative anti-HCV antibodies and negative plasma HCV-RNA may have occult HCV infection. Therefore, in the absence of a liver biopsy, the testing of PBMCs for the detection of genomic HCV-RNA may be beneficial. ***J. Med. Virol. 85:235–240, 2013.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: hepatitis C virus; occult HCV infection; peripheral blood mononuclear cells; lymphoproliferative disorders

INTRODUCTION

Oncogenesis is a process in which viral infections as well as environmental and genetic factors play a critical role [Pena et al., 2000]. In approximately 15% of human cancers, a role has been suggested for certain viruses, such as human papillomavirus (HPV), Epstein-Barr virus (EBV), and human T-cell leukemia/lymphoma virus (HTLV) [Ferri et al., 1997; Pena et al., 2000]. Several studies found numerous examples of the development of neoplastic diseases in patients with chronic hepatitis C and hepatitis B virus (HBV) infection, especially hepatocellular carcinoma (HCC) [Zignego et al., 1992; Ferri et al., 1993].

Hepatitis C virus is a single-strand RNA virus with positive polarity that belongs to the family Flaviviridae and genus hepacivirus [Zignego et al., 1992]. Hepatitis C virus infection affects more than 170–200 million people globally and its prevalence ranges from 0.2% to 40% in different countries [Brown and Gaglio, 2003; Alavian, 2008]. The hepatitis C virus is hepatotropic and lymphotropic (i.e., the virus replicates in peripheral blood mononuclear cells, PBMCs) [Zignego

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et al., 1992; Ferri et al., 1993; Bokharaei Salim et al., 2010]. The lymphotropism of this virus may explain the relationship between infection by HCV and some lymphoproliferative disorders, particularly mixed cryoglobulinemia and B-cell non-Hodgkin's lymphoma [Pena et al., 2000].

A new form of chronic HCV infection, occult HCV infection, has been described by Castillo et al. (2004). Occult HCV infection is characterized by the presence of HCV-RNA in liver biopsy specimens in patients without detectable anti-HCV antibodies or HCV-RNA in the serum. It has been reported that approximately 70% of patients with occult HCV infection have HCV-RNA in their PBMCs. Genomic and anti-genomic HCV-RNA strands have been detected in liver biopsy and PBMCs of patients with HCV infection. The presence of the HCV negative strand in these samples indicates that the virus is replicating in these cells [Quiroga et al., 2006; Laskus et al., 2007].

The most accurate and gold standard method for the identification of occult HCV infection is the detection of HCV-RNA in liver biopsy specimens, but when a liver biopsy sample is not available, testing for HCV-RNA in PBMCs is informative and beneficial for diagnosis [Bartolome et al., 2007; Barril et al., 2008]. The detection of occult HCV infection has not been investigated in patients with lymphoproliferative disorders. The purpose of this study was to determine the presence of occult HCV infection in patients with lymphoproliferative disorders.

MATERIAL AND METHODS

Study Patients

One hundred and four consecutive patients affected by lymphoproliferative disorders admitted to Firouzgar Hospital from January 2010 to March 2011 were enrolled in this cross-sectional study. Written informed consent was obtained from the patients and the study was approved by the local ethics committee of Gastrointestinal and Liver Disease Research Center (GILDRC) of Tehran University of Medical Sciences.

All of the patients were negative for anti-HCV antibodies, HCV-RNA, hepatitis B surface antigen (HBsAg), hepatitis B core antibody (anti-HBc), HBV-DNA, and anti-human immunodeficiency virus (HIV) antibodies.

Collection of Samples and Isolation of RNA From Plasma and Peripheral Blood Mononuclear Cells

A 6-ml peripheral blood sample was taken from the patients into a sterile EDTA-containing Vacutainer tube. After the plasma was isolated, the PBMCs were separated from the whole blood using a standard method by centrifugation over a density gradient (Lymphoprep, Oslo, Norway). The plasma was kept at -80°C, whereas the PBMCs were washed three times with phosphate-buffered saline (pH = 7.4 ± 0.2) and then stored in 300 μl of RNAlater (Ambion, Austin, TX) at -20°C until HCV-RNA testing. RNA was isolated from the plasma samples and the PBMC pellet using a high pure viral nucleic acid kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Detection of HCV-RNA Using RT-Nested PCR

Genomic HCV-RNA was amplified in the plasma and PBMCs by reverse transcriptase-nested polymerase chain reaction (RT-nested PCR). The cDNA synthesis and a two-step PCR with nested primers were performed as described previously [Kwok and Higuchi, 1989; Choo et al., 1991; Schroter et al., 2001; Schroter et al., 2003; Bokharaei-Salim et al., 2011].

HCV Genotyping by INNO-LiPA™ HCV II Kit, Cloning, and Sequencing

The total RNA was isolated from the plasma and PBMCs as described above. The 5′-untranslated region (5′-UTR) genotyping was performed with the INNO-LiPA™ HCV II kit (Innogenetics, Ghent, Belgium) according to the manufacturer's instructions. The HCV genotyping was also confirmed via sequencing of the 5′-UTR fragments after the PCR products were cloned into the pJET1.2/blunt cloning vector (Fermentas, St. Leon-Rot, Germany). Briefly, the 5′-UTR of HCV was amplified using RT-nested PCR as described previously [Bokharaei-Salim et al., 2011], but 3.8 U of *pfu* DNA polymerase was used rather than *Taq* DNA polymerase. The PCR products were purified using the high pure PCR product purification kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions and then cloned into the pJET1.2/blunt cloning vector. To sequence the PCR products, four clones from each specimen were sequenced, and then the retrieved sequences were aligned with the 5′-UTR sequences corresponding to the HCV reference sequences taken from the HCV database as described previously [Bokharaei-Salim et al., 2011].

Serology Tests

Anti-HCV antibody detection was performed according to the manufacturer's instructions using two different commercial enzyme-linked immunosorbant assay (ELISA) kits on the plasma samples: a third generation test (ACON Laboratories, San Diego, CA) and a fourth generation test (ETI-AB-HCVK4; Diasorin, Spain).

Statistical Analysis

The data were analyzed using SPSS version 17 (SPSS, Chicago, IL). Descriptive statistical indices such as the mean and standard deviation (SD), 95% confidence interval (CI) as well as the Student's *t*-test and Mann-Whitney U test were used. $P < 0.05$ was considered to be statistically significant.

TABLE I. Occult HCV Infection Among Patients With Lymphoprolifetative Disorders

Lymphoproliferative disorders	Patients no.	Mean age	Male/female	Positive	Negative	*P*-value
Non-Hodgkin's lymphoma	38 (36.5%)	49.2 ± 1.6	20/18	1 (50%)	37 (36.3%)	0.999+
Multiple myeloma	12 (11.5%)	58.5 ± 1.1	4/8	0 (0%)	12 (11.8%)	
Hodgkin's disease	21 (20.2%)	37.9 ± 1.6	12/9	0 (0%)	21 (20.6%)	
Chronic lymphocytic leukemia	21 (20.2%)	62.5 ± 1.2	16/5	1 (50%)	20 (19.6%)	
Acute lymphoblastic leukemia	12 (11.5%)	28.3 ± 8.6	11/1	0 (0%)	12 (11.8%)	
Total	104 (100%)	48.3 ± 1.8	63/41	2 (1.9%)	102 (98.1)	

RESULTS

One hundred and four participants without detectable anti-HCV antibodies and plasma HCV-RNA and with established lymphoproliferative disorders were enrolled in this cross sectional study. The non-Hodgkin's lymphoma, multiple myeloma, Hodgkin's disease, chronic lymphocytic leukemia, and acute lymphoblastic leukemia patient groups consisted of 38 (36.5%), 12 (11.5%), 21 (20.0%), 21 (20.0%), and 12 (11.5%) cases, respectively (Table I). The mean age of the patients was 48.3 ± 1.76 years (range: 16–83). Among the 104 patients, 41 (39.4%) were male. All of the patients were negative for anti-HCV antibodies (checked with two different ELISA commercial kits) and HCV-RNA (tested with RT-nested PCR). The complete data for these participants have been summarized in Table II.

HCV-RNA was detected in PBMCs from 2 (1.9%) of the 104 patients with lymphoproliferative disorders using RT-nested PCR. One patient suffered from non-Hodgkin's lymphoma and the other suffered from chronic lymphocytic leukemia. Patients with positive HCV-RNA results in the PBMCs underwent two extra peripheral blood samplings every 2 months. Genomic HCV-RNA strands were detected in the plasma and PBMCs again by an RT-nested PCR assay that confirmed the previous positive results. The genotyping analysis of the HCV-RNA was performed using the INNO-LiPA^{TM} HCV II kit in the positive PBMCs and showed that 2 (1.9%) patients were infected with HCV subtype 1a (Fig. 1). The HCV genotyping results of the patients with occult HCV infection were confirmed via nucleotide sequencing of the HCV 5′-UTR.

DISSCUSION

Hepatitis C virus is a hepatotropic virus that can also replicate in lymphocytes. The lymphotropism of this virus may explain the association between HCV infection and certain lymphoproliferative disorders; for example, the infection of T and B lymphocytes with HCV is likely responsible for extrahepatic disorders that are often observed in patients chronically infected with HCV [Zignego et al., 1992; Ferri et al., 1993]. Occult HCV infection is a type of chronic infection that has been identified in a group of patients with chronic liver disease of unknown etiology. This infection is characterized by the presence of HCV-RNA in liver biopsy specimens of patients who were negative for anti-HCV antibodies and plasma HCV-RNA.

In the current study, 2 (1.9%) of 104 patients who had lymphoproliferative disorders had occult HCV infection as demonstrated by RT-nested PCR detection of the 5′-UTR of HCV. The genotyping of genomic HCV-RNA isolated from the PBMCs revealed that two patients had HCV genotype 1a. Sequence analysis of

TABLE II. Epidemiological and Laboratory Data of the Patients With Lymphoprolifetative Disorders

Parameters	Total	Positive	Negative	*P*-value
No. of patients	104	2	102	
Age (year)	48.3 ± 17.6	48.0 ± 17.5	54.0 ± 33.9	0.674[b]
Sex male/female	41:63	1:1	40:62	0.999[a]
Body mass index (kg/m^2)	22.3 ± 3.8	20.0 ± 0.3	22.4 ± 3.8	0.335[b]
Laboratory test:				
White blood cell	13,540 ± 24.2	19,900 ± 15.1	13,410 ± 24.4	0.182[b]
Red blood cell	4.09 ± 1.42 × 10^6	4.16 ± 0.05 × 10^6	4.07 ± 1.44 × 10^6	0.762[b]
Hemoglobin (g/dL)	11.0 ± 2.75	11.9 ± 0.07	11.0 ± 2.78	0.553[b]
Platelet	215.7 ± 161.9 × 10^3	245.5 ± 96.9 × 10^3	215.2 ± 163.2 × 10^3	0.713[b]
Alkaline phosphatase (IU/L)	220.3 ± 197.9	145.7 ± 3.83	221.8 ± 199.6	0.830[b]
Alanine aminotransferase (IU/L)	29.5 ± 42.8	26.0 ± 19.8	29.6 ± 43.2	0.776[b]
Aspartate transaminase (IU/L)	27.1 ± 22.9	19.8 ± 3.9	27.3 ± 23.1	0.773[b]
Epidemiological features				
History of transfusion	79	2	77	0.056[a]
History of endoscopy	25	1	24	0.425[a]
History of jaundice	0	8	8	0.999[a]

[a]*P*-value base on Fisher Exact test.
[b]*P*-value base on Mann-Whitney U test.

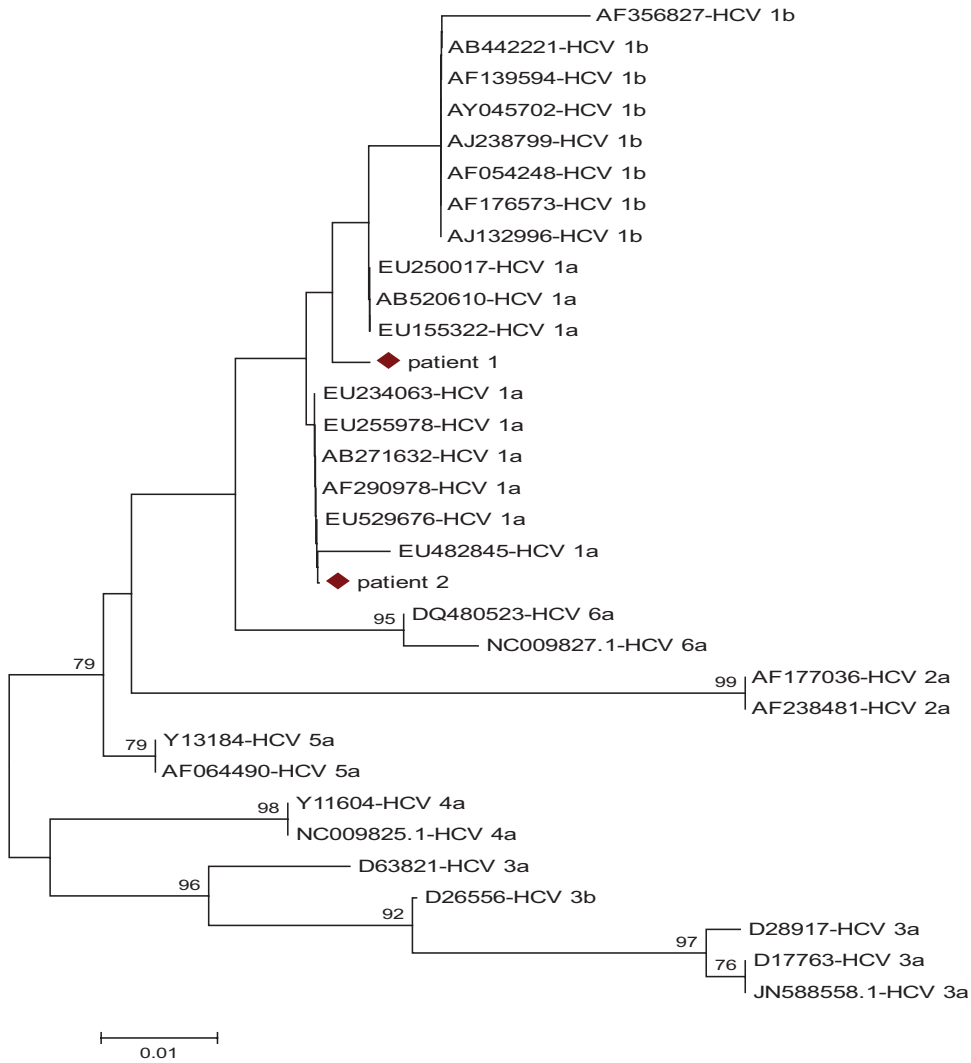

Fig. 1. A neighbor-joining tree constructed with HCV 5′-untranslated region (5′-UTR) nucleotide sequences of the clones obtained from the two patients with occult HCV infection and those corresponding to various HCV genotypes. Bootstrap values ≥70 obtained after 1,000 replicates of the data sheet, are shown in the nodes of the tree.

the 5′-UTR confirmed that the HCV isolates belonged to genotype 1a.

The prevalence of HCV infection in the general population is <0.5 in Iran (1.0 in men and 0.1 in women) [Merat et al., 2010]. In the present study, the prevalence of occult HCV infection (1.9%) in patients with lymphoproliferative disorders was approximately 4 times the rate in the general population. In a 2011 study on patients with chronic liver disease of unknown etiology, the prevalence of occult HCV infection was approximately 10.1% [Bokharaei-Salim et al., 2011]. Although the detection of HCV-RNA in a liver biopsy specimen is the gold standard for the diagnosis of occult HCV infection, testing for genomic and antigenomic HCV-RNA strands in PBMCs is an alternative procedure that can be performed when the liver biopsy specimen is not available [Barril et al., 2008; Thongsawat et al., 2008]. In previous reports, HCV-RNA was detected in the PBMCs of 70% of patients with occult HCV infection [Castillo et al., 2004]. Thus, a negative result for genomic HCV-RNA strand detection in PBMCs does not rule out the presence of hepatitis C infection in hepatocytes. Therefore, it is possible that the prevalence of occult HCV infection in patients with lymphoproliferative disorders was actually higher than that reported in this study.

Several reports have indicated a correlation between chronic HCV infection and the development of lymphoproliferative disorders; for example, the prevalence of lymphoproliferative disorders in individuals with chronic HCV infection has been found to be 4.3% [Idilman et al., 2004] and 11.4% [Paydas et al., 1999]. Therefore, the possibility of occult HCV infection

should be considered in patients suffering lymphoproliferative disorders.

Hepatitis C virus is a blood-borne pathogen that can be transmitted through exposure to infected blood products or through needle sharing among drug abusers [Alavian et al., 2005]. Our patients with occult HCV infection may have acquired the infection from blood transfusions because despite noteworthy progress in blood screening, the risk of infection via transfusion still exists [De Marco et al., 2009]. It should be noted that the transfusion history of the participants did not significantly ($P = 0.056$) affect the frequency of positive genomic HCV-RNA in the PBMCs. Some reports have shown a correlation between blood transfusion and occult HCV infection [Castillo et al., 2004; Thongsawat et al., 2008; De Marco et al., 2009; Bokharaei-Salim et al., 2011], therefore, it appears that a study with a large study population is needed to clarify this issue.

Some studies have shown an association between HCV viral load reduction and anti-viral therapies for low-grade B-cell non-Hodgkin lymphoma and mixed cryoglobulinemia [Zignego et al., 2007; Martyak et al., 2009]. However, more studies are needed to determine the effectiveness of antiviral therapy in these patients. Patients with occult HCV infection are known to have a low level and very low level of genomic HCV-RNA in the PBMCs and plasma, respectively, such that the RNA can only be detected using sensitive and reliable RT-PCR methods [Bartolome et al., 2007; Bokharaei-Salim et al., 2011; Castillo et al., 2011]. Pardo et al. [2006] treated patients with occult HCV infection with pegylated-interferon (PEG-IFN) plus ribavirin treatment for 24 weeks and then followed them for 24 weeks after treatment. The virological, histological, and biochemical response to the therapy was reported to be effective [Pardo et al., 2006]. Therefore, it is possible that the treatment of patients with occult HCV infection using antiviral therapies can provide an effective clinical response for lymphoproliferative disorders with suppression of HCV. A study with a large population is needed to determine the influence of antiviral therapy in these patients.

In conclusion, the current study shows that 1.9% of patients attending the Firouzar Hospital who suffer lymphoproliferative disorders have occult HCV infection. Therefore, the possibility of this infection should be considered in patients with lymphoproliferative disorders.

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