

Association of Interferon Alpha Receptor 1 with sustained virological response in hepatitis C and B co-infected patients

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Abstract

Objective: To determine the association of interferon alpha receptor-1 with success rate of interferon therapy in patients co-infected with hepatitis C virus and hepatitis B virus.

Methods: The study was conducted at the Army Medical College, Rawalpindi, Pakistan, from December 2013 to November 2014, and comprised patients with hepatitis C and hepatitis B co-infection. The patients were treated with pegylated-interferon-2b plus ribavirin therapy for six months. With respect to interferon therapy, patients with undetectable hepatitis C virus-ribonucleic acid along with normal alanine aminotransferase were considered responders and patients with detectable hepatitis C virus-ribonucleic acid at week 48 were considered as non-responders. SPSS 20 was used for data analysis.

Results: Of the 86 patients, there were 50(58%) males and 36(42%) females. The presence of high pre-treatment interferon alpha receptors 1-messenger ribonucleic acid in peripheral blood mononuclear cells was significantly associated with sustained virological response (85.7% vs. 64.7%, $P = 0.031$). Multiple regression analysis showed that females ($p < 0.001$), lower hepatitis C virus-ribonucleic acid levels ($p < 0.001$) and lower hepatitis B virus-deoxyribonucleic acid levels ($p < 0.001$) were associated with expression level of interferon alpha receptors 1 in the co-infected patients.

Conclusion: Interferon alpha receptors 1-messenger ribonucleic acid may be useful for predicting response to interferon plus ribavirin therapy in hepatitis C virus/ hepatitis B virus co-infected patients who were females with lower hepatitis C virus-ribonucleic acid and hepatitis B virus-deoxyribonucleic acid levels.

Keywords: IFNAR1, Peg-Interferon therapy, Co-infection, Hepatitis C virus, Hepatitis B virus, Polymerase chain reaction, Peripheral blood mononuclear cells. (JPMA 67: 980; 2017)

Introduction

Hepatitis C virus (HCV) and hepatitis B virus (HBV) infections are the major causes of chronic liver disease worldwide leading to greater incidence of developing cirrhosis and hepatic decompensation.¹ According to the World Health Organisation (WHO), 2-10% of chronic HCV patients found to be hepatitis B surface antigen (HBsAg) positive and 5-7% of chronic hepatitis B (CHB) patients are positive for anti-HCV.² Approximately 7-20 million people are HCV/HBV co-infected with 1.5 million deaths annually.³ Pakistan is considered one of the worst afflicted nations with high risk of chronic liver disease and maximum mortality by hepatitis C and B co-infection.⁴

The current standard of care (SOC) is still combination therapy with peg-interferon and ribavirin, especially in Pakistan. Interferon is a pleiotropic cytokine with immunomodulatory and antiviral activities. Antiviral

activity of interferon is mediated by its binding to cell-specific interferon alpha receptors (IFNAR). IFNAR can be expressed constitutively and ubiquitously as a heterodimer consisting of two subunits encoded by the IFNAR1 and IFNAR2 genes.⁵ The human IFNAR1 gene encodes a transmembrane (tm) protein composed of a 409 amino-acid extracellular domain (ECD) featuring four SD100 repeats, fibronectin and immunoglobulin domains. IFNAR1 significantly affects differential ligand selection and binding affinity for interferon-alpha (IFN- α).⁶ Receptor-ligand interaction then induces activation of Janus kinase-1 (JAK) and tyrosine kinase 2 (Tyk2) which phosphorylate IFNAR1 subunit. The phosphorylated IFNAR1 leads to recruitment and activation of the signal transducers and activators of transcription (STATs) signalling pathway. The entire signalling pathway leads to direct inhibition of viral replication by induction of gene expression through binding to IFN-stimulated response element (ISRE) within the promoters of IFN-stimulated genes.⁷

Several factors are extensively studied regarding poor response to interferon therapy in hepatitis C patients, such as advanced age, male gender, genotype 1 and high

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baseline alanine aminotransferase (ALT) levels.⁸⁻¹⁰ HBV co-infection in hepatitis C patients is also very common in Pakistan. These patients have been considered to be less responsive to interferon-based therapies and represent a therapeutic challenge due to reciprocal viral interference.⁸ It appears that high levels of HBV-deoxyribonucleic acid (DNA) contributes to chronic liver damage and development of cirrhosis in HCV-infected patients.¹¹ Therefore, it is important to determine a relation between pre-treatment variables and its effect on interferon therapy in HCV/HBV co-infected patients. Host's genetic factors that are involved in interferon signalling or effect or functions may play an important role in determining IFN therapy effectiveness.¹² Negative regulation of cellular signalling among therapy-resistant patients is mainly due to ligand-induced down regulation of IFNAR1.⁵ Similarly, the presence of high transcription level of IFNAR1 in peripheral blood mononuclear cells (PBMCs) of HCV-infected patients have been associated with successful response to interferon therapy.¹³ In addition, HCV/HBV co-infected patients showed unfavourable response to interferon therapy due to trivial PBMCs expression of IFNAR1-messenger ribonucleic acid (mRNA) than non-co-infected patients.¹⁴

The current study was planned to determine the expression level of IFNAR1 mRNA in PBMCs and influence of other variables on possible association of IFNAR1 mRNA with response to interferon therapy.

Patients and Methods

This cross-sectional study was carried out at Department of Biochemistry and Molecular Biology Centre for Research in Experimental and Applied Medicine (CREAM-1), Army Medical College, Rawalpindi, Pakistan, from December 2013 to November 2014. We recruited patients with HCV and HBV co-infection admitted to the liver clinic of General Hospital, Rawalpindi. A total of 11 patients discontinued the therapy due to adverse effects and could not be evaluated.

Untreated adults aged 18 years or older with seropositivity for anti-HCV by a third-generation enzyme-linked immunosorbent assay (ELISA) and for serum HCV-RNA determined by Cobas® Amplicor HCV Monitor v2.0 (Roche Molecular systems, Pleasanton, California, United States; detection limit 20 IU/ml) along with HBsAg positive and serum HBV-DNA levels of 2,000 IU/mL or higher quantified by using Roche Cobas® Amplicor HBV Monitor assay (Roche Molecular Systems, Branchburg, US) were included.

Approval for the study was obtained from the institutional ethics committee. Written informed consent was

obtained from each participant. The study protocol was done in accordance with the principles of the Declaration of Helsinki.¹⁵ Exclusion criteria included hepatocellular injury (as evidenced by the presence of metabolic, primary biliary cirrhosis or autoimmune liver disease), infection with HIV or hepatitis (A, D or E) or pre-existing psychiatric disease.

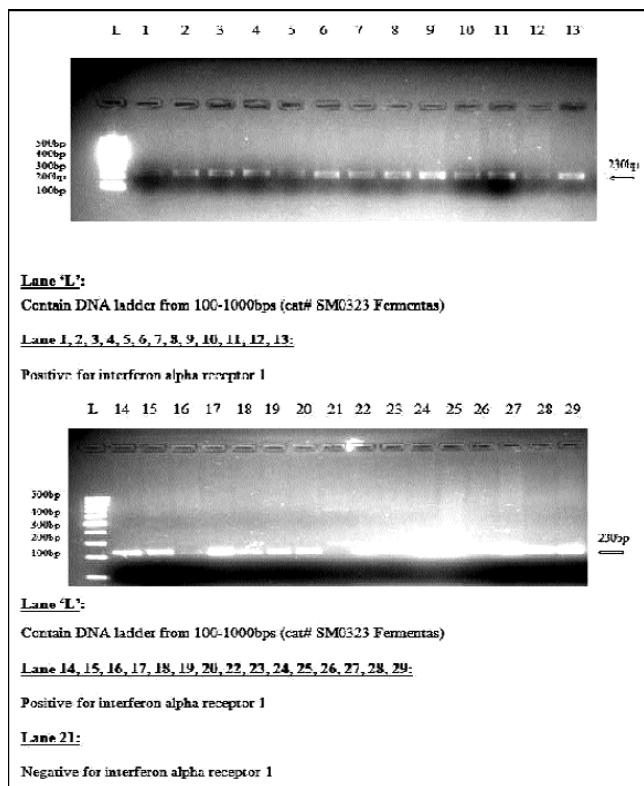
All patients were treated with Peg-IFN- α -2b (Pegintron®, Schering Plough Pharmaceutical Co. Ltd., Tokyo, Japan), intramuscularly in weekly doses adjusted to body weight according to the manufacturer's instructions (less than or equal to 45 kg = 60 μ g/dose, 46-60 kg = 80 μ g/dose, 61-75 kg = 100 μ g/dose, 76-90 kg = 120 μ g/dose, and more than or equal to 91 kg = 150 μ g/dose) along with oral ribavirin (600-1000 mg daily) for six months.

Pre-treatment biochemical and serological variables (such as age, gender, body mass index (BMI), serum ALT, virological and histological data) of all the patients were assessed. In order to evaluate the degree of histopathological lesion, pre-treatment liver percutaneous needle biopsies were performed in all patients. Liver biopsy specimens were formalin fixed, paraffin-embedded and eosin-haematoxylin stained. Histological response was assessed for inflammation and fibrosis according to the Ishak histological activity index (HAI).¹⁶ The Ishak fibrosis scores are classified as hepatic stage of low fibrosis (Ishak score 0-2) and high fibrosis (Ishak score 3-4). The grade of hepatic inflammation scores are categorised into group 1 (minimal to mild changes) and group 2 (moderate to severe changes).

Patients were designated as sustained responders having normal serum alanine aminotransferase and undetectable HCV-RNA level after 24 weeks from the end of the therapy, and non-sustained responders they were having abnormal serum alanine aminotransferase along with detectable HCV-RNA level at the end of therapy or HCV-RNA reappearance during the follow-up period.

Blood samples from all patients were collected and subjected to RNA extraction by RNA Miniprep Super Kit (Bio Basic Inc, Canada). First-strand complementary DNA (cDNA) was synthesised by Revert-Aid Premium First Strand cDNA Synthesis Kit (Thermo-scientific Inc, #K1652, US), using RNA as a template.

For quantitative real-time polymerase chain reaction (PCR) amplification, the primers were designed by using Primer3 software based on a previously available sequence of IFNAR1 on the National Centre for Biotechnology Information (NCBI). The properties of primer were then evaluated using Oligonucleotide



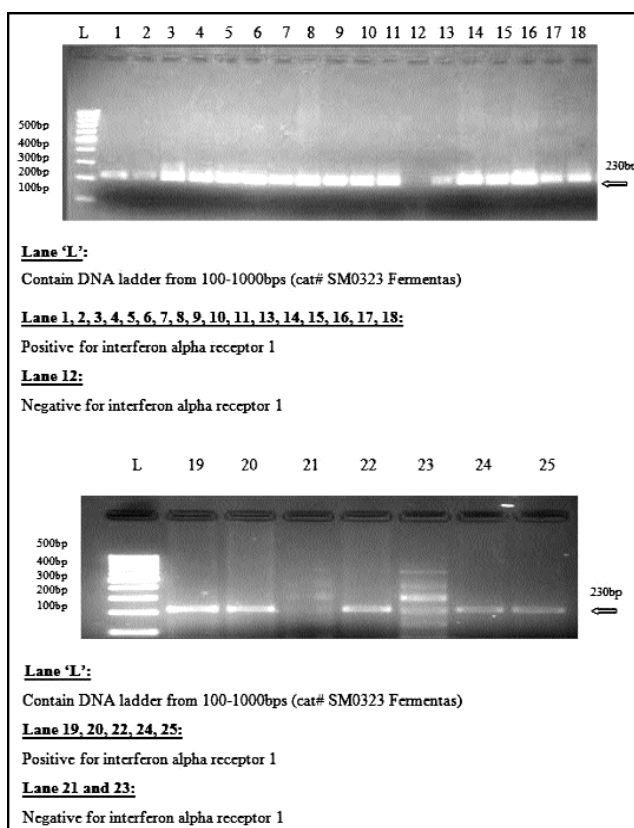
DNA: Deoxyribonucleic acid
IFNAR1: Interferon alpha receptors
PCR: Polymerase chain reaction.

Figure-1: Pretreatment electropherogram of ethidium bromide stained 2% agarose gel showing PCR based detection of IFNAR1 at 230bp of responders except patient no. 21.

Properties Calculator and electronic PCR. The sequence of the IFNAR1 primer (Thermo-scientific Inc, US) is as follows: 5' GGAACAGGAGCGATGAGTCT 3'; guanine-cytosine (GC)-content =55%; temperature (TM) =59.18°C; length =235 and R1 IFNAR1: 5' TGAGCTTTGCGAAATGGTGT 3'; GC-content =45 %; TM= 58.68°C; length =235.

Standardisation of PCR-based IFNAR1 was done by using cDNA from HCV/HBV co-infected patients. The following protocol was used for the amplification of IFNAR1: cDNA (xng), 10x PCR buffer (1x), magnesium chloride (MgCl₂) (25mM), deoxyribose-containing nucleoside triphosphates (dNTPs) (2mM), forward primer (1pmol), Reverse primer (1pmol), Taq polymerase (1 unit) and autoclaved distilled water (xµl) with total PCR of 25µl.

IFNAR1 sequence amplification was carried out by using the following cycling conditions: 35 cycles, hot start at 95°C for 5 minutes, denaturation at 93°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, final elongation at 72°C for 7 minutes and then hold at 4°C. For each sample, individual detection of IFN-



DNA: Deoxyribonucleic acid
IFNAR1: Interferon alpha receptors
PCR: Polymerase chain reaction

Figure-2: Pre-treatment electropherogram of ethidium bromide stained 2% agarose gel showing PCR based detection of IFNAR1 at 230bp of non-responders except patient no. 12, 21 & 23.

α receptor mRNA was performed separately using the same PCR conditions and specific primers for each of them. The amplified PCR products of IFNAR1-mRNA were loaded with a loading buffer of bromophenol blue and run on 2% agarose gel for analysis. Electrophoresis was carried out for 30 minutes at 90-100 volts. Then the product was visualised on gel documentation (Bio-Rad). A visualised DNA band at the length 230 bp was considered positive for IFNAR1 (Figures-1 and 2).

The primary efficacy end point was detection of IFNAR1-mRNA among HCV/HBV co-infected patients in relation to serum viral loads and stage of hepatic fibrosis. Secondary efficacy end point was attainment of sustained virological response, defined as a serum HCV RNA level below the limit of detection of the assay (< 20 IU/mL) at the end of 6-month follow-up.

Data was analysed using SPSS 20. Quantitative variables

were expressed as mean with standard deviation (SD) and compared through independent t-test. Qualitative variables were described as frequencies along with percentages and analysed using chi-square or Fisher's exact tests. Multivariate logistic regression was applied to determine significant association between independent variables and expression level of IFNAR1 mRNA in PBMCs. $P < 0.05$ was considered statistically significant.

Results

Of the 86 patients, there were 50(58%) males and 36(42%) females. The overall mean age was 37.4 ± 7.1 years (range: 21-55 years). The overall mean BMI was 23.6 ± 4.4 kg/m². After treatment with pegylated-IFN/ribavirin for 24 weeks and a 24-week post-therapy observational period (week 48), 35(41%) patients showed clearance of serum HCV-RNA and were considered as sustained responders. The remaining 51(59%) patients were HCV-RNA positive at week 48 and were classified as non-sustained responders.

Analysis of variables showed that frequency of males was significantly higher among non-responders than females ($p=0.005$). Correlation between serum ALT level and systemic vascular resistance (SVR) was also analysed. Patients with SVR had lower pre-treatment serum ALT levels ($p < 0.001$). Pre-treatment analysis of mean HBV-DNA concentration showed that non-responders to interferon therapy had considerably higher viral load than responders, i.e. 35(68.6%) vs. 11(31.4%); ($p=0.001$).

Table-1: Comparison of baseline variables between HCV/HBV responders and non-responders to therapy.

Patients Characteristics	HCV/HBV responders (n = 35)	HCV/HBV Non-responders (n = 51)	P value
Mean Age in years	37.31 ± 6.8	36.8 ± 7.4	0.745
BMI	23.96 ± 3.7	23.8 ± 4.3	0.815
Serum ALT (ULN)	48.14 ± 13.8	93.82 ± 38.8	< 0.001
Gender (Male)	14 (40%)	36 (70.6%)	0.005
High HCV-RNA $> 4 \times 10^6$ IU/ml	09 (25.7%)	33 (64.7%)	0.001
High HBV-DNA $> 2 \times 10^4$ IU/ml	11 (31.4%)	35 (68.6%)	0.001
Moderate to Severe HAI Inflammation	12 (34.3%)	24 (47.1%)	0.238
High HAI Fibrosis	10 (28.5%)	34 (66.6%)	0.001
Positive IFNAR1-mRNA	30 (85.7%)	33 (64.7%)	0.031

ALT: Alanine aminotransferase

ULN: Upper limit of normal range

IFNAR1: Interferon alpha receptor 1

BMI: Body mass index

HCV: Hepatitis C virus

HBV: Hepatitis B virus

HAI: Histological activity index

Responder: Normal ALT and undetectable HCV-RNA & HBV-DNA in serum

Non-responder: Abnormal ALT and detectable HCV-RNA & HBV-DNA after therapy

Data presented as n (%), mean \pm standard deviation (SD).

Table-2: Correlation between IFNAR1 mRNA detection and baseline variables.

Variables	R	P value
Gender	0.499	0.001
ALT	-0.048	0.42
HCV-RNA level	0.618	0.001
HBV-DNA level	0.458	0.001
HAI Fibrosis	0.590	0.001
HAI Inflammation	-0.087	0.291

Pearson's and Spearman's correlation

ALT: Alanine aminotransferase

IFNAR1: Interferon alpha receptor 1

HCV: Hepatitis C virus

HBV: Hepatitis B virus

HAI: Histological activity index.

Table-3: Independent factors influencing IFNAR1 mRNA detection.

Variables	P value
Gender (female)	< 0.001
HCV-RNA level ($< 4 \times 10^6$ IU/ml)	< 0.001
HBV-DNA level ($< 2 \times 10^4$ IU/ml)	< 0.001

$R^2 = 0.643$

IFNAR1: Interferon alpha receptor 1

HCV: Hepatitis C virus

HBV: Hepatitis B virus

RNA: Ribonucleic acid

DNA: Deoxyribonucleic acid.

Similarly, 33(64.7%) non-responders had concentration of HCV-RNA compared to 9(25.7%) responders ($p=0.001$). Besides, 34(66.6%) non-responders were at an advanced stage of hepatic fibrosis compared to 10(28.5%) responders ($p=0.001$). IFNAR1 sequence amplification was also carried out to detect its association with response to therapy. Significant difference was observed in the detection rate of IFNAR1 mRNA between sustained responders and non-sustained responders, i.e. 30(85.7%) vs. 33(64.7%) ($p=0.031$). All other variables, such as age, body weight and HAI inflammatory score showed no statistically significant difference among responders and non-responders to pegylated-IFN therapy (Table-1).

Univariate analysis was applied to detect influence of other variables on detection rate of IFNAR1 mRNA. It was found that female gender (odds ratio (OR) =12.85; 95% confidence interval (CI) =3.82-23.2; $r=0.499$; $p=0.001$); baseline HCV-RNA level $< 4 \times 10^6$ IU/ml (OR =2.2; 95% CI =1.58-3.08; $r=0.618$; $p=0.001$); baseline HBV-DNA level $< 2 \times 10^4$ IU/ml (OR =15.96; 95% CI =3.43-34.12; $r=0.458$; $p=0.001$) and low HAI fibrosis (OR =2.09; 95% CI =1.53-2.85; $r=0.590$; $p=0.001$) were significantly correlated with detection rate of IFNAR1 in HCV/HBV co-infected patients.

Age, body weight, serum ALT level and HAI inflammatory score failed to affect the detection rate of IFNAR1 mRNA in PBMCs (Table-2).

We also studied the multivariate model (using the variables that were significant in the univariate analysis) to assess the influence of independent factor on strength of association between detection rate of IFNAR1 and response rate to therapy. It showed that females ($p < 0.001$) with low HCV-RNA ($p < 0.001$) and low HBV-DNA ($p < 0.001$) with $r^2 = 0.643$ were independent variables influencing the detection rate of IFNAR1 mRNA (Table-3).

Discussion

Concurrent infection with HCV and HBV is common in Pakistan. HBV/HCV co-infection is a challenging clinical condition due to its unpredictable interferon therapy response and health care costs. Several viral and cellular factors have been identified as predictors of virological response to interferon therapy.¹⁷

IFNAR1 is one of the main preliminary factors responsible for response to interferon therapy and establishment of antiviral state in HCV/HBV co-infected patients. Detection of IFNAR1 mRNA in PBMCs can help to understand the pathophysiology of viral interaction, particularly in terms of IFN resistance.¹⁸ According to Massirer et al., PBMC expression of IFNAR1 mRNA reflects the hepatic status of IFNAR1-mRNA and interferon therapy responders have higher IFNAR1 mRNA levels than non-responders.¹⁹ To understand the importance of IFNAR1, the present study was conducted to evaluate correlation between significant detection rate of IFNAR1-mRNA and sustained virological response. We also assessed the impact of pre-treatment variables on strength of association between detection rate of IFNAR1 and response rate to therapy in HCV/HBV co-infected patients.

Viral load in a body indicates association between replication and clearance of virus. It also reflects the interferon therapy outcome. Patients with high pre-treatment viral loads are at increased risk of interferon therapy failure. Our results highlighted a significant relation between low basal levels of viral load and achievement of virological response in co-infected patients. Among non-responders, $> 2 \times 10^4$ IU/ml level of HBV-DNA (68.5% vs. 31.3%, $p = 0.001$) and $> 4 \times 10^6$ IU/ml level of HCV-RNA (64.7% vs. 25.7%; $p = 0.001$) was observed as compared to responders. We also found lower rate of SVR in males as compare to females (40% vs. 70.6%), ($p = 0.005$). Histological parameters also represent significant risk factor for poor response to interferon therapy. In this study, higher stage of hepatic fibrosis was more pronounced among non-sustained responders as

compared to sustained-responders (66.6% vs. 28.5%; $p = 0.001$), which is similar to the results of other studies.²⁰ However, there was no correlation between SVR and HAI grade of inflammation ($p = 0.238$).

In this study, of the 35 patients who responded to pegylated interferon therapy, 30 patients were detected with positive IFNAR1-mRNA which appeared to be significant as compared to non-responders (85.7% vs. 64.7%, $p = 0.031$). Based on these evidences, high expression rate of pre-treatment IFNAR1 mRNA levels in PBMC leads to valuable response to interferon therapy in HCV/HBV co-infected patients. Similarly, female patients ($p = 0.001$) with higher HBV-DNA levels ($p = 0.001$), higher HCV-RNA levels ($p < 0.001$) and severe hepatic stage of fibrosis ($p = 0.001$) leads to poor response to therapy by suppressing the expression rate of IFNAR1 mRNA. Previous studies confirmed that suppression of IFN alpha receptors can be appreciated among males with high HBV-DNA and HCV-RNA replication rate leading to suppression of cell mediated immunity, poor response to pegylated-IFN therapy and increase susceptibility of progression to hepatocellular carcinoma in co-infected patients.²¹

Our study also confirmed the results of Fukuda et al. that the absence of virological response is mainly due to higher viral loads leading to down regulation of IFNAR1.²¹ Mechanism of IFNAR1 downregulation by HBV-DNA is not yet clear, however, according to other studies, increased level of serum HBV-DNA can be considered directly related to hepatic cell injury leading to severe hepatic stage of fibrosis which makes hepatocytes unable to express IFNAR1 mRNA and may interfere with the clinical outcome in HCV/HBV co-infected patients.²²

By multivariate logistic regression analysis, neither the grade of inflammation nor the stage of fibrosis were found to have influence on strength of association between IFNAR1 mRNA and SVR among HCV/HBV co-infected patients ($r = -0.087$ and 0.590 , respectively). A few other studies also found no significant association of IFNAR1 mRNA with hepatic histological findings.²⁰ Female gender with lower viral loads were the only independent variable significantly associated with expression level of IFNAR1 leading to achievement of sustained virological response.

Of the non-responders, 33(64.7%) patients showed detection of IFNAR1-mRNA but failed to respond to interferon therapy. The likely cause was either defect in interferon signalling cascade after binding to receptor or a change in binding affinity of interferon to its functional receptor due to the presence of higher HCV-RNA and

HBV-DNA levels.^{23,24}

The current study had a few limitations as well. First, we only included patients with detectable HBV-DNA in blood, but possible occult HBV infection was not considered. Secondly, this study can be further extended by finding mutations and variations in IFNAR1 among the Pakistani population.

Conclusion

Higher expression level of IFNAR1 mRNA was significantly associated with effectiveness of pegylated interferon plus ribavirin therapy in patients with hepatitis C and hepatitis B co-infection. Females with low pre-treatment HCV-RNA and HBV-DNA levels were independently but positively associated with high expression level of IFNAR1 in PBMCS.

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