ORIGINAL ARTICLE

INTERFERON SIGNALLING PATHWAY: INTRAHEPATIC EXPRESSION OF STAT1 IN INTERFERON RESISTANT HEPATITIS C PATIENTS

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ABSTRACT

OBJECTIVE: to investigate the role of STAT1 in treatment failure in interferon resistant hepatitis C virus (HCV) infected patients.

METHODOLOGY: Liver biopsies and blood from patients infected with HCV, resistant to interferon therapy were collected under sterile conditions in the laboratory of Army Medical College, Rawalpindi. Study included HCV infected patients who were positive for serum HCV RNA, and were non-responders to two courses of interferon therapy, IFN-α injection and ribavirin oral for 24 weeks periods. Study also included 10 controls i.e. 5 normal healthy subjects with normal LFTs who were negative for anti-HCV antibodies and 5 responder patients who responded to first course of interferon treatment and had normal LFTs. The liver biopsy specimens were subjected to PCR based detection of STAT1 and liver histopathology status evaluation. GeneJET™ RNA Purification Kit# K0731, Fermentas was used for total RNA isolation from liver biopsy specimen. The study was approved by institute's ethical committee.

RESULTS: STAT1 mRNA was detected in 96.2% (n=25/26) of the HCV patients resistant to interferon therapy and in 100% of the normal as well as responders. Patient's distribution for histological activity index along with STAT1 mRNA expression was 68% in the score of 4-7 and 28% in the score of 8-12.

CONCLUSION: STAT1 mRNA is being expressed by all the HCV infected patients resistant as well as responders to interferon therapy. STAT1 expression is not a major factor responsible for interferon resistance in HCV patients.

KEY WORDS: HCV, Interferon Resistance, STAT1, Intrahepatic Expression.

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INTRODUCTION

Signal transducers and activators of transcription (STATs) play important role in antiviral immune defence, inflammation development, apoptosis and antitumor responses. Insufficient expression can hinder interferon (IFN) signalling cascade resulting in interferon resistance in HCV patients.[1]

STATs, a family of cellular proteins, mediate extracellular signals transduction as a result of growth factors and cytokines for example IFNs and Interleukin 6 (IL-6) resulting in direct regulation of transcription. Cytokines interaction with receptors on cell surface results in STATS phosphorylation by JAK (Janus kinase). On phosphorylation, STATs homo/heterodimerise, translocate to the nucleus and regulate the STAT responsible genes transcription. STAT1, STAT2 and STAT3 activation is essential for IFN induced antiviral action[2].

IFN- α/β interacts with the receptor resulting in its dimerization which in turns activates JAKs that phosphohorylates cytolpasmic domain of IFNAR1/2. STAT1 and STAT2 recruit to IFNAR1/2 receptor, get phosphorylated, associate with interferon regulatory factor-9 and form interferon-sensitive gene factor-3 (ISGF3) which then after nucleus translocation regulates expression of interferon-sensitive genes (ISGs) by trans-activating interferon-sensitive response elements (ISRE)[3]. IFN-inducible genes/ proteins i.e., RNA-dependent protein kinase (PKR), Major histocompatability complex (MHC), 2',5'-oligoadenylate synthetase (OAS) and Mx proteins inhibit viral replication and enhance host's antiviral immune responses[4].

About 2-3% of world's population (5-8) and ∼4% Pakistani population is victim of HCV5-8. Chronic infection develops in about 85% acutely infected HCV patients[9]. IFN-α plus ribavirin is currently approved standard therapy for HCV treatment4 with ~50% success rate[4,10]. This marks the importance to understand underlying mechanism resulting in interferon resistance. The findings can prove to be helpful in predicting the treatment response thus reducing patient's suffering and making therapy cost effectiveness.

Keeping in view the importance of the cellular factors, a study was conducted where qualitative PCR was used as a tool to analyse the expression of STAT1 in HCV patients resistant to interferon therapy.

Objective of the study was to detect STAT1 mRNA in liver biopsies of HCV patients resistant to interferon therapy to figure out whether presence or absence of STAT1 mRNA is playing any role in treatment failure?

METHODOLOGY

Ethical Committee Approval

Research was conducted after Institutional Ethics Committee approval. Written informed consent was obtained from each patient participating in the study.

Samples and Patients

In order to analyse the qualitative expression of STAT1 in interferon resistant HCV patients, a study was conducted on liver biopsy samples of interferon resistant HCV infected patients that were stored at -80°C at CREAM laboratory, Army Medical College, Rawalpindi. Conventional PCR was carried to determine whether the STAT1 mRNA is being produced in the liver biopsy samples of patients included in the study.

Study also included 10 controls i.e. 5 normal healthy subjects with normal LFTs who were negative for anti-HCV antibodies and 5 responder patients who responded to first course of interferon treatment and had normal LFTs. Samples were taken from both male and female with age range between 20 to 69 years. All the patients had elevated alanine amino tranferase (ALT) levels as compared to the healthy and responders subjects. The HCV patients were positive for serum HCV RNA, and were non-responders to two courses of interferon therapy (IFN-α injection of 3 million IU dose three times per week and ribavirin 10 mg/kg body weight/ day dose for 24 weeks periods)[11].

Primer Designing

Primer sequences designed specifically for STAT 1 (expected fragment size: 198bp) are:

Forward Primer:
5'GTCGGGGAATATTCAGAGCA 3'

Reverse Primer:
5'TGATCACTCTTTGCCACACC 3'

RNA Extraction and Reverse Transcription

GeneJET™ RNA Purification Kit (Cat# K0731, Fermentas) was used for total RNA isolation from liver biopsy specimen (weight up to 30 mg). Purified RNA (1pg - 5μg) was used for first strand cDNA synthesis using Fermentas Revert Aid Premium First Strand cDNA Synthesis Kit (Cat# K1652) using Oligo (dT) 100 pmol, 0.5 mM final concentration of dNTP Mix (Cat# R0192, Fermentas), 4μl 5X RT Buffer, 1μl RevertAid Premium Enzyme Mix making final volume of the reaction mixture up to 20μl by adding Nuclease-free Water. Gentle mixing was done. For oligo (dT)18 primer and gene-specific primer, incubation was done for 30 minutes at 50°C. Reaction was terminated by heating the tube at 85°C for 5 minutes. The first strand cDNA synthesized was then directly used for PCR reaction.

TABLE 1: CYCLING CONDITIONS

Cycle Step	Temperature	Time	Cycles
Initial denaturation	98°C	5 minutes	1
Denaturation	98°C	30 seconds	30
Annealing	51°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C 4°C	10 minutes Hold	1

PCR

A 5μl volume of first strand cDNA synthesized was used as template for 50μl total volume of PCR reaction. Taq DNA Polymerase (Cat # EP0402, Fermentas) was used to amplify STAT1. The reaction mixture contained final concentrations of 20μM each of the forward and reverse primer, 1X PCR buffer, 1.5mM MgCl2, 0.25mM each dNTPs and 0.5Units Taq DNA polymerase.

The following cycling conditions (Table 1) were used to amplify STAT1 sequence from cDNA previously synthesized.

To visualize STAT1 PCR products, 2% agarose gel stained in 0.1% ethidium bromide (EB) solution was used. Bromophenol blue (BPB) was used as loading dye. Data obtained was analyzed statistically using SPSS Version 16.

RESULTS

The mean age of both the control groups i.e., normal and responder was lower as compared to non-responders. Males in non-responder group were two times as compared to females i.e., 17 males and 9 females. BMI, liver enzymes, and liver ultrasound reports were normal in both control groups as compared to the non-responders who have advanced liver pathology.

Intrahepatic expression of STAT1 was detected in all (100%) of the normal (n=5) as well as responders (n=5) and 25/26 (96.1%) non-responders were also positive for intrahepatic expression of STAT1 mRNA as shown in fig 2. Among 26 non-responders, 5 patients were of liver cirrhosis and only one was negative for intrahepatic expression of STAT1. The STAT1 expression along with liver histopathology data is shown in the Table II and III.

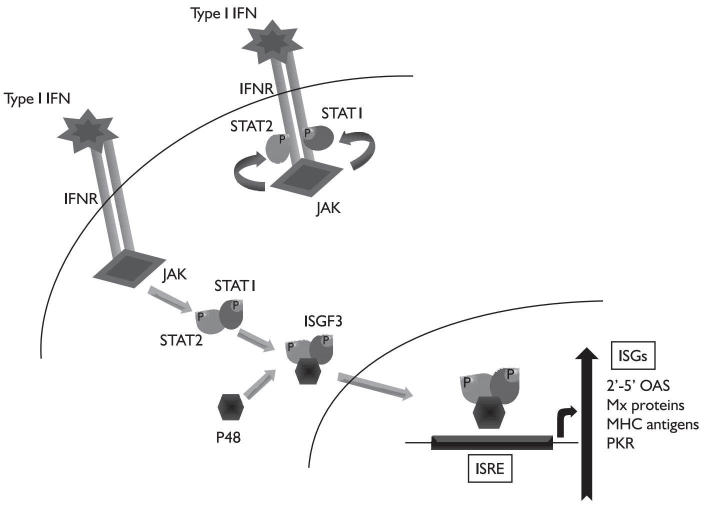

Fig1. Interferon Signaling Pathway

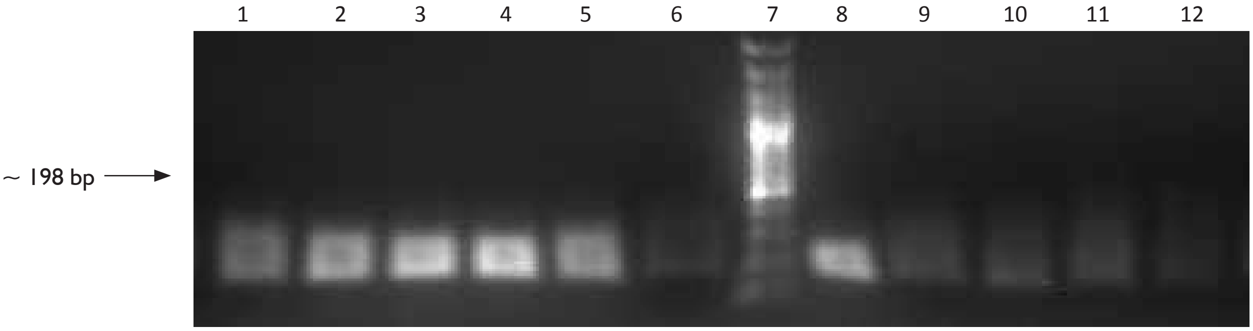

Fig 2: Qualitative PCR analysis of STAT1 gene expression in patients infected with HCV.

Lanes 1-5 contain samples from the HCV infected patients responders to IFN treatment. Lane 7 contains 100bp DNA Ladder (Cat # SM0323 , Fermentas). Lanes 6, 8-12 contain samples from HCV patients non-responders to IFN treatment.

TABLE II: PATIENTS DISTRIBUTION FOR FIBROTIC STAGES ALONG WITH STAT1 MRNA EXPRESSION DATA

Fibrotic Stages	STAT1 Positive		STAT1 Negative	
	Frequency (n=25)	%Age	Frequency (n=1)	%Age
Stage 1	1	4	0	0
Stage 2	21	84	0	0
Stage 3	3	12	0	0
Stage 4	0	0	1	100
Stage 5	0	0	0	0
Stage 6	0	0	0	0

TABLE III: PATIENTS DISTRIBUTION FOR HISTOLOGIC ACTIVITY INDEX ALONG WITH STAT1 MRNA EXPRESSION DATA

Histologic Activity Index (HAI)	STAT1 Positive		STAT1 Negative	
	Frequency (n=25)	%Age	Frequency (n=1)	%Age
HAI Score: 0-3	1	4	0	0
HAI Score: 4-7	17	68	0	0
HAI Score: 8-12	7	28	1	100
HAI Score: >12	0	0	0	0

DISCUSSION

Standard interferon treatment has failure rate in ~50% of the HCV infected patients and treatment failure can be contributed by viral or host factors or can be due to molecular mechanism induced by HCV proteins that inhibit IFN signalling cascade[10]. The purpose of this study was to investigate a cellular factor i.e., STAT1. The expression analysis was carried out on liver biopsies of genotype 3 infected HCV patients already stored in the laboratory. The former investigations we conducted showed that sex (2/3 of the patients being males), age (mean age of ~41years), elevated LFTs and BMI in overweight category can be the reasons responsible for interferon treatment response apart from STAT1[11] and our data was supported by the studies conducted earlier[10,12-15].

STAT1 expression was detected in 25 out of 26 HCV patients non-responders to interferon therapy. The STAT1 expression was detected in all the responders and normal controls. There was no association between serum levels and STAT1 expression. The results were similar to the study conducted by Manal[2]. Only one of the liver cirrhosis patients was negative for STAT1 expression. There can be other reasons e.g. STAT1 phosphorylation disruption by HCV NS5A[16], HCV core protein or STAT1 ubiquitin-mediated proteasome-dependent degradation by the HCV core protein[17]. So the study at protein level considering the protein phosphorylation status in particular can further confirm whether or not STAT1 is playing any role in interferon resistance in HCV infected Pakistani subjects.

The STAT1 expression as well as protein degradation are the steps where HCV can influence to block Jak-STAT pathway. We observed that there was no expression of STAT1 in only one out of 26 patients. Our study is supported by another study conducted by Lin et al[18]., 2005 who observed that STAT1 expression and P-STAT1 accumulation in nucleus was decreased as result of HCV expression. HCV core protein degrades STAT1. STAT1 plays crucial role in innate immune response against HCV expression. In turn, HCV supresses Jak-STAT kinase by STAT1 degradation[18]. This study also supports our findings that if STAT1 is expressing then HCV might be influencing at the protein degradation level.

There was expression observed in most of the patients in our study which was supported by other studies conducted by Bautista et al[19] and El-Saadany et al[20]. The studies showed that the STAT1 expression was normal among the responders and the non-responders but the PIAS1 i.e., protein inhibitor of activated STAT1 over expression may result in defective response to IFN therapy[19,20].

This study has limitation as it does not explain any single nucleotide polymorphism. It is the qualitative PCR analysis of the STAT1 showing either STAT1 is expressing or not and to which extent the expression hinder interferon signalling pathway. Our study has shown that STAT1 is being expressed by almost all patients but there can be some other factors e.g., degradation at protein level etc. that can hinder the interferon signalling cascade.

A broad study emphasizing analysis of multiple factors at the same time i.e., viral and host factors at both RNA and proteins levels can help figure out the significant factors contributing interferon resistance in HCV infected Pakistani subjects.

CONCLUSION

STAT1 expression is not a major factor responsible for interferon resistance in HCV patients. STAT1 mRNA is being expressed by all the HCV infected patients resistant to interferon therapy as well as respondents to interferon therapy and normal controls. There can be other factors STAT1 protein degradation, viral and cellular factors that can affect IFN stimulated signalling pathway.

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AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under:

QB, AR & AKN:	Conception and design, acquisition of data, drafting the manuscript, final approval of the version to be published
RSAK:	Critical revision, drafting the manuscript, final approval of the version to be published
SR:	Analysis and interpretation of data, final approval of the version to be published
AY:	Critical revision, final approval of the version to be published

CONFLICT OF INTEREST

Author declares no conflict of interest

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NIL

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