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Original Article

Comparison of HCV Plus-and Minus-Strand RNA in PBMCs of Responders and non-Responders of Chronically Infected Patients Receiving Ribavirin and Interferon Therapy

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Abstract

Background and Aims: Hepatitis C virus (HCV) can cause hepatocellular carcinoma (HCC) in a significant proportion (≈ 20 %) of individuals with chronic HCV infection (CHC). Currently, CHC is treated with peginterferon and ribavirin, which depending on genotype approximately 50 to 70% of patients are cured. The so-called "extrahepatic HCV infection" or viral replication in regions of the body other than liver, e.g. peripheral mononuclear cells (PBMCs), is likely to contribute to the lack of response to treatment in non-responders. In this study, HCV infection of PBMCs was compared between responders- and non-responders HCV-infected patients.

Materials and Methods: RT-nested PCR was utilized to detect the plus- and minus- strands of HCV RNA in PBMCs using type-specific primers specific for the HCV core region.

Results: Both the plus- and the minus strands of HCV genome were significantly lower in PBMCs of responder- than non-responder patients.

Conclusion: The presence of both plus and minus HCV strands in PBMCs was associated with the treatment outcome, such that HCV infection of PBMCs was identified in higher proportion of non-responders relative to responders.

Keywords: Responder Groups; (End of treatment) ETR; Ribavirin; Interferon; Hepatitis C Virus

Introduction

HCV is classified within the hepacivirusgenus in theFlaviviridae family. It is responsible for approximately 90% of bloodborne non-A, non-B hepatitis (1). HCV is considered as one of the most common viral infectious agents in humans and the second factor in human hepatitis (2-5). It possesses a positive-sense single-stranded RNA (+ssRNA) genome with approximately 9.6 kb in length (2). The +ssRNA genome contains a single large open reading frame (ORF), which encodes for a polyprotein that is cleaved by the viral and host cell proteases during and after translation,

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producing the structural and non-structural viral proteins (1, 6, 7). The HCV genome was initially isolated from the blood of HCV infected patients and classified by Chooet al, in 1989 (8).

Hepatitis C vius infection is a major global disease (9), with approximately 170 million people are currently living with the HCV infection worldwide, from which approximately 70-80 % are suffering from the chronic form of disease which may ultimately lead to severe consequences such as cirrhosis and HCC (10-12). HCV prevalence varies considerably by country and region. For instance, according to the estimates by WHO, the rate of HCV infections in developed countries such as the US, Australia, and most countiers in Western Europe is <2%, as compared to a frequency of >3% in many countries in Eastern Europe, Latin America, the Middle East, Africa and South Asia. Egypt with >10% HCV infections continues to be the most heavily affected country in the world. The use of shared needles during the treatment of Schistosoma in this country is believed to be the cause of such high prevalence (13).

HCV is divided into six distinct genotypes (1 to 6) and >52 subtypes, with genotype 1 being the most difficult to treat. The overall distribution of genotypes in the world is different such that genotype 1 is the most common HCV genotype in the US (75%) and Europe, whereas genotype 3 is predominant in Asia. Genotype 4 and 6 are the most prevalent genotypes in the Middle East and Hong Kong, respectively. According to the previous reports, the rate of HCV infection in Iran has been estimated about 1%, with genotype 1 dominating the infections (14, 15).

Acute HCV infection is often asymptomatic (2, 16). Studies have shown that approximately 55 to 85% of patients with acute infection will develop CHC. Five to twenty percent of these individuals will experience cirrhosis and HCC after 20 to 25 years of infection (17). HCV is cleared spontaneously from plasma in about15-45% of patients during the first year of infection (16, 18).

HCV infection has been recently associated with several extrahepatic diseases such as mixed Cryoglobulinamia Vasculitis (MC), Membranous Glomerulonephritis, Porphyria Cutaneatarda and non-Hodgkin's Lymphoma (19). These observations therefore reinforce the hypothesis that virus infections occur in places of body other than liver (20). Extrahepatic HCV infections may lead to failure of therapy. PBMCs are proposed as one of the extrahepatic sites of HCV infection. Therefore, in this study the presence of HCV RNA in PBMCs were compared in responder- and non-responder patients to determine the role of PBMCs infection in therapy outcome.

Methods

The study population

The study subjects included 49 CHC patients with genotypes 1a and 1b, who were admitted to the Digestive Disease Research Center (DDRC) at Shariati Hospital in Tehran, and treated with PEG-INF and ribavirin. From these, 38 (77.5%) patients were men and 11 (22.5 %) patients were women. The patients were aged between 26 and 63 years old. While 34 patients (69%) responded to the therapy, 15 patients (31%) did not. The study subjects had no underlying diseases.

Isolation of PBMCs of blood

Ten milliliters blood was collected from each patient in EDTA tube. The blood samples were transported to the laboratory and PBMCs were isolated using Ficoll as described previously (1). Briefly, samples were diluted with an equal volume of normal saline. The same volumes of diluted blood and Ficoll were then added to a 15 ml Falcon tube. Samples were centrifuged for 30 minutes at 650g at room temperature, and then the plasma supernatant was discarded. The lymphocyte layer (milky color layer) was removed and immersed in 10 ml cold 1X PBS solution containing 1% fetal bovine serum (FBS) and 0.2 mM EDTA. Samples were centrifuged at 450g for 10 min at 4°C. The supernatant was discarded and the PBMSc were washed once in washing solution. The PBMCs were then resuspended into 1 ml of 1X PBS and were divided into 200

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Table 1. The sequence of specific primers and internal control.

Primers used in the first round of PCR	External Reverse	(730)5'-AAG CCG CAC GTA AGG GTA TCG-3'(710)
	External Forward	(324)5'-TCT CGT AGA CCG TGC ACC ATG AGC3'(347)
	Product length	386bp
Primers used in the second round of PCR	Internal Reverse	(643)5'-CGG GGA GAC AGG AGC CA-3'(627)
	Internal Forward	(423)5'-GGT CAG ATC GTT GGT GGA GTT TAC-3'(446)
	Product length	204bp
Sequence of GAPDH primers		
	Reverse	5-CCTGCTTCACCACCTTCTTGAT-3
	Forward	5-ACCTGACCTGCCGTCTAGAAA-3
	Product length	63bp

microliter aliquots and stored at -70°C for later use (18, 21, 22).

Specific Primer design

RT-nested-PCR method was used to identify HCV RNA in PBMCs. Primers were designed for the Core HCV according to the HCV sequences obtained from the NCBI and those identified in Iran, using Oligo7, Mega4, NCBI Blast and Oligo analyzer 3.1 softwar*es* (23).The designed primers were synthesized by AnaSpec (Table 1).

Internal control primer

The successful DNA extraction was verified by amplifying GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene (Table 1).

RNA extraction

RNA was extracted from PBMCs using the High Pure RNA Isolation Kit (Roche) according to the manufacture's protocol.

cDNA synthesis

The RT-PCR reaction was carried out in a total volume of 20 µl, containing 10 µl of the extracted RNA, 2 µl of specific primers, 4µl M-MuLV (5X) buffer, 2µl dNTP, 1µl RNase inhibitor and 1µl M-MuLV 200 U/µL. The mixture of RNA (10 µl) and primers (2 µl) were first incubated for 5 minutes at 70°C in a Thermal Cycler before preparing the reactions. The RT-PCR program included the following cycles: 5 min at 70°C, 10 min at 37°C, 60 min at 42°C and 10 min at 70°C. Negative control included RNA genome extracted from PBMCs of healthy donors. The synthesized cDNA was used as template for PCR amplification.

First and second rounds of PCR and sensitivity of nested-PCR

PCR reactions were carried out in a total volume of 25 µl, containing 2.5 µl of 10X

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reaction buffer, 0.8µl of 10 mM dNTP mix, 0.8µl of each 10 µM reverse and forward primers (Table 1), 3 µl cDNA, 0.5µlTaq DNA polymerase, 0.7 µl of 50 mM $MgCL_2$ and water to the volume. Amplification was performed as follows: initial denaturation for 2.5 min at 95˚C, followed by 95˚C for 25, 58˚C for 20, 35 cycles of 72˚C for 50 seconds, and a final extension of 7 minutes at 72˚C. After the first round of PCR, optimization was done on the second round of PCR. Optimization was performed on temperature, magnesium ion, primer, template, buffer and enzyme. After optimization, the sensitivity of the nested-PCR results was analyzed.

Detection of plus HCV genome

RNA genome was fist extracted from the PBMCs of responders- and non-responders HCH patients, and the presence of the extracted RNA was verified using GAPDH PCR. cDNA was then produced using reverse primers (Table 1) which are specific for the plus strand HCV genome. The cDNA product was utilized as template in the first and second rounds of PCR reactions. Positive and negative controls (d-H2O) were included in the experiment.

Detection of minus HCV genome

The presence of the minus HCV strand RNA was investigated in patients who were positive for plus HCV RNA genome. cDNA was constructed using forward primers (table 1) which are specific for the minus strand RNA genome. The generated cDNA was then used as template for the first and second rounds of PCR reactions (specific primers were used in each round). d-H2O and positive sample were included in the experiment as negative and positive controls, respectively.

Results

The sensitivity of the nested PCR

Once the second round of PCR was optimized, the sensitivity of the nested PCR was determined using different dilutions of 50 International Unit in each milliliter (IU/ml), 100 IU/ml, 200 IU/ml, 500IU/ml and 1000 IU /ml of a serum with known viral load . The sensitivity was estimated to be 110 IU/ml using SPSS Probit Regression Analysis

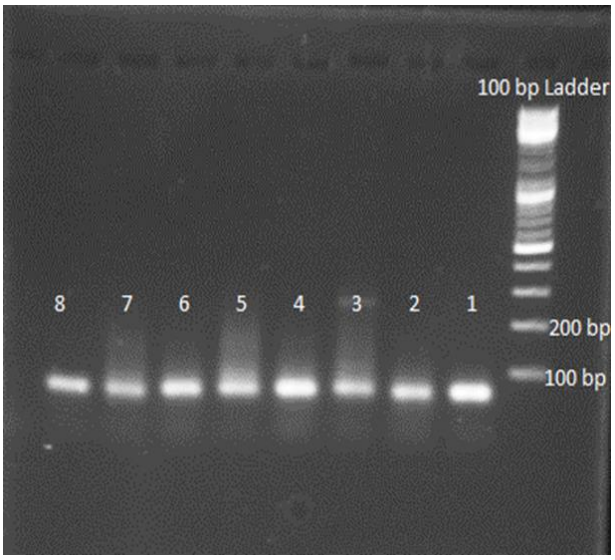

Fig. 1. Bands of GAPDH primers on genome extracted from PBMCs of patients. 1- Positive control 2-8 Patient samples

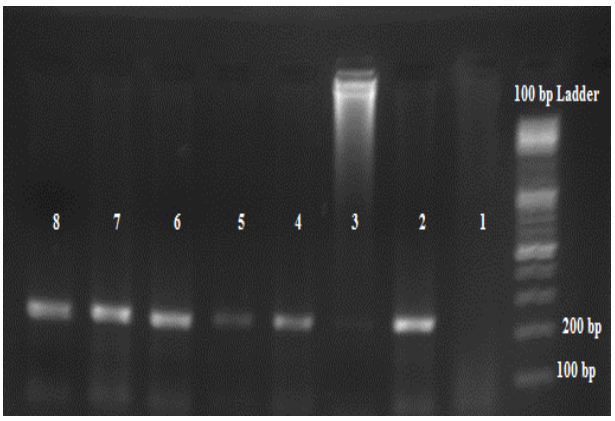

Fig. 2. Identification of positive strand HCV RNA in patients sample. 1- Negative control 2-positive control 3-8 Patient samples

Confirmation of the viral RNA genome in PBMCs from patients by GAPDH primers

To confirm genome extracting from PBMCs internal controls were used (figure 1).

The detection of the plus and minus strands HCV RNA in the PBMCs of HCH patients

The plus strand RNA genome was identified in 47% of patients who responded to the therapy as opposed to 73.3% in non-responder patients (Figure 2). Similarly, the minus strand RNA genome was identified in higher proportion of

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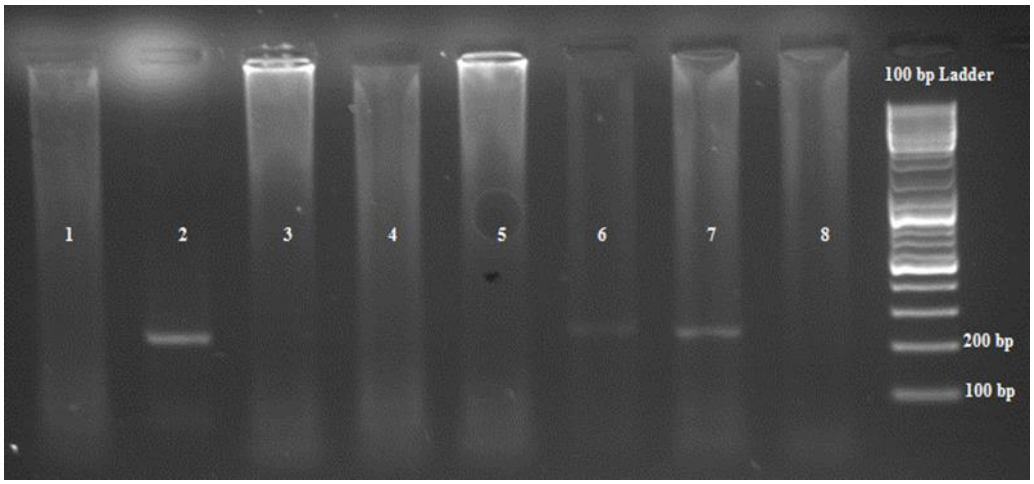

Fig. 3. Detection of negative strand HCV RNA in extracted genome
1- Negativecontrol 2-positive control 3-8 Patient samples

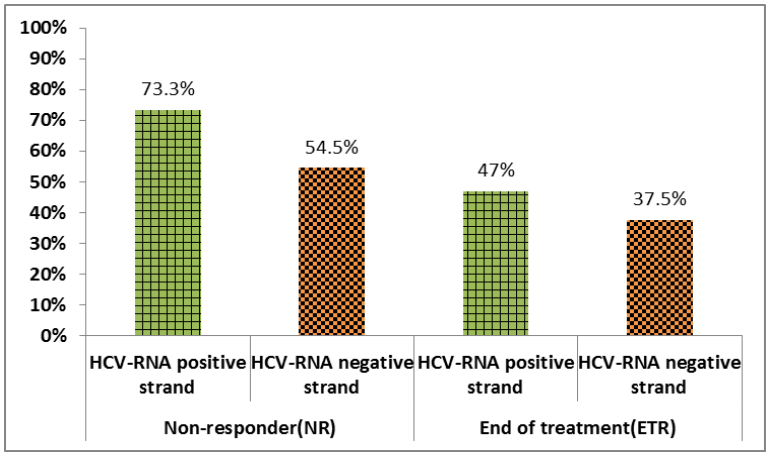

Fig. 4. The comparison of the plus and minus strands of HCV genome in responder and non-responder patients.

non-responders (54.5%) compared to responders (37.5%) (pv: 0.002) (Figure 3).

Comparison of percent the positive and negative strand RNA viruses in both groups of responders and non-responders

With comparison of existence of plus and minus HCV genome in PBMCs between responder and nonresponder groups, it was found that there is 37/5 % minus strand and 47% plus strand in responder against 73/3% positive strand and 54/5 % minus strand in nonresponder (figure 4). The difference between the two groups is significant of plus strand (PV= 0.000) and miusstrand (PV= 0.002).

Discussion

The expression of eukaryotic proteins using Although HCV is essentially a hepatotrophic virus, recent studies have shown that HCVcan actively infect and replicate in PBMCs (17, 21). For example, some HCV proteins such as core, NS3 and NS5 have been shown in PBMCs (33, 34). The presence of HCV RNA in extrahepatic tissues may have important implications for virus transmission, treatment and also progression to cirrhosis. High expression of the HCV core protein in PBMCs, for instance, has been associated with advanced chronic type of HCV infection (35). In addition, the presence of HCV RNA in

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PBMCs has been related to virus concentration in serum, which may facilitate virus transmission. Infected PBMCs may also act as HCV reservoirs (2). This is supported by the evidences which have shown the presence of the minus strand of HCV genome in PBMCs (25). The minus strand of HCV RNA is an intermediate form in viral replication and is not found in the serum, and as such it can be considered as a suitable indicator of virus replication. These findings therefore suggest that the infected PBMCs are likely to shed HCV particles leading to recurrence of liver infection upon discontinuation of therapy (17).

The plus strand HCV RNA has been frequently isolated from PBMCs of patients with either active or chronic HCV infections. In contrast, the minus strand HCV RNA is more frequently observed in chronically infected patients as opposed to acute infections. In a previous study, the minus strand HCV RNA was detected in approximately 40% of patients with HCV (26).

It has been shown that patients with acute HCV but not chronic infections are more responsive to interferon treatment. The presence of the minus strand HCV RNA in PBMCs indicates active viral replication and may act as a barrier to treatment with interferon (26, 27). Although several studies have demonstrated the presence of both the plus and the minus strands of HCV RNA in PBMCs of HCH patients (33, 38-42), some studies argue against the infection of PBMCs by HCV (28, 32).

The results of this study confirm and support the idea that PBMCs can act as an extrahepatic reservoir for HCV. Furthermore, the absence of HCV genome in serum in ETR stage may not necessarily indicate the absence of virus in blood stream. Our results also suggest that HCV infection of PBMCs may influence the treatment outcome.

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