

HCV Positive Peripheral Blood Mononuclear Cells : Role in Treatment Personalization

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Abstract: Background: Hepatitis C virus (HCV) is a lymphotropic virus, around 70% of Egyptian HCV patients harbor HCV in their Peripheral blood mononuclear cells. This study aimed to assess the sensitivity of intracellular HCV in PBMC to different antiviral treatments. Materials and methods: 20 chronic HCV patients were tested for genomic and antigenomic strands in PBMC. Patient's PBMC were individually subjected to *in vitro* treatment with either: interferon/Ribavirin, herbal extract or algae extract. Amplification of HCV by RT PCR strands is used to investigate the antiviral effect. Results: In *in vitro* tested HCV patients, differential inter individual response to IFN/RBV was reported and this matched their *in vivo* response. Intra individual response variation was also detected. Inter and intra individual response variation was also shown in results obtained from both the herbal extract and algae extract. Moreover, the herbal extract showed stronger antiviral effect compared to IFN/RBV represented by its eradication of viral strand(s) that were still present upon treatment by IFN/RBV. In contrary, the algae extract couldn't clear the virus strand(s) that IFN/RBV did. Of the most important results, testing IFN from two different sources on the cells of the same patient showed differential response to them.

Conclusion: this *in vitro* antiviral assay can be used for best treatment selection, offering a new tool for treatment personalization.

Key words: Hepatitis C virus, pretreatment response, IFN/RBV, HCV in PBMCs, antiviral assay, virus-host interaction.

INTRODUCTION

HCV infects an estimated 3% or 170 million of the world's population (Alter H.J. 2000). In Egypt, the HCV prevalence rate is the highest of the world, with approx. 25% of the population positive for HCV. This is the unfortunate result of campaigns of intravenous mass administration of tartar emetic against schistosomiasis, without disposable needles and syringes (Strickland G.T. 2002). HCV is transmitted mainly through exposure to contaminated blood or blood derivatives, i.e. by transfusion or intravenous drug administration in drug users or by pricking with HCV-soiled needles. HCV is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, and hepatitis C is also the most frequent indication for liver transplantation.

A protective vaccine is not available yet, and therapeutic options are still limited. Current treatment consists in the administration of pegylated recombinant IFN- α (interferon- α) associated to ribavirin. The successful eradication of HCV, defined as a sustained virological response (SVR), is associated with a reduced risk of developing hepatocellular carcinoma. However, sustained virological response is achieved in roughly 50% of patients and toxic side effects can be extremely severe (Picardi A.; Feld J. J. and Hoofnagle J. H. 2005).

Because PEG-IFN/RBV therapy is costly and often accompanied by adverse effects, pre-treatment predictions of those patients who are unlikely to benefit from this regimen enables ineffective treatment to be avoided.

Both virus and host-related elements have been reported as factors correlated to therapeutic effects of combination therapy (Akuta N. 2007; Shirakawa H. 2008; Asahina Y. 2008). A particular focus has been placed on virus mutations, age, gender, fibrosis of the liver, lipid metabolism, and degree of fatty metamorphosis of the liver.

The most reliable method for predicting the response is to monitor the early decline of serum HCV-RNA levels during treatment (Davis G.L. 2003) but there is no established method for prediction before treatment (Kurosaki M 2011).

Recently it was discovered that a single nucleotide polymorphism (SNP) of the host gene IL28B is significantly involved in the therapeutic response to the PEGIFN and RBV combination therapy (Ge D. 2009; Tanaka Y. 2009). The possibility of becoming a non responder (NR) is high in cases of the minor allele carriers of IL28B. Since then a trend is growing to adapt an individualized treatment approach to optimize treatment outcomes among chronic hepatitis C patients. However, it is not possible to routinely measure an SNP of IL28B

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in the clinical setting (Izumi N. 2010). Moreover, nearly half of patients achieving SVR did not show favourable genotype (Romero-Gomez M. 2011)

Studies have suggested that HCV infects not only hepatocytes but also peripheral mononuclear lymphocytes, particularly B cells. The idea that B cells may serve as HCV reservoirs was advocated by Muller et al. 1993. Several subsequently published papers also favored the notion of HCV lymphotropism (Ducoulombier D. 2004; Blackard J. T. 2006; Pal S. 2006).

In this study we described a method for pretreatment prediction of response based on short term culture of PBMC from HCV patients harboring the virus and showing the inter and intra response variation.

Patients and Methods

Patients:

In this study 20 patients were selected to be HCV positive by ELISA test (third generation), naïve for treatment, and to be negative for HBV and HIV. Clinical data of the patients is shown in table (1). Serum and PBMCs of the patients were then tested for the presence of the HCV-RNA plus and minus strands. Only patients that are positive for HCV-RNA in both serum and PBMCs were included in the study (36 patients), while those patients positive for HCV-RNA in serum only were excluded (4 patients).

Reagents:

Ribavirin for in vitro treatment was provided by ICN (virazole 100 mg/ml) Virazole: ICN (100 mg/ml). Roferon: Schering- Plough (3MiU/ml). Ismaferon®: ACAP1-Egypt. A potential antiviral is provided by department of parasitology, NRC. The extract is made from the herbal plant *Ecbalium Elatirium* in the form of a cell sap and is referred to as drops.

Cell Separation and Culture:

Ten ml of blood were collected from each patient [approved by the medical ethical committee of the National research center (NRC)] and PBMC were separated from whole blood using Ficoll separating solution. Cells were washed with PBS three times. Mixtures were then centrifuged at 1600 rpm for 10 min to collect the washed cell pellet. After the last wash, cells were re-suspended in 1 ml RPMI-1640, supplemented with 10% FCS, counted and adjusted with RPMI to be 0.75 million cells/ml media. Cells were then plated into a 6 well plate at 2 million cells per well.

Then IFN/RBV (500U/0.17mg) was added to one of them named (T) and the other well was kept as control non treated (C) cells. wells with same number of cells were included in the same plate when needed for other antiviral agents Cells were cultured for 48 hr at 37°C, 5% CO₂, then harvested, counted and subjected to RNA extraction. Cellular RNA was tested for the presence of HCV RNA strands by RT-PCR. INF/RBV was tested on all HCV/PBMC positive patients(18) while each of the algae extract and the herbal extract were tested on 6 out of 18 patients only.

Reverse Transcription – Polymerase Chain Reaction (RT-PCR):

Total cellular RNA was reverse transcribed to cDNA in 25 µl reaction mixture containing 20 U of AMV reverse transcriptase (promega, Madison, WI, USA), 200 µM of each dNTP, 25 pmoles of either antisense primer (1CH: 5' -GGT GCA CGG TCT ACG AGA CCT-3') for plus strand or sense primer (2CH: 5' -AAC TAC TGT CTT CAC GCA GAA -3') for minus strand. The first round PCR was done in a total volume of 50 µl containing 200 µM of each dNTP, 1 × reaction buffer (10 mM Tris-HCl pH 8.8, 1.5 mM MgCl₂, 50 mM KCl and 0.1% triton X-100), 2 U Taq polymerase (Finnzyme, Finland), 50 pmoles from each primer (2 CH and P2: 5' -TGC TCA TGG TGC ACG GTC TA -3'). Amplification was performed by 35 cycles of thermal cycling, each consists of denaturation at 94°C for 1min, annealing at 55°C for 1 min and extension at 72°C for 1 min followed by a final extension step for additional 10 min. The reaction was then cooled to 4°C. Twenty percent of the reaction was taken for a second amplification round (35 cycles) with the internal pair of primers (D1: 5'-CGC AGA AAG CGT CTA GCC AT -3' and D2: 5' -ACTCGG CTA GCA GTC TCG CG -3'). Cycling conditions on the thermal cycler were the same as the first round. Products of PCR were analyzed on 2% agarose gel electrophoresis and photographed.

The criteria proposed in this study for patients classification into in vitro responders or non responders to IFN/RBV were as follows : a) responders : are those patients who show disappearance of preexisting HCV-RNA strand (s) post in vitro treatment. b) non responders: are patients who retain preexisting HCV-RNA strand (s) after treatment in vitro. c) Partial responders: those patients who show loss of either one of the initially present viral strands after in vitro treatment.

Results:

RT-PCR Detection of HCV Strands in PBMC:

All enrolled patients (table 1) were HCV positive by ELISA test (third generation). Two out of the 20 patients included in the study lacked either forms of the viral strands so they were excluded from the study. In the remaining 18 patients, the genomic and/or the antigenomic strand of the virus was detectable (Fig1).

Effect of Culture on PBMC Count and HCV Strands:

Culturing Patient’s PBMCs for 72 hr under the used conditions resulted in increase in the PBMCs count in all cases from 1.3-2.3 fold increase, mean 3.04 ± 0.5 . while *in vitro* replication of HCV was affected as follows: a) 20 % of the patients lost both viral RNA strands upon culturing, b) 45% retained viral replication, c) 5% lost the positive RNA strand, d) 30% lost the negative strand of the virus.

In vitro Effect of Different Antivirals on HCV in Different Patients:

1- IFN/RBV:

Differential response to IFN/RBV from different patients have been recorded Fig 2. This included either elimination of preexisting HCV strand/s (*In vitro* responder), elimination of only one strand (*In vitro* partial responder) or no change (*In vitro* non responder). Matching between *In vitro* and *In vivo* results was available for 6 patients only (table 1) who showed concordant results.

2- IFN/RBV from Two Different Sources:

The effect of IFN α -2b from two different sources (Schering Plough & Acapi) was tested. It was shown that HCV in patient’s PBMCs showed differential response to different preparations of interferon, as it showed response to IFN of Schering while showed no response to IFN of Acapi (figure 3).

3- Effect of a Herbal Extract Named (Drops):

This compound showed strong antiviral effect as it resulted in loss of both RNA-strands of the virus in 4 HCV positive patients (Figure 3) while IFN /RBV didn’t. Nonetheless in other patients it wasn’t effective to eradicate the virus *in vitro* (figure 4).

4- Effect of an Algae Extract:

It was tested on PBMCs from 6 HCV patients included in the study. One of them showed loss of one of the viral RNA strands detected in control untreated cells. According to the proposed response criteria this patient was classified partial responder to the effect of this agent but was non responder to IFN/RBV, another patient showed no response to this drug although responsive to IFN/RBV, while the remaining patients were non responders to both algae and IFN/RBV (figure 5).

Table 1: Clinical data of HCV patients.

Characteristics	Data
Age range	23-46 years
Sex	M: 15 F: 5
Anti-HCV in serum	All positive
GOT range AST range	21-98 U/l 26-287 U/l
HCV-RNA	100% positive
Bilharziasis %	22.5%
Diabetes %	7.5%

ALT: alanine aminotransferase, AST: aspartate amino transferase
F:female, M: male.

Table 2: Matching between *in vitro* and *in vivo* response to IFN/RBV.

Patient’s code No.	0 month			6 month after treat end			<i>In vitro</i> response
	Serum +ve	PBMC +ve	PBMC -ve	serum +ve	PBMC +ve	PBMC -ve	
3	+	+	+	+	-	+	NR
13	+	+	+	+	-	+	NR
18	+	+	+	+	+	-	NR
11	+	+	+	+	+	-	NR
15	+	+	-	-	-	-	R
19	+	+	+	+	-	-	NR

+ve: plus-RNA HCV strand, -ve: minus-RNA HCV strand
R: responder, NR: non responder, PR: partial responder

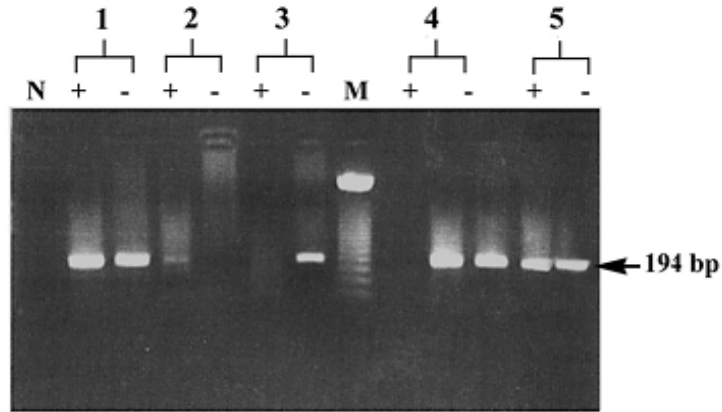


Fig. 1: Demonstration of HCV RNA in circulating PBMCs. PCR products from amplification of HCV plus-RNA strand (+) and minus RNA strand (-) from total RNA extracted from PBMCs of 4 patients (lanes 2, 3, 4, 5). Lane N represents the negative control of the PCR. Lane 1 represent the positive control of the PCR. Lane M, is the DNA Mwt marker ϕ x174 HaeIII digest.

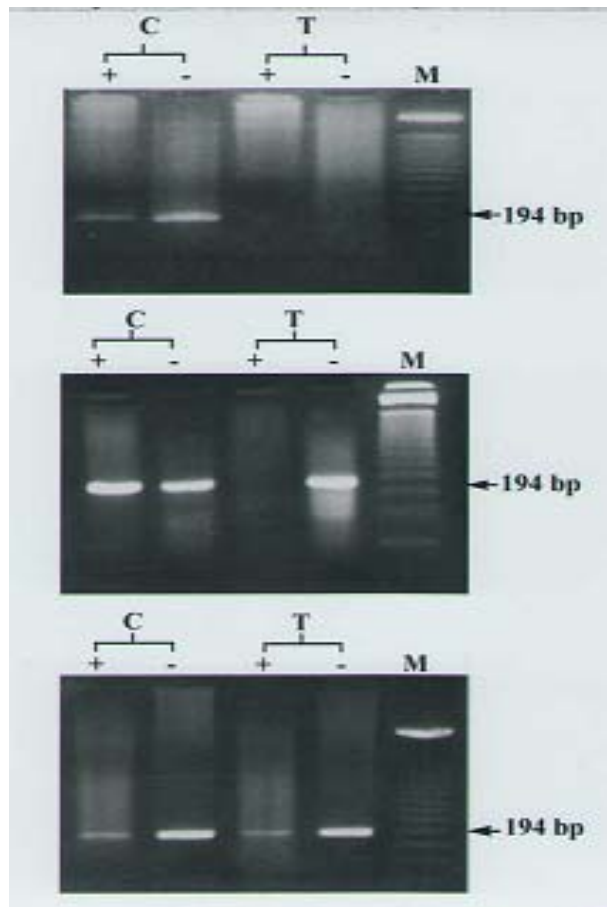


Fig. 2: Effect of INF/RBV on HCV replication in PBMCs *in vitro*

A) Example of a responder patient:

The figure shows PCR amplification product of HCV RNA strands from control PBMCs (lane C), INF/RBV treated cells (lane T). Lane M is the Mwt DNA marker pGem.

B) Example of a partial responder patient:

The figure shows amplified PCR products of HCV plus -strand (+) and minus -strand (-), lane (T) represents products from PBMCs treated with INF/RBV, lane (C) represent positive and negative strand PCR product from RNA of the control PBMCs. M is the Mwt DNA marker M50.

C) Example of a non responder patient:

The figure shows bands representing PCR amplification of HCV plus- strand (+) and minus- strand (-) in lane (T) from RNA extracted from PBMCs treated with INF/RBV, While lane (C) represents positive and negative strand PCR product from RNA of control PBMCs. M is the Mwt DNA marker M50.

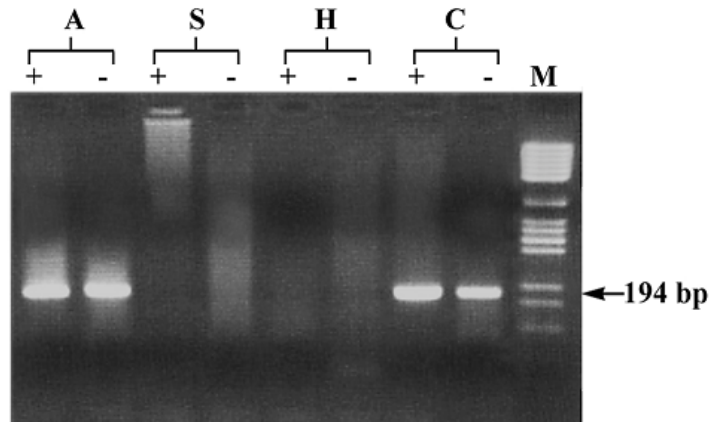


Fig. 3: Effect of herbal extract and IFN/RBV from two sources on HCV replication *in vitro*. PCR products amplified from RNA extracted from an HCV patient's PBMCs. The plus RNA strand of HCV is illustrated as (+) and the minus strand as (-). Control cells are in lane (C), cells treated with INF/RBV from Acapi are in lane (A), cells treated with INF/RBV from Schering Plough are in lane (S), cells treated with herbal extract of *Ecbalium Elaterium* are in lane (H). lane (M) is the Mwt DNA marker pGem

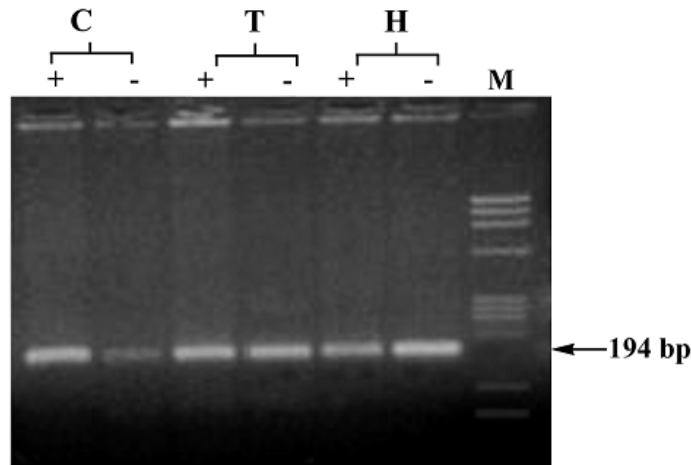


Fig. 4: Effect of herbal extract on HCV replication *in vitro*.

Results of PCR amplification of HCV from cultured PBMCs treated with herbal extract in lane H (+, -) compared to PBMCs treated with INF/RBV in lane T (+, -) and control untreated cells in lane C (+, -). The plus- and minus- strands are illustrated as (+, -) respectively. M is the DNA Mwt marker ϕ x174 Hae III digest

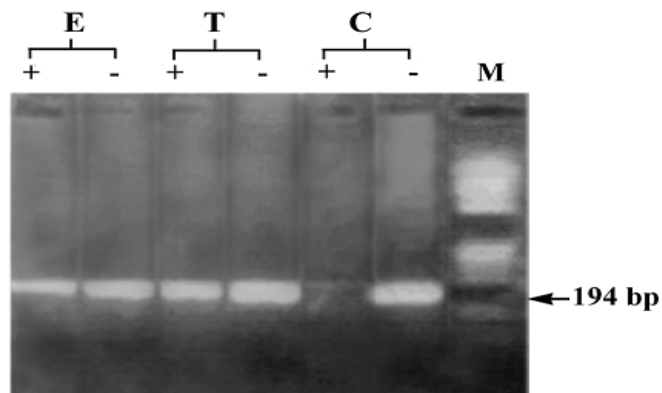


Fig. 5: Effect of algae extract on HCV replication *in vitro*

The figure shows the PCR products for the plus (+) and minus (-) RNA strands of HCV amplified from cultured PBMCs of an HCV patient. PCR products from control cells are shown in lane C, from cells treated with INF/RBV are shown in lane T, from cells treated with algae extract in lane E. M is the DNA Mwt marker ϕ x174 Hae III digest

Discussion:

Significant numbers of patients do not achieve an SVR, or are intolerant/have contraindications to therapy and no other treatment options are currently available (Ito M. 2011) many individuals have turned to herbal remedies. Two recent studies have reported that as many as 13% to 23% of U.S. patients with chronic liver disease are using herbal remedies (Davis G.L. 2003; Strader D.B. 2002). Pretreatment prediction of response is a tremendous need but there is no established method for prediction before treatment.

Studies on Pretreatment prediction of response based on testing antiviral effect on intracellular HCV in infected patient's PBMC are very limited, only one previous study showed that the *in vitro* effect of IFN-b on HCV in PBMC reflects clinical response and would be taken into account as a predictive marker of IFN therapy for chronic hepatitis C (Seeff L.B. 2008). This study evaluated the effect of IFN only without RBV, and didn't check for any other antiviral agents.

In this study we investigated the possibility to predict response of HCV to standard antiviral therapy (IFN/RBV) using short term culture of patient's PBMC harboring the virus. Moreover, to detect the inter and intra individual *in vitro* variation in response of intracellular HCV in patient's PBMC to specific antiviral agent of HCV.

Initially we assessed the *in vitro* antiviral effect of IFN/RBV on different patients (fig 2), which showed differential response from different patients, proving that this assay is sensitive to monitor intra individual variations in response to IFN/RBV. Moreover, these patients were followed during *in vivo* treatment and they showed *in vivo* response to IFN/RBV that matched the *in vitro* one. Thus indicating that this *in vitro* assay can predict pre treatment response to IFN/RBV, this is consistent with the previous study of Mochizuki K et al., 2004.

In order to test the sensitivity of this assay to predict response to other antiviral agents than the standard IFN/RBV, we used it to investigate the antiviral effect of a herbal extract (fig 3, 4) and that of an algae extract (fig 5) on HCV individually compared with IFN/RBV. Results of testing the herbal extract showed response to it in patients (fig 3) and non response (fig 4) in others, notably, in certain patients it eradicated the virus although not eradicated by IFN/RBV (fig 4) indicating better response of these patients to the herbal extract compared to IFN/RBV and reflecting the sensitivity to detect differential response of each patient to more than one antiviral agent. Results of testing the algae extract also showed differential response to it with non responders number greater than responders and no preference response on IFN/RBV (fig 5).

Worth mentioning that when this *in vitro* assay was used to test the efficiency of IFN preparation from different sources in which one of them was known to have weaker antiviral activity, results showed viral eradication with the stronger IFN preparation versus non response with the weaker IFN, proving the sensitivity any change in IFN preparation.

Evidently this *in vitro* assay can be used to predict pretreatment response to IFN/RBV, and to select the best antiviral agent for each patient, in other words, it can help personalize treatment for chronic HCV patients. This work was supported by NRC.

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