RESEARCH ARTICLE

Interferon alpha receptors and STAT1 as therapy predictors of a sustained virological response in hepatitis C/B co-infection

Maleha Asim,[1] Amir Rashid,[2] Saleem Ahmed Khan[3]

Abstract

Objective: To determine the expression of interferon alpha receptors 1 and 2 along with signal transducer and activator of transcription-1 in peripheral blood mononuclear cells of both hepatitis C mono-infected and hepatitis C and B co-infected patients, and to assess whether these targeted genes predict sustained virological response to interferon therapy.

Methods: This cross-sectional study was carried out at the Army Medical College, Rawalpindi, Pakistan, from January 2012 to December 2015, and comprised hepatitis C mono-infected and hepatitis C and B co-infected patients. The patients were divided into groups 1 and 2. Group-1a and group-2a consisted of mono-infected and co-infected sustained responders, while group-1b and group-2b had mono-infected and co-infected non-sustained responders. Peripheral blood mononuclear cells from healthy controls were also quantified for these subunits. Target gene expressions were studied by retro-transcription of respective messenger ribonuclieic acid extracted from the cells followed by polymerase chain reaction amplification.

Results: Of the 191 subjects, there were 20(10.5%) in group-1a, 35(18.3%) in group-2a, 65(34%) in group-1b and 51(26.7%) in group-2b. The remaining 20(10.5%) were controls. Overall, 106 (55.5%) were males and 85 (44.5%) were females. Interferon alpha receptor 1 expression in groups 1a and 2a was significantly higher compared to groups 1b (p=0.018) and 2b (p=0.031). Signal transducer and activator of transcription-1 protein expression showed no significant difference (p=0.062 and p=0.519). No difference in expression was measured between the two sets of groups with regard to interferon alpha receptor 2 expression (p=0.278 and p=0.590).

Conclusion: Our results show that levels of IFNAR-1 mRNA expression may be a good predictor for IFN-related anti-viral activity in both HCV mono-infected and HCV/HBV co-infected patients.

Keywords: Hepatitis C virus, Hepatitis B virus, Interferon alpha receptor 1, Interferon alpha receptor 2, Interferon therapy response, Signal transducer and activator of transcription 1.
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Introduction

Due to shared mode of transmission, hepatitis C virus (HCV)/hepatitis B virus (HBV) co-infection represents significant public health issues worldwide. Owing to hepatotropic nature, HCV and HBV co-infection is common in highly endemic areas and among subjects with increased possibility of parental infections.[1] HCV causes persistent infection in 60-65% of HCV mono-infected and 70-80% of HCV/HBV co-infected patients.[2] Exogenous interferons (IFNs) are the main anti-viral cytokines with pleiotropic activities that act as a crucial factor in the innate immune system which provide mechanisms to defend the host from infection by other organisms. Pegylated interferon-α (Peg-IFN-α) in combination with ribavirin is currently the standard-of-care therapy for HCV mono-infected and HCV/HBV co-infected patients.[3] However, it produces sustained viral clearance in only 40-50% of treated patients and is associated with significant intimidating effects.[4] IFN therapy response rate varies in all populations and the reasons for treatment failure are associated with host, virus and immune response variables. Identifying the biomarkers predicting response to IFN therapy would also assist clinician to treat patients infected with HCV-

[1]Department of Biochemistry, Foundation University Medical College,
[2]Department of Biochemistry, [3]Department of Pathology, Army Medical College, NUMS, Islamabad.
Correspondence: Dr Maleha Asim. e-mail: dr.maleha1@gmail.com

associated cirrhosis or hepatocellular carcinoma.[5,6] It is now known that IFN-α demonstrates its anti-viral activity via cognate receptors, including IFN-α receptor 1 (IFNAR-1) and IFNAR-2. The IFN-α receptors are single-membrane spanning proteins and responsible for signal transduction by increasing binding of ligands.[7] Many researchers assessed the amount of IFN receptors' expression and their significant role in response to IFN treatment and patients with sustained virological response (SVR) were found to have significantly higher expression levels of IFNAR-1 and IFNAR-2 compared to non-sustained responders.[8]

The molecular event of the signalling pathway is triggered by different cytokines such as IFN receptors and Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling pathway. These act together in a relatively simple and direct signalling cascade capable of activating gene transcription. Signal transduction is mediated by rapid tyrosine phosphorylation of IFNAR-1 and JAK proteins, leading to activation of downstream STATs by phosphorylating critical serine and tyrosine residues. STAT proteins are the key mediators of cytokine-induced gene expression and exert active role in anti-viral, anti-mycobacterial and anti-tumour responses against hepatitis.[9] A recent review described multiple mechanisms related to JAK-STAT signalling pathway for modulation of HCV infection.[10] Activated STAT-1 and STAT-2 bind IFN-regulatory factor 9 and form a heterotrimeric complex called IFN-stimulated gene factor3 (ISGF-3). Consequently, ISGF-3 binds to IFN-stimulated response elements in the promoters of IFN-stimulated genes, thereby activating transcription.[11] IFN receptors' expression and STAT may be directly involved in response to therapy and pathogenesis of viral hepatitis. There is evidence that IFNs can activate non-STAT signalling pathways and modulate gene expression to shape IFN-induced bioactivities.[12]

The molecular mechanism of viral persistence and the pathogenesis of hepatitis C and hepatitis B are poorly understood. To learn more about molecular mechanisms underlying IFN treatment failure, the current study was planned to investigate the relationship between trans-membrane receptor genes IFNAR-1 and IFNAR-2 and cytoplasmic protein STAT-1 expression to Peg-IFN-α plus ribavirin responsiveness among patients with HCV mono-infection and HCV/HBV co-infection.

Patients and Methods

This cross-sectional study was conducted at the Centre for Research in Experimental and Applied Medicine (CREAM), Department of Biochemistry and Molecular Biology, Army Medical College, Rawalpindi, Pakistan, from January 2013 to December 2015. Patients were enrolled from the Armed Forces Institute of Pathology, Benazir Bhutto Hospital and Holy Family Hospital, Rawalpindi, over a one-year period. The sample size was estimated using World Health Organisation's (WHO) calculator using prevalence of 4.8% for HCV mono-infection and 1.3% for HCV/HBV co-infection among patients seeking hospital care in Islamabad, Pakistan, along with 95% confidence interval (CI) and 5% margin of error.[13] Research approval was obtained from the institutional ethics committee of Army Medical College and the study followed the principles of Declaration of Helsinki.[14] The purpose and benefits of the study were explained to each participant and informed written consent was obtained.

By using non-probability convenience sampling, we enrolled treatment-naïve HCV mono-infected and HCV/HBV co-infected patients along with healthy controls. Out of the eligible patients, some were also positive with hepatitis B surface antigen (HbsAg) along with serum HBV-deoxyribonucleic acid (DNA) level of ?2000 IU/mL determined by using Roche Cobas® Amplicor HBV Monitor assay (Roche Molecular Systems, Branchburg, USA).

Those enrolled were untreated adults aged 20 years or older with seropositive both for HCV antibodies (anti-HCV) along with serum HCV-RNA of $\geq$200IU/mL determined by third-generation enzyme-linked immunosorbent assay (ELISA) and Cobas® Amplicor HCV Monitor v2.0 (Roche Molecular systems, Pleasanton, CA, USA) respectively. Patients with hepatitis (A, D or E), human immunodeficiency virus (HIV) infection or decompensated liver disease, and those with pre-existing psychiatric illness were excluded.

Participants were treated with subcutaneous Peg-IFN-α-2b; (Pegintron®, Schering Plough Pharmaceutical Co. Ltd., Tokyo, Japan) in weekly doses adjusted to body weight along with oral ribavirin 800mg/day if body weight was $\leq$75 kg and of 1000 mg/day if >75 kg for duration of 24 weeks. The efficacy end-point was SVR,

and patients were defined as HCV-sustained responders if they had normal serum alanine aminotransferase (ALT) along with undetectable serum HCV-RNA levels 6 months after the cessation of therapy. All other patients were defined as non-sustained responders (NRs).[4] Subjects were divided into groups. Group 1 contained HCV mono-infected patients who were further subdivided into two groups depending upon achievement of SVR; group 1a had sustained responders and group-1b had NRs. Group 2 contained HCV/HBV co-infected patients and was subdivided into group 2a that had sustained responders and group 2b had NRs. Group 3 contained healthy subjects that were negative for both anti-HCV and HBsAg and showed serum ALT levels within normal limits. These volunteers were recruited from Army Medical College.

For expression analysis of IFNAR-1, IFNAR-2 and STAT-1, blood samples were collected from all subjects before the start of IFN therapy. GeneJET™ RNA Purification Kit (Thermo Fermentas, USA) was used to purify total RNA. Synthesis of first strand complementary DNA (cDNA) was carried out using Revert-Aid Premium First Strand cDNA Synthesis kit (ThermoFermentas, USA), using RNA as a template.

The sequences of the IFNAR-1 primer used in the study were as follows: Forward Primer, 5'GGAACAGGAGCGATGAGTCT3'; Reverse Primer, 5'TGAGCTTTGCGAAATGGTGT3'; GC Content, 45%; TM, 58.68°C and Length, 235. The sequences of the IFNAR-2 primer are: Forward Primer, 5'ACAAGTGGCGGTGGCTATAC3'; Reverse Primer, 5'TCAGGATCCTCTGGGTCAAC3'; GC Content, 55%; TM, 54°C and Length, 20. The sequences of the STAT-1 primer were: Forward Primer, 5'GTCGGGGAATATTCAGAGCA3'; Reverse Primer, 5'TGATCACTCTTTGCCACACC3'; TM, 51°C and Length, 200.

The following protocol was used for the amplification of IFN-α receptors and STAT-1: cDNA/RNA (xng), 10x polymerase chain reaction (PCR) buffer (1x), magnesium chloride (MgCl2) (25mM), deoxyribonucleotide triphosphates (dNTPs) (2mM), Forward primer (1pmol), Reverse primer (1pmol), Taq polymerase (1unit) and autoclaved distilled water (xµl) with total PCR of 25µl. The reactions were performed on corbet Inc. PCR machine.

IFNAR-1 sequence amplification was carried out by using following cycling conditions: 35 cycles, hot start at 95°C for 5 minutes, denaturation at 93°C for 30 seconds, followed by annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, final elongation at 72°C for 7 minutes and hold at 4°C. IFNAR-2 sequence amplification was carried out by using following cycling conditions: 35 cycles, hot start at 95°C for 6 minutes, denaturation at 94°C for 45 seconds, followed by annealing at 54.5°C for 45 seconds, extension at 72°C for 1.3 minutes, final elongation at 72°C for 10 minutes and hold at 4°C. STAT1 sequence amplification was carried out by using following cycling conditions: 30 cycles, hot start at 98°C for 5 minutes, denaturation at 98°C for 30 seconds, annealing at 51°C for 30 seconds, 30 seconds extension at 72°C, final elongation step at 72°C for 10 minutes and hold at 4°C.

Data was expressed as mean ± standard deviation (SD) or frequencies along with percentages. Continuous data was statistically analysed using student's t test and for categorical data, chi-square test or Fischer's exact test was performed on SPSS 20. P<0.05 was considered statistically significant.

Results

Initially, 194 patients were enrolled, but treatment had to be discontinued in 23(12%) cases due to adverse effects. Of the eligible 171(88%) patients, 86(50.3%) were also positive with HbsAg. The final study sample had 191 subjects. Of them, there were 20(10.5%) in group-1a, 35(18.3%) in group-2a, 65(34%) in group-1b and 51(26.7%) in group-2b. The remaining 20(10.5%) were controls.

Table-1: Characteristics of patients and control included in the study.

Characteristics	Group 1 HCV mono-infected (n= 85)	Group 2 HCV/HBV co-infected (n= 86)	Group 3 Control (n = 20)
Mean Age, years	31.20 ± 5.04	36.33 ± 8.61	31.70 ± 6.78
Gender			
Male	47 (55.3%)	48 (55.8%)	11 (55%)
Female	38 (44.7%)	38 (44.2%)	09 (45%)
BMI, kg/m^2	23.32 ± 2.07	24.72± 1.81	22.60 ± 2.68
AST, U/L	45.20 ± 15.10	38.12 ± 8.56	26.55 ± 6.6
ALT, U/L	61.89 ± 20.44	65.07 ± 21.03	31.20 ± 7.19
HCV-RNA, >4×10^6 IU/ml	5.48 ± 1.2	5.84± 1.3	Not detected
HBV-DNA, >2×10^4 IU/ml	Not detected	4.18 ± 1.90	Not detected
IFNAR-1 mRNA	47 (55.29%)	63 (73.25%)	20 (100%)
IFNAR-2 mRNA	72(84.7%)	54 (62.7%)	20 (100%)
STAT-1	75 (88.23%)	54(62.7%)	14 (70%)

BMI: Body mass index, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase
HCV: Hepatitis C virus, HBV: Hepatitis B virus, IFNAR: Interferon alpha receptor
STAT: Signal transducer activator of transcription,
Data presented as mean ± Standard Deviation or n (%)

Overall, 106 (55.5%) were male and 85 (44.5%) were female. Comparison of demographic and clinical characteristics among the groups was done (Table-1). Mean age and serum ALT were significantly higher in group 2 compared to the other two groups ($p<0.05$), while no difference was observed with respect to gender and body mass index (BMI ($p>0.05$). STAT-1 expression level was significantly increased in group 1 compared to the other two groups ($p<0.05$). However, expression rates of IFN-α receptors were significantly higher in group 3 compared to the patient groups with regard to IFNAR-1 mRNA and with regard to IFNAR-2 mRNA ($p<0.05$).

Table-2: Characteristics of HCV mono-infected responders and non-responders.

Characteristics	HCV mono-infected (n= 85) Responders (n = 20)	Non-responders (n = 65)	P value
Age, years	32.92 ± 5.6	37.61 ± 8.4	0.010
Gender	0.318		
Male	13 (65%)	34 (52%)	
Female	07 (35%)	31 (48%)	
BMI, kg/m^2	23.60 ± 2.1	23.23 ± 2.06	0.489
AST, U/L	40.05 ± 12.24	46.78 ± 15.62	0.081
ALT, U/L	49.05 ± 17.65	65.84 ± 19.71	0.001
HCV-RNA, $>4\times10^6$ IU/ml	4.79 ± 1.47	5.72 ± 1.11	0.001
IFNAR-1 mRNA	20 (100%)	27 (41.5%)	0.018
IFNAR-2 mRNA	20 (100%)	52 (80%)	0.278
STAT-1	20 (100%)	55 (84.6%)	0.062

Statistically significant (P<0.05), BMI: Body mass index, ALT: Alanine aminotransferase
AST: Aspartate aminotransferase, HCV: Hepatitis C virus, HBV: Hepatitis B virus
IFNAR: Interferon alpha receptor, STAT: Signal transducer activator of transcription
Data presented as mean ± Standard Deviation or n (%)

Table-3: Characteristics of HCV/HBV co-infected responders and non-responders.

Characteristics	HCV/HBV co-infected (n= 86) Responders (n = 35)	Non-responders (n = 51)	P value
Age, years	34.77 ± 7.21	38.56 ± 7.93	0.027
Gender	0.498		
Male	18 (51.4%)	30 (58.8%)	
Female	17 (48.6%)	21 (41.2%)	
BMI, kg/m^2	22.82 ± 2.8	24.14 ± 3.6	0.078
AST, U/L	36.11 ± 6.29	39.50 ± 9.63	0.071
ALT, U/L	59.00 ± 21.62	69.24 ± 19.77	0.026
HCV-RNA, $>4\times10^6$	5.25 ± 1.13	6.25 ± 1.33	0.001
HBV-DNA, $>2\times10^4$	3.49 ± 1.90	4.66 ± 1.76	0.004
IFNAR-1 mRNA	30 (85.7%)	33 (64.7%)	0.031
IFNAR-2 mRNA	23 (65.7%)	31 (60.7%)	0.590
STAT-1	20 (57.1%)	34 (66.6%)	0.519

Statistically significant (P < 0.05), BMI: Body mass index, ALT: Alanine aminotransferase
AST: Aspartate aminotransferase, HCV: Hepatitis C virus, HBV: Hepatitis B virus
IFNAR: Interferon alpha receptor, STAT: Signal transducer activator of transcription
Data presented mean ± SD or n (%).

Comparison between groups 1a and 1b showed significant difference with respect to mean age (p=0.010), serum ALT (0.001), serum AST (p=0.081) and HCV-RNA (p=0.001) levels (Table 2). Activated STAT-1 was more detectable in peripheral blood mononuclear cells (PBMCs) of group 1a patients compared to group 1b patients, but the result was not statistically significant (p=0.062). IFNAR-1 mRNA expression showed significant difference between groups 1a and 1b (p=0.018). However, reverse transcriptase PCR of IFNAR-2 mRNA expression revealed no statistically significant difference (p=0.278).

Differences in clinical examination and gene expression rates were also assessed in HCV/HBV co-infected patients in groups 2a and 2b. Serum ALT (p=0.026), HCV-RNA (p=0.001), HBV-DNA (p=0.004) levels as well as the age of the patients (p=0.027) were significantly increased in group 2b. IFNAR-1 gene expression was significantly higher in group 2a compared to group 2b (p=0.031). However, regarding IFNAR-2 mRNA and STAT-1 protein expressions, no difference was observed between groups 2a and 2b (p=0.590 and p=0.519) (Table-3).

Discussion

HCV and HBV are global public-health problems and affecting a significant proportion of Pakistani population. Since IFN therapy is expensive and often poorly tolerated by the patients, it would be clinically valuable to assess the biomarkers that predict the efficacy of IFN therapy in HCV mono-infected or HCV/HBV co-infected patients. IFN-α receptors and STAT-1 proteins are critical components of the cellular anti-viral and anti-proliferative responses induced by IFNs.[15-16]

The present study was aimed at understanding the significance of IFN receptor-mediated JAK-STAT molecular mechanism, primary end-point included determination of expression rates of STAT-1, IFNAR-1 and IFNAR-2 mRNA in HCV mono-infected and HCV/HBV co-infected responders in comparison with non-responder patients to justify which gene expression was more down-regulated among non-responders to therapy and failed to predict SVR.

The mechanism of IFN-α and ribavirin (RBV) resistance is likely connected to host-related factors, which is supported by numerous studies.[17] The demographic and clinical characteristics of 20 control subjects showed significantly lower serum AST and ALT levels compared to 85 HCV mono-infected and 86 HCV/HBV co-infected patients. All

control subjects showed expression of IFN-α receptors (100%) and 30% failed to express STAT-1.

In this study, pre-treatment parameters significantly associated with sustained virological response were lower age (p=0.010), lower AST level (p=0.081), lower ALT levels (p=0.001) and lower serum HCV-RNA levels (p=0.001) among HCV mono-infected patients. According to our results, all HCV mono-infected responder patients showed expression of IFNAR-1 relative to 27 NRs (100% vs. 41.5%; p=0.018). This data verifies that the SVR in HCV mono-infected patients is due to induction of IFNAR-1 leading to substantially improved responses of JAK-STAT signalling pathway to IFN-α and RBV combination therapy. Study data (Table-2) indicates a direct link between expression rate of IFNAR-1 and inhibition of HCV replication mediated by IFN therapy. The IFNAR-1 significance can be observed from a study where restored IFNAR-1 expression alone was able to restore defective JAK-STAT pathway, STAT-1 and STAT-3 phosphorylation and anti-HCV response in IFN-α resistant cell lines.[18] In response to HCV replication, IFNAR-1 expressions are down-regulated in PBMCs of HCV mono-infected non-responders (n=38; 58.4%) resulting in poor response to IFN therapy and only 27(41.5%) patients showed expression of IFNAR-1. Several other studies have also shown expression of IFNAR-1 in IFN-resistant cell lines and IFN-resistant HCV patients.[19] The reason of IFN resistance in patients with positive IFNAR-1 expression may be other mutations in IFNAR-1 sequence as observed earlier.[20-21]

Among HCV mono-infected patients, IFNAR-2 and STAT-1 protein were highly expressed in both responders as well as non-responders. Despite high levels of expressions, most non-sustained responders failed to achieve SVR (p=0.278 and p=0.062 respectively). The reason may be attributable to other factors such as viral and host factors that inhibit IFN signalling mechanism. The data reported herein is in accordance with a model in which no significant association was found between expression levels of IFNAR-2, STAT-1 and virological response to IFN therapy.[22] The high level of these proteins may inhibit IFN-alpha therapy and contribute to IFN therapy failure in these patients.

Co-infection with HCV and HBV is common and has a detrimental influence on the natural history of chronic hepatitis C. Among HCV/HBV co-infected patients, 30/35 patients expressed IFNAR-1 and significantly responde to IFN therapy compared to 33/51 patients, who failed to achieved virological response (85.7% vs. 64.7%; p=0.031) (Table-3). According to a study, presence of HBV protein-X (HBX) may down-regulate IFNAR-1, leading to avoidance of extracellular type-I IFN-mediated signalling.[23] Persistence of HBV-DNA is another factor leading to poor response to IFN therapy by suppression of cell mediated immunity and increasing susceptibility to progression of hepatocellular carcinoma in co-infected patients.[24,25] Similarly, lower expression of IFNAR-1 in our study may be the reason behind weak binding affinity of ligand, leading to IFN resistance in HCV/HBV non-responder patients.[26]

Lastly, we assessed IFNAR-2 mRNA and STAT-1 expression levels and their association with IFN therapy outcomes in HCV/HBV co-infected patients. Our data showed that although IFNAR-2 and STAT-1 expressions are induced by IFN in both responders and non-responders, no significant difference was observed between two groups following IFN-α stimulation (p=0.590 and p=0.519 respectively). Accumulating evidence suggested that IFNAR-2 and STAT1 activation plays a pro-inflammatory role in the hepatic disease pathogenesis and may contribute to IFN therapy failure.[27]

Our results highlighted a direct link between IFNAR-1 mRNA expression and activation of host innate immunity leading to inhibition of HCV replication. IFNAR-1 mRNA represent a significant predictor in both HCV mono-infected and HCV/HBV co-infected patients compared to STAT-1 and IFNAR-2 mRNA. This observation is consistent with a previous study reporting microsatellite polymorphism of the IFNAR-1 promotor region but not the IFNAR-2 promotor region having an influence on the levels of type I IFN-mediated signalling and, thus an association with positive response to IFN therapy. On the other hand, although IFNAR-2c is associated with signal transduction, both IFNAR-2a and IFNAR-2b act in a dominant-negative manner leading to suppression of IFN signaling.[28]

Similarly, the lack of correlation between IFNAR-1 phosphorylation and STAT activation potency suggests that IFN-α responsiveness must be achieved through JAK-independent pathway.

There are a few limitations in our study. First, intrahepatic expression of IFNAR and STAT-1 was not performed in the patients. Second, the exact molecular mechanisms for the influence of IFNAR on SVR in HCV mono-infected and

HCV/HBV co-infected patients have not been clarified. Thirdly, patients with occult HBV infection were not included in this study. Hence, further studies are required to find out the concrete mechanism.

Conclusion

In HCV mono-infected and HCV/HBV co-infected patients, expression rate of IFNAR-1 mRNA maybe useful index for predicting long-term efficacy of IFN therapy compared to STAT-1 and IFNAR-2. Replication of HBV-DNA is not the main factor leading to down-regulation of IFN-α receptors or STAT-1.

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