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Research article

Assessment of ubiquitin specific Peptidase-18 gene in peripheral blood of chronic hepatitis C patients treated with direct-acting antiviral drugs

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ABSTRACT

Hepatitis C virus (HCV) infection remains one of the leading causes of liver complications globally. Ubiquitin Specific Peptidase-18 (*USP18*) is a ubiquitin-specific protease that cleaves interferon-stimulated gene 15 (ISG15) from ISGylated protein complexes and is involved in regulating interferon responsiveness. To study the effect of direct-acting antivirals (DAAs) on the *USP18* gene using qPCR, 132 participants were recruited and classified into different groups based on treatment duration. *USP18* expression was raised compared to rapid virologic response (RVR) and early virologic response (EVR) groups with $P = 0.0026$ and $P = 0.0016$, respectively. *USP18* was found to be 7.36 folds higher in naïve patients than those with RVR and sustained viral response (SVR). In RVR and SVR groups where patients had cleared HCV RNA after treatment with direct-acting antiviral agents (DAA) therapy, the expression of *USP18* was found to be low, with a fold change of 1.3 and 1.4 folds, respectively. Expression of *USP18* was significantly higher in the non-RVR group than in the RVR group. In the No EVR group, gene expression was significantly higher than in the EVR group. It is concluded that targeting HCV proteins using DAAs can cause *USP18* expression to be normalized more effectively. Moreover, *USP18* is a vital marker indicating treatment resistance and distinguishing responders from non-responders during DAA therapy.

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1. Introduction

Chronic hepatitis C is a major global health problem, with an estimated 58 million infected people and 290,000 deaths due to cirrhosis, hepatocellular carcinoma (HCC), and other liver complications in 2019 [1]. It is the leading cause of liver diseases and is responsible for the mortality and morbidity of millions of people worldwide. HCV belongs to the family *Flaviviridae,* and its genome comprises 9500 nucleotides in a positive single-stranded RNA molecule. The single large open reading frame (ORF) encodes a single polyprotein precursor of 3000 amino acids [2]. HCV genome has a high degree of genetic heterogeneity. Due to the lack of a proof-reading mechanism for HCV RNA-dependent RNA polymerase, seven closely related genotypes have a high degree of sequence similarity and unique clinical implications [3]. Genotype 3 is the second most abundant type of HCV genotype globally, accounting for more than 40 % of HCV infections in Asia. In the Pakistani population, genotype 3a is the most common genotype responsible for HCV infections [4–6].

The development of treatment strategies for HCV progressed steadily since the discovery of the virus in 1989. Before the arrival of direct-acting antiviral agents (DAA) into the market, Interferon-α and ribavirin were used to treat HCV. Interferon (IFN) does not act directly on the virus or viral replication but induces an antiviral state by activating a certain set of genes called interferon-stimulated genes (ISGs) [7]. Ribavirin is a guanosine analog and is phosphorylated into monophosphate, diphosphate, and triphosphate. These phosphate molecules are then misincorporated by RNA polymerase and result in early chain termination, thus inhibiting viral replication [8]. A large proportion of patients achieve sustained virological response (SVR) with pegIFN-α therapy in combination with ribavirin. The major drawback of IFN-α and ribavirin therapy is the non-responsiveness of patients towards therapy in almost 50 % of the cases. The response to treatment depends on many factors, including HCV genotype, the patient's physiological stage, the infection's severity, etc. [8].

The processing of HCV polyproteins and the replication of viral RNA are the potential targets of DAA. The DAA acts directly on viral enzymes essential for HCV replication, making them more effective against the virus [9]. In recent years, SVR of up to 100 % has been attained for non-cirrhotic HCV patients using different combinations of DAA. Daclatasvir is a direct-acting antiviral agent and a potent viral NS5A (non-structure 5 A) inhibitor. It is administered at a dose of 60 mg/day in a 12 weeklong treatment course. A high rate of SVR is reported for daclatasvir after completion of HCV therapy [10]. Sofosbuvir (SOF) is a nucleotide analog and inhibitor of the viral NS5B polymerase, and it is equally effective against all the genotypes of HCV. An SVR rate of 87–92 % was achieved by patients who were infected with HCV genotype 1 after 12 weeks of treatment. HCV patients with genotype 2 and genotype 3 also achieved SVR after 12 weeks of therapy when Sofosbuvir was used in combination with ribavirin with or without peg-IFN. Until now, no virologic breakthrough has been reported while treating patients with Sofosbuvir [11].

The ubiquitin-specific peptidase 18 (*USP18*) gene, formerly known as UBP43, is a special member of the ubiquitin-specific peptidases (USPs) enzyme family. The two of the most significant functions of the *USP18* protein are its isopeptidase activity and the inhibition of the interferon signalling pathway [12]. The *USP18* gene is 25 kb long and is present on chromosome 22 in humans. The transcript (1771bp) is translated into a 368 amino acid protein. The Cys box, along with His box and Asn residue of the catalytic domain, is responsible for the isopeptidase activity of the protein [13]. The other functional domain of *USP18* is present between exon 9 and exon 11 and is responsible for the binding of the *USP18* to the interferon-alpha/beta receptor 2 (IFNAR2) intracellular domain to regulate the interferon signalling pathway [14,15]. High *USP18* expression occurs in many tissues, mainly in the liver, thymus, and spleen. Low-level expression of the gene is also observed in bone marrow, adipose, and lung tissue. The expression of *USP18* is upregulated after a viral attack and also by type 1 and type 3 interferons [12].

USP18 is considered to be the negative regulator of the IFN pathway. During viral infection, binding of IFN-1 occurs to the interferon receptor, which is dimeric in nature, and both of its subunits (IFNAR1 and IFNAR2) are involved in the kinase activity. The binding of IFN-1 to its receptor results in the phosphorylation of Janus kinase 1 (JAK1) along with tyrosine kinase 2 (TYK2) kinases, which in turn initiates the transcription of genes involved in antiviral activity by phosphorylating the signal transducer and activator of transcription (STAT) proteins [16]. In the presence of interferons, the expression of *USP18* is upregulated and independent of its isopeptidase activity. It blocks the JAK-STAT pathway by binding to the IFNAR2 subunit of the IFN receptor, thus inhibiting the phosphorylation of JAK1. In the absence of *USP18*, the STAT1 and STAT2 signalling pathways are activated; therefore, the expression of many antiviral genes is enhanced, as indicated by different studies [12].

USP18 is involved in resistance to interferon therapy as its high expression level can be seen in interferon non-responders. In vitro, silencing of this gene results in the liver cells being more sensitive to interferon treatment, thus clearing the viral RNA more efficiently and effectively [17]. *USP18* also makes the hepatic cells resistant to interferon-alpha treatment, while its silencing leads to sensitivity to the said interferon treatment [18]. Moreover, IFN-λ4 induces the *USP18* levels, leading to desensitization to Interferon I therapy in HCV genotype 1b [19]. The correlation of *USP18* expression with interferon treatment is well known, as discussed above, but its relation with the DAA therapy response has not been studied before.

Likely, a treatment strategy that includes the viral replication limiting agent Sofosbuvir may normalize the expression of the *USP18* gene, prolonging the JAK-STAT pathway and the subsequent expression of antiviral genes. The main purpose of this study is to evaluate the expression of the *USP18* gene using real-time PCR in HCV-infected patients who are treated or undergoing treatment with DAA and also study its correlation with different clinical characteristics. We hypothesize that the expression of *USP18* in response to DAA regimen can predict the patient's treatment response and differentiate responders from non-responders. This study used human peripheral blood mononuclear cells (PBMCs) from HCV-infected and healthy individuals to investigate *USP18* expression because their acquisition was easier and safer than that of liver tissue.

2. Materials and methods

2.1. Patient cohort

The flowchart in Fig. 1 shows the inclusion of study participants. A total of 147 individuals were enrolled in the current study. 25 HCV patients who had not yet received any medication (naive group), 87 HCV patients who had received direct-acting antiviral therapy for 3–6 months, and 15 IFN relapse patients who were now being treated with DAAs. Ten HCV-induced hepatocellular carcinoma (HCC) patients were also recruited in the study. Both naive and DAA-treated patients were positive for anti-HCV antibodies while tested by enzyme-linked immunoassay (ELISA). Ten healthy individuals were also included in this study. Patients co-infected with HBV and HDV were excluded from the study criteria. Of 87 DAA-treated HCV patients, 47 were females, and 40 were males. In the naive group, out of 25 patients, 14 were males, while 11 were females. In the HCC group, six male and four female patients were included.

2.2. DAA treatment strategy

In the DAA experienced group, 87 patients who were taking Sofosbuvir (400 mg) along with weight-based ribavirin for 4–24 weeks out were recruited. In the current study, 11 out of 87 patients showed failure of response towards DAA treatment at the end of 24 weeks of treatment. They were categorized into non-responders (NR). Naïve patients refer to those individuals who were positive for HCV viral load and had not started therapy with anti-HCV drugs. Rapid virologic response (RVR) refers to patients who achieved viral clearance after four weeks of treatment with anti-HCV drugs, while no RVR refers to those who did not achieve viral clearance after four weeks. EVR (early virologic response) refers to those who have cleared HCV viral load after 12 weeks (3 months) of therapy, while no EVR refers to those who did not achieve viral clearance after three months. SVR (sustained virologic response) refers to patients who remain negative for HCV RNA 24 weeks (6 months) after completion of therapy with direct-acting antivirals.

2.3. Laboratory tests and collection of blood samples

The task of patient enrollment, data collection via developed Performa, and blood sample collection was undertaken at Akhtar Mubarrak Referral Centre, Lahore, Pakistan, during the study period from November 2017 to March 2018. After informed consent was taken from the patient, blood was collected from the bend of the elbow/anticubital fossa with aseptic techniques. Ethylenediaminetetraacetic acid (EDTA) vials collected 5 mL of blood from the subjects. Peripheral blood mononuclear cells (PBMCs) were extracted from blood samples following standard procedure using Histopaque (Sigma Aldrich).

The viral load in the plasma of patients was checked using a Real-time PCR (Sacace Biotechnologies) based assay with low base detection of 20 IU/mL. Other clinical parameters like alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), Hemoglobin (Hb), bilirubin (BLB) levels, white blood cells (WBCs), and platelet count were assessed from patient's reports and observations were recorded as mean ± and standard error of the mean. The molecular analysis of blood samples from HCV patients was performed at the Molecular Virology laboratory, Centre for Applied Molecular Biology (CAMB), University of the Punjab, Lahore, Pakistan.

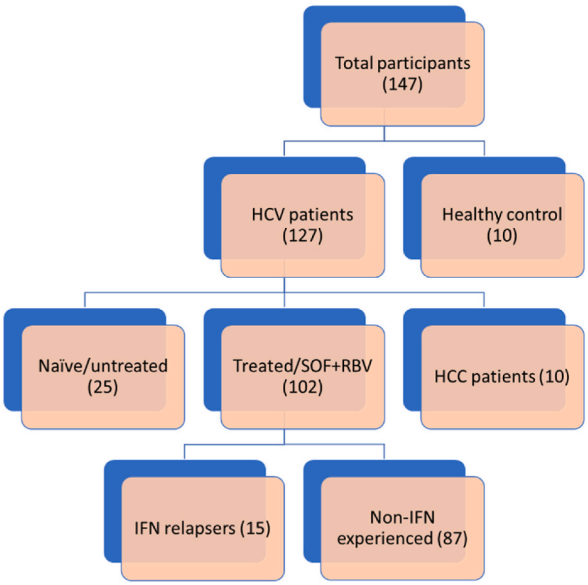

Fig. 1. Schematics of recruitment of patients.

2.4. cDNA preparation

In order to extract total RNA from PBMCs, trizol (Wizol™ reagent) was used according to the manufacturer's instructions. Using Nanodrop™ 2000 spectrophotometer (Thermo Scientific), the RNA samples with absorbance ratios at 260 and 280 nm of 1.8–2.2 were further used for cDNA synthesis. MMLV reverse transcriptase enzyme (Invitrogen) was used to transcribe cDNA from each RNA sample in a Thermocycler. The cDNA was quantified using a Spectrophotometer (NanoDrop 2000c; Thermo Scientific). The samples were stored at −20 °C for further experiments.

2.5. Primer designing and Optimization

Primers for USP-18 were designed using Primer 3 software and checked for specificity using NCBI Primer-BLAST and UCSC *in silico* PCR. Primers for amplifying the *USP18* gene and housekeeping gene (*GAPDH*) are shown in Table 1.

2.6. Real-time PCR and statistical analysis

The cDNA was amplified using Thermo Scientific Maxima SYBR Green/ROX Master Mix (2 ×) in Rotor-Gene Q Real-Time PCR Detection System (QIAGEN). The cycling conditions for *USP18* were 95 °C for 10 min for polymerase activation followed by 35 cycles at 94 °C for 30s, 58 °C for 60s, and 72 °C for 30s. *GAPDH* was also amplified as a housekeeping gene for normalization of USP-18 expression. To check the expression of the target gene in relation to endogenous control GAPDH (housekeeping gene), the Livak method, also known as $C_t = 2^{\sim\Delta\Delta Ct}$ was used as reported by Ref. [20]. Expression of this gene in healthy control was used as a reference gene. Relative fold change in gene expression was calculated for different study groups with GraphPad PRISM software (version 8.0.1). The level of significance among different study groups was calculated with ordinary one-way ANOVA, and a P value less than 0.05 was considered statistically significant. Clinical and demographic characteristics of different study groups were presented as the mean and standard error of mean (±) using descriptive statistics with Microsoft Excel 2016. In order to ensure the accuracy and reliability of our results, all experiments were conducted in triplicates to minimize error and enhance the precision of our data.

3. Results

3.1. Patient selection and laboratory tests

Table 2 presents the clinical and demographic features of different studied groups. Chronic HCV patients showed elevated levels of liver enzymes Alanine transaminase (ALT), Aspartate aminotransferase (AST), and Alkaline Phosphatase (ALP) as compared to the control group.

3.2. Real-time PCR expression of USP18 in different study groups

USP-18 gene expression was analyzed in all the patients treated with sofosbuvir and ribavirin, and responders were categorized into different groups based on the treatment duration. $2^{\sim\Delta\Delta Ct}$ value for expression of *USP18* was calculated for patients who achieved SVR, RVR, EVR, and untreated patients. *USP18* was found to be significantly upregulated (7.36 folds) in treatment-naive patients as compared to RVR (P=<0.0001), EVR (P = 0.0001), and SVR (P=<0.0001) groups, as seen in Fig. 2. While in RVR, EVR, and SVR groups where patients had cleared HCV RNA from their blood after treatment with DAA therapy, the expression of USP-18 was comparatively low, i.e., 1.3, 2.1, and 1.4 folds. Ordinarily, one-way ANOVA was used to compare gene expression in both RVR and SVR groups, but no significant difference was found, suggesting that viral clearance leads to lower expression of the *USP18* gene.

As shown in Fig. 3a, expression of *USP18* was significantly higher in the No RVR group than in the RVR patients (P = 0.0026). Similarly, gene expression in the No EVR group was significantly higher than in the EVR group (P = 0.0016), as shown in Fig. 3b. These results suggest that viral clearance affects the expression level of *USP18* in HCV DAA-treated patients.

3.2.1. Comparison of gene expression in IFN relapse patients

Fig. 4 represents the comparison of *USP18* expressions in different patient groups who were previously treated with IFN but relapsed later. IFN relapse patients who received DAA therapy were categorized into different groups. All these patients showed a significant reduction in the expression of *USP18* levels as compared to healthy control (P = 0.0001, P = 0.0001 and P = 0.0001).

Table 1
Primer sequences used in the current study for PCR reaction include the housekeeping gene GAPDH.

Gene	Primer	Sequence
USP18	Forward	5′-CAGACCCTGACAATCCACCT-3′
	Reverse	5′-AGCTCATACTGCCCTCCAGA-3′
GAPDH	Forward	5′- CGGATTTGGTCGTATTGGG -3′
	Reverse	5′- CTCGCTCCTGGAAGATGG-3′

Table 2
Different clinical parameters for all the study groups.

Study groups	M/F	Average Age	Platelet count/ µl	TLC/µl	Hb g/dl	Bilirubin mg/dl	ALT IU/L	AST IU/L	ALP IU/L	P-value
Healthy controls	5/5	38.2 ± 2.5	212161.5 ± 54324.5	7863.5 ± 757.5	13.35 ± 0.99	0.618 ± 0.23	42.5 ± 9.53	35.3 ± 4.4	125.52 ± 10.6	0.005
Naïve	14/11	42 ± 6.43	180636.5 ± 2554.55	6549.5 ± 130.5	13.1 ± 1.24	0.74 ± 0.28	56.7 ± 4.5	52.5 ± 2.1	236.5 ± 7.31	0.001
RVR	11/9	56.5 ± 8.5	191700 ± 44676.2	6800.5 ± 332.5	12.8 ± 0.6	0.68 ± 0.295	53.0 ± 3.5	41.45 ± 13.1	228.63 ± 14	0.004
EVR	10/10	55.8 ± 5.81	179005 ± 46722.5	7601.5 ± 511.8	13.2 ± 0.67	0.56 ± 0.523	45.17 ± 5.81	39.21 ± 5.1	195.30 ± 13.5	0.008
SVR	8/12	41 ± 5.213	219177.2 ± 18922.8	6830.5 ± 141.5	12.75 ± 0.95	0.68 ± 0.285	55.7 ± 0.8	41.75 ± 12.1	220.9 ± 16.25	0.023
IFN relapsers	7/8	50.85 ± 8.355	155002.5 ±	7691 ± 114.4	12.975 ± 0.225	0.65 ± 0.09	49.1085 ± 6.5915	47.125 ± 5.375	237.955 ± 17.055	0.006
NR	6/5	48 ± 7.1	141091 ± 48163.25	8833 ± 436.50	14.5 ± 0.45	1.29 ± 0.325	63.18 ± 4.55	50.27 ± 15.1	242.35 ± 2.11	0.056
HCC	6/4	53 ± 6.5	131000 ± 43650.1	5660 ± 52621	11.7 ± 0.32	0.84 ± 0.422	56.4 ± 0.5	53.4 ± 0.75	255.01 ± 8.5	0.043

Abbreviations: TLC: Total leukocyte count; Hb: hemoglobin; ALT: Alanine transaminase; AST: Aspartate aminotransferase; ALP: Alkaline Phosphatase.

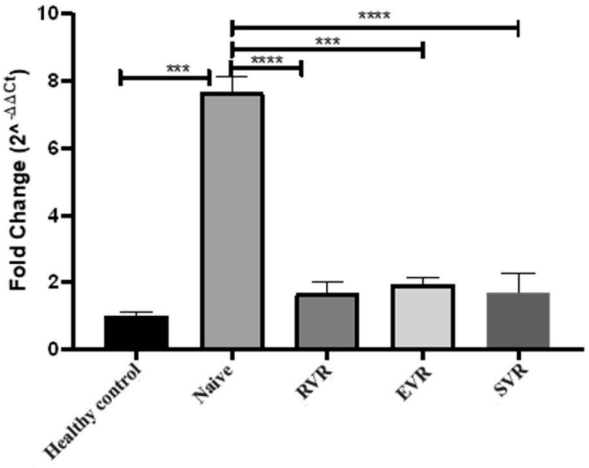

Fig. 2. *USP18* expression in naïve, RVR, EVR and SVR HCV patients. Naive represents the subjects before treatment, while RVR and EVR represent the treatment response in HCV patients after 4 weeks and 12 weeks, respectively. SVR represents the group having SVR with no detectable virus at the end of treatment and after 24 weeks of therapy. Columns and error bars indicate the mean values with their standard errors, respectively ($P < 0.001$, $P < 0.0001$).

3.2.2. USP-18 expression in DAA non-responders and HCC patients

USP18 gene expression was analyzed in 11 patients who showed response failure towards DAA treatment. The expression of *USP18* was upregulated in DAA non-responders (NR) compared to healthy controls ($P = 0.0092$). DAA non-responders also show upregulated expression of this gene compared to SVR patients, which suggests that clearance of HCV RNA from the host causes the expression of *USP18* to downregulate. We also compared the expression of *USP18* in HCC patients and chronic hepatitis C patients. HCC patients exhibit remarkably high expression of USP-18 in their blood (8.69 folds) compared to healthy controls ($P = 0.0014$). Compared to chronic hepatitis C (CHC) patients, there was no significant difference in *USP18* expression between the HCC and CHC groups ($P = 0.59$), as seen in Fig. 5.

3.2.3. Comparison of USP18 gene expression in interferon relapse patients treated with Sofosbuvir + Ribavirin (SOF + RBV) regimen

In the PBMCs of interferon relapse HCV patients, the expression of *USP18* mRNA was compared with the different study groups. In naive patients, seven-fold higher expression was seen compared to RVR, EVR SVR, and healthy controls (Fig. 6).

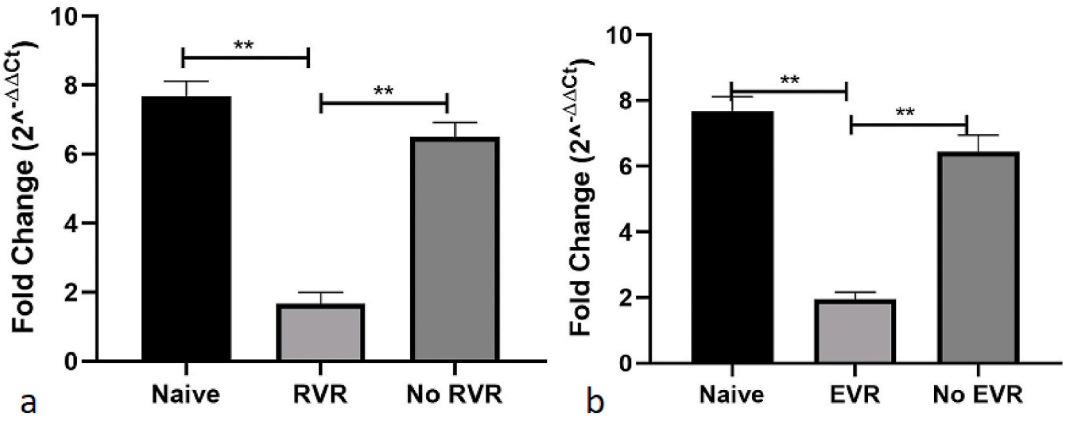

Fig. 3. **(a)** *USP18* expression in RVR and No RVR patients. Patients who had cleared HCV after 4 weeks were placed in RVR group, and those who had not cleared HCV RNA from their blood after one month or 4 weeks were placed in the No RVR group. **(b)** *USP18* expression in EVR and No EVR patients. EVR refers to patients who had no detectable HCV RNA in their blood after 12 weeks of therapy, while those who had not cleared the virus after 12 weeks were classified as the No EVR group. Columns show mean values, while bars represent the standard error of the mean ($P < 0.01$).

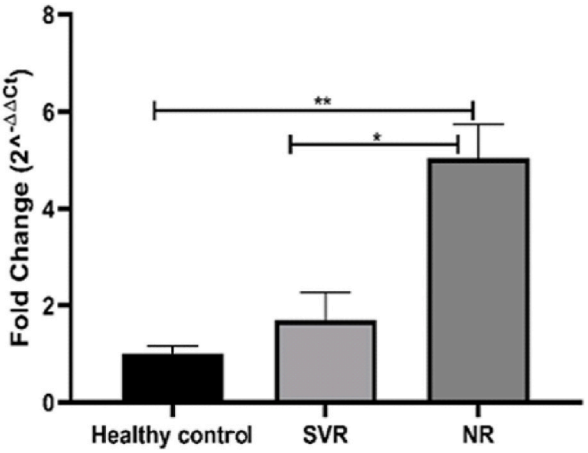

Fig. 4. Comparison of *USP18* expressions in non-responders and SVR group. Non-responders refer to the subjects who did not show any response to the DAA therapy. The SVR group contains patients who remained negative for HCV RNA 24 weeks (6 months) after completion of therapy. Columns depict the mean fold change with bars showing mean standard error ($P < 0.05$, $P < 0.01$).

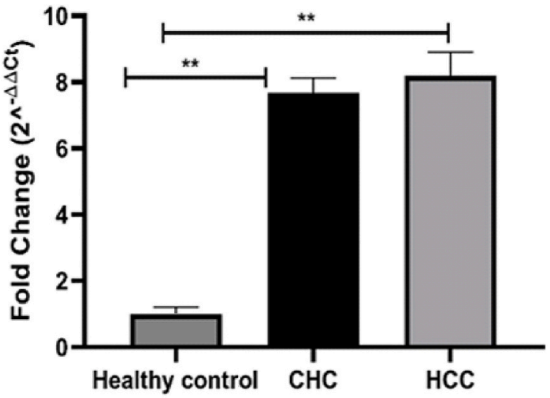

Fig. 5. Comparison of *USP18* expression in chronic hepatitis C patients (CHC) and hepatocellular carcinoma (HCC) patients. Mean values of fold change in groups are shown by columns, and the standard error of means by bars ($P < 0.01$).

4. Discussion

Chronic hepatitis C is a principal health problem affecting more than 58 million people in the globe [1]. The introduction of DAA therapy has brought a revolutionary change in HCV therapeutics, and a very high response rate has been reported for DAA therapy against HCV, with very few relapse cases. An SVR rate of 85 % has been reported for patients infected with genotype 3 of HCV, who were treated for 24 weeks with Sofosbuvir + Ribavirin therapy [21]. In the current study, 87.5 % of HCV patients (genotype 3a) who received Sofosbuvir + Ribavirin therapy achieved SVR. In the Pakistani population, DAA therapy is becoming standard care therapy against HCV. However, DAA therapy is more expensive than IFN therapy. In the case of genotype 3 infections, patients achieved SVR with 24 weeks of DAA therapy [22,23]. An SVR rate of 90 % was achieved after 12 weeks of combination therapy (Daclatasvir/Sofosbuvir/Ribavirin) in a study reported by Nelson et al. [23], as compared to the SVR rate of 87.5 % achieved for combination therapy of Sofosbuvir + Ribavirin in this study.

It has been reported that HCV replicates in the peripheral blood mononuclear cells (PBMCs) produce type I interferons (IFNs). It is reported that the gene expression profile of PBMC is very similar to that of the liver. It is well known that the pattern of ISG expression is the same in the liver hepatocytes and PBMCs of HCV-infected patients [24,25]. *USP18* is a member of ubiquitin-specific proteases and cleaves ISG15 from ISGylated protein complexes [26]. *USP18* is a significant regulator of IFN-I signalling. Under specific circumstances, *USP18* tends to bind with IFNAR2, one of the subunits of the IFNAR dimer, and compete with JAK, preventing proper activation of the pathway [27].

When USP-18 is continuously expressed in cultured cells, it inhibits interferon-induced JAK/STAT signalling and is a negative regulator of the IFN pathway [28]. Moreover, USP-18 expression is considered a poor prognostic marker for IFN-α therapy for HCV-infected patients [29]. In a study by Frankova et al., the initial expression of USP-18 in PBMCs predicted the likelihood of SVR achievement and was also associated with the interleukin−28-beta subunit (IL28B) genotype in HCV-infected patients [26].

The current study observed an elevated transcription level of *USP18* for untreated HCV patients compared to healthy controls. This might be because viral infections stimulate the production of interferons, and these interferons, in turn, stimulate the expression of the *USP18* gene. In a study by Meissner et al., *USP18* expression was downregulated after Sofosbuvir/ledipasvir therapy, irrespective of cirrhosis and pretreatment ISG levels, and an SVR rate of 90 % was achieved [30]. Similar results were observed in our study, where the expression of USP-18 was downregulated in the SVR, EVR, and RVR group compared to the untreated patients, and the level of expression of *USP18* was almost similar to that of healthy controls. These results confirm that type 1 interferon sensitivity will likely be restored during DAA therapy in HCV patients [25]. We have also observed the downregulation of *USP18* in IFN relapse patients who were later treated with the DAA regimen. *USP18* upregulation stimulates HCV production and dulls the anti-HCV effects of Interferons [31].

We also saw elevated levels of *USP18* in those patients who were being treated with a SOF/RBV combination but could not clear viral RNA from their blood after one or three months of therapy, i.e., No EVR and No RVR groups. Most of the patients in our study rapidly responded to DAA/RBV combination therapy and achieved viral clearance after a few weeks of treatment, and their *USP18* level also declined, as discussed in the results section. In those few who failed to do so, their gene expression levels did not decrease. Further, follow-up studies are required to ascertain the role of *USP18* in viral clearance in the case of HCV. *USP18* was suggested as a possible therapeutic target for HCV treatment by Randall et al., so we can assume that targeting this gene can make DAA therapy more effective in HCV patients [17]. The presence of resistance-associated variants (RAS) also affects the treatment response to DAA in genotype 3 patients [32], so there is a need for further analysis of those patient samples who do not show a response to sofosbuvir treatment. We also compared the expression of USP-18 in SVR and non-responders, and the transcription level of *USP18* was significantly higher in non-responders than that of patients who achieved SVR.

Our results are also consistent with Frankova et al., who suggested a positive role of *USP18* in treatment resistance, as we also observed higher expression of *USP18* in the non-responders group [33]. We also evaluated the expression of *USP18* in the RVR group, i.e., patients who achieved viral clearance at week 4 of therapy. We could surmise from these findings that viral clearance results in decreased expression of the USP-18 gene. In a study by Frankova et al., *USP18* expression in the SVR group increased continuously until week 12 of triple therapy with boceprevir/telaprevir/peg-IFN. In the non-SVR group, the expression level of *USP18* declined at week 12 [26], which is inconsistent with our findings, where non-responders show elevated expression of *USP18* than the SVR group. The difference can be attributed to HCV genotype (3a) and antiviral therapy, which in our case is a combination therapy of sofosbuvir and ribavirin.

Patients who are chronically infected with HCV show abnormal alanine aminotransferase (ALT) enzyme activity [34]. Moreover, different studies have linked Aspartate transaminase (AST) to HCV viral load and liver damage [35–37]. In the current study, liver profiles (ALT, AST, bilirubin levels) of different study groups were found to be consistent with the studies mentioned above. ALT and AST levels of the patients reverted to normal ranges upon therapeutic clearance of the virus.

The expression of *USP18* was highly upregulated for patients suffering from HCC in the current study. These results support that *USP18* has a prominent role in the development of many cancers as it upregulates many growth factors and oncogenes involved in the development and progression of different types of cancers. Epidermal growth factor receptor (EGFR) has been implicated in the development and progression of human cancers, and *USP18* is one of the regulators of EGFR protein expression. In a previous study, it was demonstrated that depletion in *USP18* levels inhibits EGFR expression through upregulation of microRNA miR-7, and in turn, tumorigenic effects of EFGR are inhibited [38].

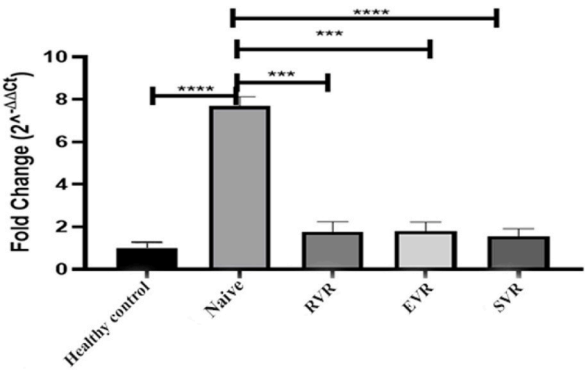

Fig. 6. Comparison of *USP18* gene expression in interferon relapse patients who were treated with SOF/RBV regimen. Ordinary one-way ANOVA calculated the level of significance, and asterisks represent the p-value.

5. Limitations of study

The current study has a few limitations: i) Our study is limited to the expression profiling of the *USP18* gene using Quantitative PCR (qPCR). Due to the limitation of time and resources, protein quantification using immunohistochemistry (IHC) or western blotting was not done, which can provide a better insight into the overexpression of the *USP18* gene in the cell lines. ii) Small sample size iii) Due to the limited resources, metabolic disorders, etc., were not tested on the studied population.

6. Conclusion

We concluded that upregulation of *USP18* can predict non-responsiveness to DAA therapy, as non-responders show higher expression of *USP18* than that of DAA responders. Moreover, *USP18* can be implicated in treatment resistance in the case of DAA therapy because it is upregulated in non-responders and downregulated in SVR and RVR patients. DAA responders show almost similar expression of *USP18* as healthy controls, indicating the reversal of type 1 IFN sensitivity during DAA therapy.

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Availability of data and materials

All datasets on which the conclusions of the manuscript rely are presented in the paper.

Institutional review board statement

The study was approved by the Institutional Ethics Committee of Hazara University Mansehra, 21120, Pakistan, (HU/ORIC/2022/1091)." for studies involving humans.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

CRediT authorship contribution statement

Sami Ullah: Methodology. **Mariam Naveed:** Methodology, Formal analysis. **Amjad Ali:** Methodology, Investigation, Conceptualization. **Sadia Bibi:** Formal analysis, Data curation. **Wafa Idrees:** Formal analysis, Data curation. **Shazia Rafique:** Software, Resources, Investigation. **Muhammad Idrees:** Resources, Investigation, Formal analysis. **Muhammad Waqas:** Writing - original draft, Software. **Jalal Uddin:** Formal analysis, Data curation. **Afnan Jan:** Software, Formal analysis, Data curation. **Ajmal Khan:** Writing - review & editing, Funding acquisition, Conceptualization. **Ahmed Al-Harrasi:** Writing - review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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