original article

Presence of HCV RNA in peripheral blood mononuclear cells may predict patients' response to interferon and ribavirin therapy

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BACKGROUND AND OBJECTIVES: Hepatitis C virus (HCV) is considered a hepatotropic virus, but it can replicate in peripheral blood mononuclear cells (PBMCs), which influence the sustained virological response (SVR) of the patients, as well as relapse in successfully treated patients. The main objective of this study was to establish the importance of PBMC HCV RNA detection as a primary test to declare the patient as a responder, and the secondary objective was to investigate the risk of non-SVR or relapse in individuals who showed an end-of-treatment (ETR).

DESIGN AND SETTINGS: Blood samples were collected after the completion of 6 months of therapy, and they were collected 6 months after the completion of treatment.

PATIENTS AND METHODS: A total 103 patients infected with the 3a genotype of HCV and those who were treated with interferon-α-2b and ribavirin for 24 weeks were selected. HCV RNA in plasma of at the end of treatment and 6 months after the completion of treatment was determined with the help of quantitative real-time polymerase chain reaction (qRT-PCR).

RESULTS: Of the 103 patients, 74.8% (number [n]=77) were end-of-treatment responders, while 25.2% (n=26) were nonresponders. Seventy-seven responders were tested for HCV RNA in their PBMCs. The HCV RNA was detected in the PBMCs of 29 patients (37.7%). After 6 months of the end of treatment, 15 (19.5%) of 77 ETR patients showed virological relapse, while 62 (80.5%) patients attained SVR. Relapse appeared significantly more often in patients with HCV RNA in their PBMCs at the ETR stage when compared to the patients who did not have the viral RNA (34.5% versus 10.4%, respectively; R2=6.67, *P*=.01; odds ratio [OR]: 1.3; 95% confidence interval [CI]=1.032–1.811).

CONCLUSION: Patients with HCV RNA in their PBMCs after attaining an ETR are more likely to show relapse as compared to patients who are negative for viral RNA in PBMCs at the ETR stage.

Hepatitis C virus (HCV) is the principal agent that causes acute and chronic infections; as such, it is a serious global health problem. The morbidity and mortality rates are also very high in chronic infection.[1] According to 1 estimate, 200 million people carry HCV, of which 70%–80% of those who are chronically infected may develop cirrhosis and hepatocellular carcinoma.[2] Pakistan has the third-highest high prevalence of HCV infection in the world.[3] Currently, there is no vaccine available to prevent HCV infection due to many factors including the ability of the virus to support chronic and persistent chances of reinfection in the body.[4] HCV is the only member of the *Flaviviridae* family of enveloped viruses with a positive-sense RNA genome of ~9.6 kb consisting of a single open reading frame that encodes 10 structural and nonstructural viral proteins.[5] There is no protective vaccine against HCV infection, and the drugs that are used are limited in action. Administration of pegylated recombinant interferon (IFN)-α and ribavirin is the

current treatment of choice, but the sustained virological response (SVR) rate is about 50%. In Pakistan IFN-α is still in practice because it is an economical choice. Moreover, these drugs also produce some toxic effects. The exact mechanism involved in chronic infection with HCV is not clear. One reason might be its viral ability to escape from the immune system because of its genetic mutation. Another reason may be that it infects the immune cells that protect the host system from the virus to eradicate the virus from the body.[6] Although the liver is the main target for HCV replication, in some cases, HCV also replicates in peripheral blood mononuclear cells (PBMCs), lymph nodes, oral epithelial cells, adrenal glands, pancreas, brain, thyroid, salivary glands, and oral epithelial cells.[7] The presence of HCV RNA has been detected in PBMCs and other blood cells by in situ hybridization.[8] HCV RNA has also been detected in the platelets of patients with undetectable HCV RNA in their serum after treatment, and it has been associated with viral relapse. Positive and negative strands of HCV RNA were found to be significantly lower in the PBMCs of responder patients than those of nonresponder patients, and these strands were associated with the treatment results.[9]

According to our knowledge, no such method for the detection of HCV RNA in PBMCs has been used in the Pakistani population. Therefore, we planned to work on HCV RNA detection in the PBMCs of hepatitis C patients using a quantitative real-time polymerase chain reaction (qRT-PCR) method. We also aimed to check viral RNA positivity in PBMCs with respect to the end-of-treatment response (ETR) to determine its influence on relapse cases.

PATIENTS AND METHODS

The study was conducted from August 2011- July 2013 at the Holy Family Hospital, Rawalpindi, Pakistan. The patients' data were collected using a questionnaire. A total of 103 patients (58 males and 45 females) who had undetectable HCV RNA in their plasma after 24 weeks of subcutaneous interferon-α-2b 3 MU thrice weekly and ribavirin 800-1200 mg orally per day were enrolled in the study. The patients were followed up for another 24 weeks. The blood samples were collected from all patients at the end of treatment, and for a further 6 months after the end of treatment. Written informed consent was obtained from each patient, and the study was approved by the institutional ethics committee. HCV genotype 3a-infected patients were selected for the present study. The patients coinfected with hepatitis B virus or human immunodefiency virus were excluded from the study. Patients with positive HCV RNA in their plasma at the end of treatment were also excluded from the study.

Extraction and measurement of HCV RNA from plasma

Quantitative detection of HCV RNA in the patients' plasma at the end of treatment and at an additional 6 months after the end of treatment was performed. Viral RNA was extracted and quantified by the RoboGene HCV RNA extraction and quantification kits according to the manufacturer's protocol (AJ Roboscreen GmbH; Analytik Jena AG, Jena, Germany). A reaction cocktail was prepared by combining 2× reaction mixture containing Mg–sulfate (50 mmol/L), everse transcription-polymerase chain reaction (RT-PCR) buffer, buffer containing 6 mmol/L of Mg–sulfate, and deoxyribonucleotide. Then, a 25× reaction mixture of primers and probes was also added, along with an RT-PCR enzyme mixture. The HCV quantification kit, RoboGene, was used for the quantification of viral RNA using the Rotor-Gene 3000 detection system from Corbett Life Science (Qiagen, Venlo, Limburg, the Netherlands). RNA was amplified by PCR using the following conditions of thermocycling: 59°C for 60 minutes, then hold for 1 minute; 95°C for 120 minutes, then hold for 2 minutes; and 45 cycles of amplification including 95°C for 15 seconds and 57°C for 1 minute. This cycle amplifies the 5' untranslated region (UTR) of the HCV genome, with standards containing the 5' noncoding HCV genome region, which has a primer and a probe that identifies the target sequence of the HCV genome. The RNA level of the virus was found with the help of a standard curve, which was obtained by amplifying the standards separately.

PBMC isolation and counting

Blood mononuclear cells (BMCs) were separated on Ficoll-Histopaque density gradients (Sigma-Aldrich Co., St Louis, MO, USA) according to the manufacturer's protocol,[9] with minor modifications. PBMCs were separated from 3 mL of whole blood using centrifugation on a Ficoll–Histopaque gradient at 2200 rpm for 30 minutes. The PBMC layer was separated and washed 3 times with 1× phosphate buffered saline with a pH of 7.4 at 2200 rpm for 10 minutes. The cells were resuspended in fresh RPMI 1640 (Cellgro; CellGenix GmbH, Feiburg, Germany), and 10 μL of the cell suspension was mixed with an equal volume of trypan blue dye. This mixture was used for cell counting and viability determination using an automatic Countess machine (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). The cells were preserved with 10% fetal bovine serum, 1% each of penicillin, streptomycin, and

gentamycin, and 1% nonessential amino acids (Sigma-Aldrich Co.), and they were stored at −80°C until further use.

Total RNA extraction from PBMCs and quantitative RT-PCR (qRT-PCR)

Total RNA from the PBMC cell suspension was extracted following the protocol developed by Chomczynski and Sacchi[10] using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Briefly, 1 mL of Tri Reagent was taken into an Eppendorf tube and 200 µL of a well-mixed suspension of PBMCs were added. The tubes were shaken for a few seconds and kept at near-room temperature for 5 minutes; then, 100 µL of bromochloropropane (BCP) was added. After adding BCP, the tubes were shaken for 15 seconds and stored for 2 to 15 minutes. The tubes were centrifuged at 14 000 rpm at 4°C for 15 minutes. The aqueous phase was added to a new Eppendorf tube and mixed with 500 µL of isopropanol and stored for 5 to 10 minutes. The tubes were centrifuged again at 14 000 rpm for 8 minutes at 4°C to 25°C and the supernatant was removed from the tubes; the RNA pellet was then washed with 1 mL of 75% ethanol. The tubes were centrifuged at 8000 rpm for 5 minutes at room temperature, and the attached ethanol drops were removed by air drying the RNA pellet for 3 to 5 minutes. The remaining ethanol was discarded with the help of a pipette tip. The RNA pellet was resuspended in 30 µL of diethylpyrocarbonate distilled water by mixing with the help of a pipette. The tubes were incubated for 10 to 15 minutes at 55°C to 60°C. Then, 1 µL of dissolved RNA was mixed with 99 µL of distilled water.

Primer design

Primers for the amplification of HCV 5′ UTR were designed by the Oligo Primer D bioinformatics tool primer sequence (Gene Bank Accession number: D10749).

Forward: 5′GAGTGTCGTGCAGCCTGGA 3′(nt 98-116); reverse: 5′CACTCGCAAGCACCCTATCA 3′(nt 313-294); and probe: 5′ (FAM) CCGCAAGACTGCTAGCCGAGTAGTGTTGG (TAMRA) 3′ (nt 238-267).

Quantitative real-time PCR

Master mixes were prepared according to the protocol of the TaqMan EZ RT-PCR Kit (Applied Biosystems; Thermo Fisher Scientific); the lowest limit of detection was 100 copies/µg of cellular RNA.[11,12] The method used was standardized, and the kit was commercially available. A total of 19 µL of reaction mixture was prepared containing 4 µL of 5× TaqMan buffer; 2.4 µL of $MnCl_2$; 0.4 µL each of dATP, dCTP, dGTP, and dUTP; 1 µL of each primer; 0.4 µL of TaqMan probe; 0.8 µL of rTth polymerase; 0.2 µL of uracil N-glycosylase; and 0.4 µL of GAPDH were used. In addition, 1 µL (20 ng) of purified RNA was added in each mixture tube. RNA was denatured at 50°C for 1 minute, reverse transcription was performed at 60°C for 50 minutes, and complementary DNA denaturation was denatured at 95°C for 5 minutes; it was further denatured at 94°C for 15 seconds, annealing at 55°C for 10 seconds, and the final extension was performed at 69°C for 60 seconds for 50 cycles.

In vitro transcription of HCV in B-cell lines

SbJFH1 plasmid was digested and purified for in vitro transcription; DNA was transcribed, and mRNA was electroporated into the B-cell line according to a previously published protocol.[13] Uninfected cells were used as a negative control and in vitro transfected Raji cells with sbJFH-1 were used as a positive control.

Statistical analysis

The data obtained were analyzed by the Statistical Product and Service Solutions (SPSS 16.0 for Windows; SPSS, Inc., Chicago, IL, USA). The results for all of the variables were given in the form of rates (%.). Chi-square and Fisher exact test were used to find an association between HCV RNA in the PBMCs with relapse. *P* values <.05 were considered significant.

RESULTS

A total of 103 patients were diagnosed with chronic hepatitis C according to positive HCV RNA and anti-HCV tests, fluctuating levels of alanine aminotransferase (ALT) levels for more than 6 months, and an absence of other hepatitis virus markers. All of these patients received subcutaneous injections of 3 MU of IFN-α–2b 3 times per week and ribavirin 3 times per day for 6 months. The HCV RNA in the plasma was tested by RT-PCR. Nonresponders were excluded from the study.

Baseline clinical characteristics of patients

The demographic characteristics of the patients that were treated are shown in **Tables 1 and 2**. A total of 103 chronic HCV-infected patients including 58 (56.3%) male and 45 (43.7%) female patients were enrolled. The male-to-female ratio was 1.28:1. The mean (standard deviation [SD]) age of the patients was 34.39 (9.15) years and the mean (SD) weight was 70.68 (5.74) kg. The mean (SD) value of ALT was 79.44 (75.05) U/L with a range of 11 to 444 U/L. The mean (SD) value

of hemoglobin was 13.89 (1.81) g/dL, with a range of 9.3 to 18 g/dL. The mean (SD) value of the white blood cell count was 7.46 (1.73×10^3)/mm^3 with a range of 4.33×10^3/mm^3 to 12.3×10^3/mm^3. The mean (SD) platelet count was 219.56 (75.00×10^3)/mm^3, with a range of 88×10^3/mm^3 to 480×10^3/mm^3. The quantitative baseline data are presented in **Table 1**. All responders were called for follow-up. Out of the 103 patients, 77 had achieved ETR and were included in this study.

Virological response

The plasma samples of 103 patients were tested for the detection of HCV RNA after 24 weeks. RNA was not detected in 77 patients (ie, 74.8%); these patients were considered end-of-treatment responders. In addition, 25 (25.2%) patients had HCV RNA in their plasma; these patients were nonresponders. Out of 58 male patients, 42 patients were responders and 16 patients were nonresponders; conversely, of a total of 45 female patients, 35 females showed a response and 10 patients did not show a response. Since 26 patients were nonresponders, they were thus excluded from further study. After 6 months of ETR, the patients were followed up for the SVR study. The plasma samples were collected and tested for HCV RNA. A total of 62 patients were SVR achievers and 15 patients were relapsers (detectable HCV RNA in their serum 6 months after ETR). Out of the 77 patients included in the study, 42 were male and 35 were female. Of the 42 male patients, 30 had achieved SVR and 12 failed to achieve SVR. Of the 35 female patients, 32 achieved SVR and the remaining 3 could not attain SVR. The non-SVR achievers were excluded from further study.

Table 1. Qualitative baseline characteristics of treated patients.

Variables	Group	Frequency	Percentage
Gender	Male	58	56.3
	Female	45	43.7
Marital status	Single	16	15.5
	Married	87	84.5
Blood transfusion	No	87	84.5
	Yes	16	15.5
Body piercing	No	69	67
	Yes	34	33
Barber shaving	No	48	46.6
	Yes	55	53.4
Surgery	No	73	70.9
	Yes	30	29.1
Dental surgery	No	61	59.2
	Yes	42	40.8

Table 2. Quantitative baseline characteristics of treated patients.

Variable	Mean	Median	Range	Standard deviation
Age (y)	34.39	35	18–55	9.15
Body weight (kg)	70.68	70	57–82	5.74
Hemoglobin (g/dL)	13.79	13.7	9.3–18	1.81
TLC (count/mm^3)	7.46×10^3	7.5	4.3×10^3-2.3×10^3	1.73×10^3
Platelet (count/mm)	219.56×10^3	198	88×10^3-480×10^3	75×10^3
Bilirubin (mg/dL)	0.91	0.8	0.3-3.5	0.45
ALT (U/L)	79.44	63	11-444	75.05

TLC: total leukocyte count; ALT: alanine aminotransferase.

Association of HCV RNA in PBMCs at the ETR stage with relapse

Out of a total of 77 patients, 62 (80.5%) had achieved SVR, while 15 (19.5%) patients showed relapse. It was further observed that 29 (37.7%) patients had HCV RNA in their PBMCs while 48 (62.3%) had no HCV RNA in their PBMCs. Of 29 PBMC HCV RNA individuals, 19 (65.5%) showed SVR response while 10 (34.5%) did not show SVR response. Out of a total of 48 PBMC viral RNA-negative patients, 43 (89.6%) patients were SVR responders while 5 (10.4%) patients were relapsers. There was a significant difference noted between the 2 groups of HCV RNA-positive and RNA-negative patients in terms of their PBMCs, with relapse occurring in 34.5% versus 10.4%, respectively (R2=6.67; odds ratio [OR]: 1.3; 95% confidence interval [CI]: 1.032–1.811; P=.01) (**Table 3**). Thus, patients positive for HCV RNA in the PBMCs at the ETR stage showed significant relapse.

On the basis of the data available at the end of treatment, the clearance of HCV RNA from the PBMCs at 6 months of treatment had a positive predictive value of 34.5% and a negative predictive value of 89.6%, with a sensitivity of 66.6% and a specificity of 69.3%.

DISCUSSIONS

Currently, the IFN and ribavirin combination therapy is given to HCV-infected patients to eliminate HCV RNA from serum and to achieve SVR. In this study, the ETR was observed in 74.8% of patients, which was

Table 3. Association of HCV RNA in PBMCs with an SVR rate.

Category	SVR	Non-SVR	χ^2	*P* value	Odds ratio	95% Confidence interval
HCV RNA-positive PBMCs (n=29)	19 (65.5%)	10 (34.5%)	6.67	.01	1.3	1.032-1.811
HCV RNA-negative PBMCs (n=48)	43 (89.6%)	5 (10.4%)				
Total (n=77)	**62 (80.5%)**	**15 (19.5%)**				

HCV: hepatitis C virus; PBMCs: peripheral blood mononuclear cells; SVR: sustained virological response; n: number.

in accordance with the results previously reported by Ahmed et al.[14] The researchers observed a similar ETR response rate (ie, 74.5%) in patients treated with the current antiviral therapy. Our ETR result was higher than the result reported by several other investigators, for the combination therapy including INF-α-2b and ribavirin for 6 months (63.4%,[15] 57%,[16] and 53%[17]).

In our study, the relapse rate was 19.5%, and our results are similar to those reported by Idrees and Riazuddin,[2] who reported a 16.5% relapse rate in Pakistan. The relapse rate in our study (19.5%) was lower when compared to the rate that was reported by Sarrazin in 2001 (43%); however, it was higher when compared to the results from the study by Khokhar in 2002 (4%).[18,19]

Our results are in accordance with those from the study by Inglot et al,[20] which reported that recurrence was significantly greater in patients with RNA in PBMCs at the end of therapy (ie, in 2 out of 15 individuals), as compared to 0 out of 33 patients (*P*=.03). HCV is known to be a hepatotropic virus, but several studies have reported that it can actively infect and replicate in PBMCs; however, the exact mechanism of HCV replication in PBMCs is not clear.[21]

Alborzi et al[22] also confirmed HCV infection in PBMCs isolated from Iranian patients, and the authors noted that these cells act as extrahepatic reservoirs; in addition, they found that the absence of HCV in sera at the ETR could not be excluded. The results of this study confirm and support the idea that PBMCs can act as an extrahepatic reservoir. Moreover, these cells may influence antiviral therapy outcomes. An interesting finding in our study is that some patients also showed relapse, although there was no HCV RNA in either the PBMCs or in the plasma, which indicates that there may be some other extrahepatic reservoirs that support viral replication and its persistence. Further, our results are not in agreement with the results of a recent study conducted in the same region, as HCV RNA in PBMCs were detected among responder patients, but none of them showed any relapse.[23] The persistence of HCV RNA in PBMCs for more than 6 months after the ETR did not exclude the chances of late relapse, as the HCV RNA in the serum was still negative in 19 patients after 6 months of therapy.[24,25] These viral-infected PBMCs could not be recognizable by the host immune system. Löhr and Goergen[26] cultured the PBMCs of HCV-infected individuals in in vitro conditions, and they observed HCV minus RNA in cultured cells and anti-HCV antibodies in a PBMC culture supernatant. These findings suggested that HCV replication in immune cells can increase the expression of viral proteins that enhance the production of anti-HCV antibodies.[26]

There is much proof that HCV replication in PBMCs can be associated with HCV infection and its relapse following the completion of therapy. Moreover, these immune cells can work as viral reservoirs for viral relapse and reinfection after liver transplantation. In HCV-treated patients, viral RNA was not present in the plasma, but its RNA was detected in PBMCs; this showed that the virus at this stage may be replicating itself and that antiviral therapies could not exert their pressure on the immune system to clear the virus.[27]

There are various causes of HCV persistence in the body, but the most important one may be a mutation in the HCV genome, which could provide support for the virus to escape from the immune action of viral clearance.[28] The existence of HCV infection in other cells may be an important factor in relapse of the virus after IFN therapy. The HCV-negative strand indicating viral replication could be identified in the PBMCs.[24] It has been reported that 75% of HCV-infected persons have HCV replication in their PBMCs.[29]

On the basis of our results, it can be concluded that viral replication in PBMCs following treatment is responsible for the recurrence of viral infection in the blood. Further, this study will not only help develop an awareness of relapse, but it will also help physicians suggest detection of HCV in PBMCs as a new diag-

nostic tool for HCV in PBMCs in ETR responders. Our study is different from others because previous studies were non-3a genotype studies. The present study recommends that HCV detection be investigated in specific types of immune cells (T-cells, B-cells, natural killer cells) using cell markers in other body tissues like bone marrow and the kidneys, as well as using HCV genotyping and mutational analysis in the serum and PBMCs. These results will strengthen the opinion that HCV RNA should be used as a marker in responders.

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