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BRIEF ARTICLE

Distribution of different hepatitis C virus genotypes in patients with hepatitis C virus infection

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Abstract

AIM: To investigate the presence of mixed infection and discrepancy between hepatitis C virus (HCV) genotypes in plasma, peripheral blood mononuclear cells (PBMCs), and liver biopsy specimens.

METHODS: From September 2008 up to April 2009, 133 patients with chronic hepatitis C referred to Firouzgar Hospital for initiation of an antiviral therapy were recruited in the study. Five milliliters of peripheral blood was collected from each patient and liver biopsy was performed in those who gave consent or had indications. HCV genotyping was done using INNO-LiPA™ HCV Ⅱ in serum, PBMCs, and liver biopsy specimens and then confirmed by sequencing of 5′-UTR fragments.

RESULTS: The mean age of patients was 30.3 ± 17.1 years. Multiple transfusion was seen in 124 (93.2%) of patients. Multiple HCV genotypes were found in 3 (2.3%) of 133 plasma samples, 9 (6.8%) of 133 PBMC samples, and 8 (18.2%) of 44 liver biopsy specimens. It is notable that the different genotypes found in PBMCs were not the same as those found in plasma and liver biopsy specimens.

CONCLUSION: Our study shows that a significant proportion of patients with chronic hepatitis C are affected by multiple HCV genotypes which may not be detectable only in serum of patients.

Key words: Chronic hepatitis C virus infection; Mixed hepatitis C virus infection; Peripheral blood mononuclear cells; Hepatocyte

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus of the family Flaviviridae and genus Hepacivirus that causes both acute and chronic hepatitis[1-3]. HCV is a major health problem affecting 170 million people worldwide[4]. It is estimated that chronic HCV infection is responsible for approximately 250000 to 350000 deaths per year, mainly related to decompensation of cirrhosis, end-stage liver disease, and hepatocellular carcinoma[5].

Phylogenetic analysis of HCV sequences resulted in a nomenclature that recognizes distinct virus types and subtypes[6]. Six large groups of viral genotypes[7-12], in addition to over 70 different subtypes (termed a, b, c, *etc.*) are distributed worldwide[6,13]. The HCV genotypes should be systematically determined before treatment, as it determines the indication, the duration of treatment, the dose of ribavirin and the virological monitoring procedure[14].

The HCV is considered as essentially hepatotropic[15], but virus sequences have also been found in other important extrahepatic sites, including peripheral blood mononuclear cells (PBMCs), the central nervous system, and bone marrow from chronically-infected patients[16-19].

HCV replicates through a negative-strand intermediary. Although hepatocytes are the primary sites for HCV replication, there is evidence of negative-strand HCV RNA in PBMCs. The HCV genomic sequences present in PBMCs have been found to differ from those found in serum and the liver biopsy specimens[20-24]. Detection of HCV RNA in extrahepatic reservoirs has important implications for transmission, disease progression, and effective treatment[25]. Furthermore, the PBMC compartment may be a privileged site for HCV, which is able to reinitiate viral replication after termination of HCV treatment when conditions again become more favorable. Re-infection of HCV after orthotropic liver transplantation has postulated that extrahepatic sites are suitable for HCV replication[26]. Thus, even if clearance of HCV from hepatocytes is achieved by treatment, re-infection from extrahepatic sites, such as the PBMC compartment, may occur[27].

The purpose of the present study is to determine the presence and frequency of different HCV genotypes in plasma, PBMC, and liver biopsy specimens of chronically infected patients.

MATERIALS AND METHODS

Patients

In this cross-sectional study, 133 consecutive patients with established chronic hepatitis C referred to Firouzgar Hospital from September 2008 to July 2009 were enrolled. Informed consents were obtained from the patients, and the study was approved by the local ethics committee of GI and Liver Disease Research centre (GILDRC) of Iran University of Medical Sciences.

Inclusion criteria were positive anti-HCV antibodies along with positive plasma HCV RNA. Also none of the included patients had been treated for HCV previously.

Collection and preparation of samples

About 5 mL of peripheral blood were collected from each patient into EDTA-containing vacutainer tubes. Plasma was stored at -70℃ until analysis. PBMCs were isolated from EDTA-treated blood by centrifugation over density gradient (Lymphoprep, Oslo, Norway). PBMCs were then washed three times with phosphate-buffered saline (pH = 7.4), counted and stored at -70℃ for later detection. Some of the patients who gave consent underwent a liver biopsy for diagnostic purpose. Liver biopsy specimens were divided into 2 portions: one used for histological diagnosis, and the second was submerged into RNALater (Ambion Inc., Austin, TX) and stored at -70℃ for HCV genotyping. Samples of serum and PBMCs were collected from all patients on the same day the liver biopsy was performed.

Isolation of RNA from serum, PBMCs, and liver

RNA was extracted from 140 μL of plasma by using the QIAamp Viral RNA Extraction kit (Qiagen GmbH, Hilden, Germany), from a pellet of approximately 3-5 $\times 10^6$ PBMCs using the RNA Virus Mini Extraction Kit (Invitek GmbH, Germany), and from approximately 2 mm^3 of liver biopsy specimens using the DNA/RNA Virus Mini Extraction Kit (Invitek GmbH, Germany), according to the manufacturer's instructions.

HCV genotyping

RNA was isolated from plasma, PBMC, and liver biopsy specimens as described above. 5'-untranslated region (5'-UTR) genotyping was done using a standard methodology with the INNO-LiPA™ HCV Ⅱ kit (Innogenetics, Ghent, Belgium) according to the manufacturer's instructions. Briefly, the 5'-UTR is amplified with biotinylated primers. Biotin-labeled PCR products are reverse hybridized to specific probes attached to nitrocellulose strips and then incubated with a chromogen. Developing results in a purple precipitate that forms a line on the strip. The HCV type is deduced on the basis of the patterns of hybridizing bands, using the line probe assay interpretation chart[28].

INNO-LiPA™ HCV Ⅱ genotyping has also been confirmed by sequencing of 5'-UTR fragments. The HCV 5'-UTR was amplified from total RNA isolated from plasma, PBMC, and liver biopsy specimens of 10 randomly selected patients by reverse transcriptase-PCR as described previously[29]. The 211-bp 5'-UTR PCR products were sequenced by dye termination method using the ABI 3130 XL sequencer. The sequencing results were compared with other sequences posited in gene bank by Blast software.

Statistical analysis

Data analysis was done by SPSS software version 16.0, using descriptive statistical indexes such as mean and standard deviation (SD), 95% confidence interval (CI) and *t* test. *P* value less than 0.05 was considered statistically significant.

RESULTS

One hundred and thirty three patients with established chronic hepatitis C were recruited in this study. Multiple blood transfusion was the most important related risk factor found in 124 (93.2%) of patients, 87 (65.4%) with thalassemia and 37 (27.8%) with hemophilia. The source of infection was unknown in 9 (6.8%) patients. The mean age of patients was 30.3 ± 17.1 years. Out of 133 patients, 100 (75.2%) were male. The mean viral load of all of the patients was $6.3 \times 10^5 \pm 7.8 \times 10^5$ (range from 45000 to 5.59×10^6). Out of 133 patients, 44 patients gave consent to undergo liver biopsy. Genotype 1a was the most fre-

Table 1 Distribution of different HCV genotypes in plasma, PBMCs, and liver biopsy specimens

No.	Gender/age (yr)	Viral load	Disease	HCV genotypes in plasma	HCV genotypes in PBMCs	HCV genotypes in liver biopsy specimen
1	F/15	76500	Thalassemia	3	3/1a	3
2	F/36	138000	Thalassemia	1a	3	1a
3	M/17	597000	Hemophilia	1a/3	1a	
4	M/23	989000	Thalassemia	1a/1b	1b	1a/1b/3
5	M/23	226000	Thalassemia	1a/1b	1b	1a/1b
6	M/33	450	Thalassemia	1a	3	
7	M/26	250000	Thalassemia	3	1a	1a/3
8	M/17	106000	Thalassemia	1a	1a/3	
9	M/25	780000	Thalassemia	1a	1a	1a/3
10	F/30	10800	Thalassemia	1b	1a/1b	1b/3
11	F/27	750000	Thalassemia	3	3	3/1b
12	M/14	5590000	Thalassemia	3	3	1a/3
13	M/28	1190000	Thalassemia	1a	1a/3	1a
14	M/19	1150000	Hemophilia	1a	1a/3	
15	M/20	890000	Thalassemia	1a	1a/1b	
16	F/22	618000	Thalassemia	3	3	3/2a
17	M/27	167000	Thalassemia	3	3/2a	
18	M/46	230000	Hemophilia	3	3/2a	
19	M/27	273000	Hemophilia	3	3/2a	
20	M/57	480000	Unknown	4	3	

HCV: Hepatitis C virus; PBMCs: Peripheral blood mononuclear cells.

Table 2 Frequency of HCV genotypes in plasma, PBMCs, and liver biopsy specimens *n* (%)

HCV genotypes	HCV genotypes in plasma	HCV genotypes in PBMCs	HCV genotypes in liver biopsy specimen
1a	85 (63.9)	81 (61.0)	24 (54.5)
1b	8 (6.0)	10 (7.5)	3 (6.8)
2	0 (0.0)	0 (0.0)	0 (0.0)
3	35 (26.2)	33 (24.8)	9 (20.0)
4	2 (2.3)	1 (0.8)	0 (0.0)
Mixed infection	3 (2.3)	9 (6.8)	8 (18.2)
Total	133	133	44

quent genotype in serum (63.9%), PBMCs (61.0%), and in liver biopsy specimens (54.5%). HCV genotypes in serum, PBMCs, and liver biopsy specimens of all patients were similar except for 20 patients (15%).

The complete data of these patients have been summarized in Table 1. As shown in Table 2, more than one HCV genotype has been found in some patients, as 3 (2.3%) patients had different HCV genotypes in their serum samples. In 9 (6.8%) patients, different HCV genotypes were found in PBMCs. In 8 (18.2%) patients (who were not exactly same as the 9 patients with different HCV genotypes in PBMCs) multiple HCV genotypes were found in liver biopsy specimens. The INNO-LiPA™ HCV Ⅱ genotyping was confirmed with sequencing of the 5'-UTR. A 100% correlation was demonstrated between INNO-LiPA™ HCV Ⅱ genotyping and sequencing of the 5'-UTR.

DISCUSSION

The present study was performed on 133 chronically HCV infected patients to evaluate the prevalence of potential mixed HCV infection in their plasma, PBMCs, and liver biopsy specimens. In 15% of these patients different HCV genotypes were found in these compartments. Multiple HCV genotypes were detected in 3 (2.3%) of 133 plasma, 9 (6.8%) of 133 PBMC, and 8 (18.2%) of 44 liver biopsy specimens.

Mixed infection is infection of an individual with two or more distinct HCV genotypes. Mixed viral infection is of great clinical importance as it may result in more severe disease, unresponsiveness to antiviral therapy or recurrence after the completion of antiviral therapy course[30].

In our study, various HCV genotypes presented in PBMCs were different from those found in plasma or liver biopsy specimens (Table 1). It means that PBMCs may present some divergent types, which are not detectable in liver biopsy specimens. It reinforces the previous proposed theories in which PBMCs have been known as an extrahepatic replication site for HCV[17,25,26,31-34].

It is suggested that infection with one HCV type doesn't make a barrier to acquisition of other genotypes, therefore multiple exposures to HCV especially in potential risk groups, might lead to several episodes of re-infection and to the establishment of mixed infection in some patients. It is also well known that super infection with a new HCV strain leads to suppression of one virus under the detection limit of PCR while the other one prevails as under antiviral therapy, the displaced strain may become viremic again and may alter the outcome of therapy[30,35-39].

In our study the prevalence of mixed HCV infection was estimated about 2.3% in plasma, 6.8% in PBMC, and 18.2% in liver biopsy specimens of chronically infected patients with HCV. Mixed infection with two HCV genotypes have been detected in 1% of HCV-positive patients,

using type-specific primers[40,41]. Also figures of 1.6% to 31% have been reported in multi-transfused hemophiliacs[42,43].

Our study demonstrated that a significant proportion of HCV infected patients have divergent HCV genotypes in their PBMCs and liver biopsy specimens which were not detectable in their plasma.

In the present study we used INNO-LiPA™ HCV Ⅱ genotyping which is currently the most applied method and has more sensitivity than RFLP. One of the problems of INNO-LiPA™ HCV Ⅱ is that it may underestimate the actual rate of mixed infection[9,30,44]. According to this, the true prevalence of mixed infection may be higher than estimated in this study.

The second shortcoming is that only 44 of our patients had indication or gave consent to undergo liver biopsy, however, 18.2% of them had multiple HCV genotypes which is a significant proportion of the total. It indicates that hepatocytes are the main reservoirs for HCV. On the other hand since performance of liver biopsy is not possible for all patients, we suggest the assessment of PBMCs as another HCV reservoir for detection of HCV mixed infection[26,34,45].

In conclusion, our study shows that patients with hemophilia and thalassemia are from the most high risk groups in whom mixed infection is relatively common. On the other hand failure to treatment and relapse of infection is also frequent in these groups. So it seems that considering the plasma genotype as the target genotype for scheduling of an anti HCV therapy may be one of the factors that leads to the failure of treatment. HCV genotyping in PBMCs or liver biopsy specimens might be beneficial.

COMMENTS

Background

Relapse and failure of antiviral therapy have been frequently seen in patients with chronic hepatitis C especially in those who are on multiple blood transfusions.

Research frontiers

It has been suggested that infection with multiple hepatitis C virus (HCV) genotypes in patients affected by chronic HCV infection might result in frequent relapse after antiviral therapy.

Innovations and breakthroughs

The authors found that a significant proportion of patients with chronic HCV infection, especially who are on chronic blood transfusion, might present more than one HCV genotype in their serum, hepatocytes and peripheral blood mononuclear cells (PBMCs). It is notable that detected HCV genotypes in serum may be different from those found in PBMCS or hepatocytes.

Applications

According to the above findings the authors suggest HCV genotyping in hepatocytes and PBMCs along with serum of patients. This may result in choosing a more appropriate antiviral therapy in these patients.

Peer review

This is a well written manuscript and it addresses a relevant topic of multiple HCV infections/superinfection/humoral protection and others. Moreover, the material itself is of some value.

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