

Transcriptional Analysis of TP53 Gene in Chronic Hepatitis C Patients Treated with Sofosbuvir, Daclatasvir, Pegylated Interferon, and Ribavirin

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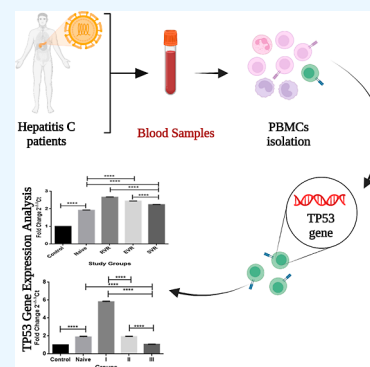
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ABSTRACT: Hepatitis C virus (HCV) is a major public health problem that affects more than 170 million people globally. HCV is a principal cause of hepatocellular carcinoma (HCC) around the globe due to the high frequency of hepatitis C infection, and the high rate of HCC is seen in patients with HCV cirrhosis. TP53 is considered as a frequently altered gene in all cancer types, and it carries an interferon response element in its promoter region. In addition to that, the TP53 gene also interacts with different HCV proteins. HCV proteins especially NS3 protein and core protein induce the mutations in the TP53 gene that lower the expression of this gene in HCV patients and leads to HCC development. In this study, we examined the transcriptional analysis of the TP53 gene in HCV-infected patients administered with different combinations of antiviral therapies including sofosbuvir + daclatasvir, sofosbuvir + ribavirin, and pegylated interferon + ribavirin. This study included 107 subjects; 15 treated with sofosbuvir + daclatasvir, 58 treated with sofosbuvir + ribavirin, 11 treated with interferon + ribavirin, 8 untreated, 10 HCC patients, and 5 were healthy controls. Total RNA was extracted from the PMBCs of HCV infected patients and reverse transcribed into cDNA using a gene specific reverse primer. The expression level of TP53 mRNA was analyzed using quantitative PCR. The expression of TP53 mRNA was notably upregulated in rapid virological response (RVR), early virological response (EVR), and sustained virological response (SVR) groups as compared to non-responders and naive groups. The expression of TP53 mRNA was seen high in HCC as compared to control groups. Additionally, it has been demonstrated that sofosbuvir + daclatasvir treatment stimulates significant elevation in TP53 gene expression as compared to (sofosbuvir + ribavirin) and (IFN + ribavirin) treatment. This study indicates that the TP53 gene expression is highly upregulated in RVR, EVR, and SVR groups as compared to control groups. Moreover, sofosbuvir + daclatasvir therapy induces significant rise in TP53 mRNA expression levels as compared to (sofosbuvir + ribavirin) and (IFN + ribavirin) treatment. According to these results, it can be concluded that sofosbuvir + daclatasvir plays a significant role in preventing HCV patients from developing severe liver complications as compared to other administered therapies. This study is novel as no such type of study has been conducted previously on the expression of TP53 in local HCV-infected population treated with different combinations of therapies. This study is helpful for the development of new therapeutic strategies and for improving existing therapies.



1. INTRODUCTION

Chronic hepatitis C virus (HCV) infection is the principal cause of cirrhosis and hepatocellular carcinoma (HCC) globally. World Health Organization reported that more than 3% of the world's population is infected with HCV.¹ In Pakistan, about 6% subjects of the total population are suffering from hepatitis C infection.² HCV is a single-stranded RNA virus and belongs to the family *Flaviviridae*.³ HCV replicates its own genome into the cytoplasm of hepatocytes and the replication rate is extremely high and ranges from 10^{10} to 10^{12} virions per day.⁴ During HCV replication, it can mutate its own genome multiple times due to the lack of proofreading ability of viral RNA polymerase.^{3,5} It has been estimated that HCV is responsible for 27% of cirrhosis and 25% of HCC cases around the globe but this ratio varies in

different countries depending upon the burden of infection.⁶ p53 gene, located on chromosome number 17, is the most frequently mutated or silenced gene in cancer that has been found to interact with various HCV proteins.⁷ Tumor suppressor p53 gene has been considered as an ISG due to the presence of interferon (IFN) stimulated gene response element

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(ISGRE) in its promoter region. It has been found that IFN- α /b stimulate the p53 gene that results in tumor suppression.⁸ So, IFN by enhancing p53 expression levels provides protection from developing carcinoma.⁹ HCV proteins have been found to directly interact with the p53 gene and considered to be responsible in down regulating its expression and transcriptional activity.^{10,11} In addition to that, HCV replication also gets enhanced in p53 knocked-down cells.¹² So, to date, there is not a final verdict available about p53 expression during HCV infection. According to our information, it is a first study in Pakistan based on the study of expression levels of the p53 gene in genotype 3a HCV-infected patients. In the past 2 decades, HCV patients were treated by a combination of IFN and ribavirin (RBV) but many studies reported its adverse effects and low number of SVR achieved patients. From 2013, with the discovery of two direct-acting antiviral agents (DAAs), NS5B polymerase inhibitor sofosbuvir (SOF), and NS5A inhibitor daclatasvir (DCV) made significant contributions in increasing the number of SVR achieved patients.¹³ Combined dose of SOF and DCV with or without RBV had shown cure rates of more than 90%.¹⁴ So, the purpose of this research is to find out the TP53 expression levels in response to IFN + RBV, direct-acting antivirals (DAAs): SOF + RBV, and double DAAs: SOF + DCV.

Specific aims are to examine an association of TP53 gene with different HCV treatments being used in Pakistan, and to investigate the relation between HCV infection and various HCV-associated clinical factors.

2. MATERIALS AND METHODS

2.1. Study Population and Collection of Blood Samples. Blood samples were collected from 102 individuals infected with 3a genotype of HCV and five samples were taken from healthy volunteers having no previous history of HCV, hepatitis B virus (HBV), or any other disease. Consent letters got signed from all individuals to seek the permission of sample collection from the enrolled patients. Clinical and demographic parameters were also noted down for each studied group through a specifically designed questionnaire. The ethical committee of the university approved this study. Blood samples were drawn using sterile disposable syringes. Samples were collected in the duration of November 21, 2017, to March 25, 2018. Approximately 5 mL of blood was drawn and transferred into pre-labeled ethylenediaminetetraacetic acid (EDTA vials). PBMCs were extracted from blood through a standard protocol using Histopaque-1077 (Sigma-Aldrich, Germany). After extraction, PBMCs were washed and resuspended in phosphate-buffered saline (PBS) and stored at $-80\text{ }^{\circ}\text{C}$ for further use (Figure 1).

2.2. Inclusion Criteria. HCV patients of 3a genotype having PCR-positive results treated with DAA, double DAA, or IFN + RBV were included, and naive, relapsed, and non-responder patients were also part of the study.

2.3. Treatment-Based Classification of the Patients. Patients were categorized into four groups

1. Rapid virologic response (RVR)—absence of detectable levels of HCV RNA in serum after 4 weeks of therapy.
2. Early virologic response (EVR)—absence of detectable levels of HCV RNA at 12 weeks of therapy.
3. Sustained virologic response (SVR)—absence of detectable levels of HCV RNA in serum after 6 months of therapy.

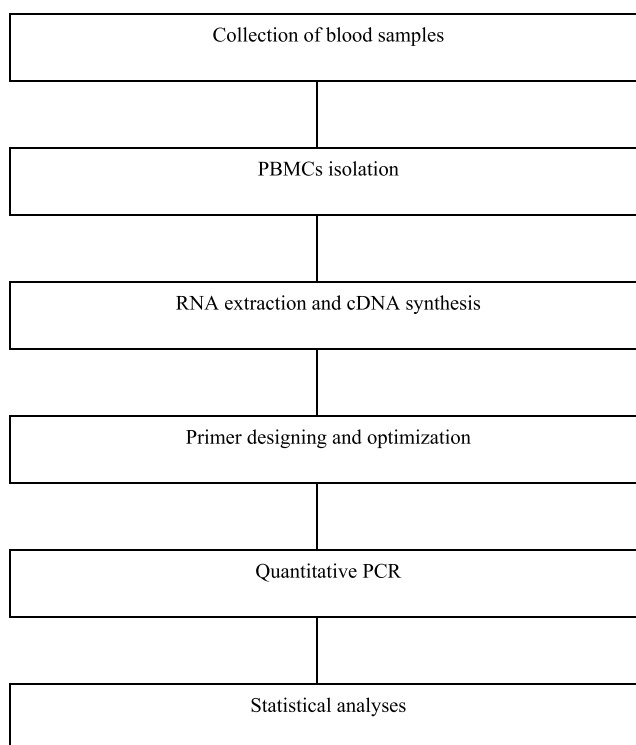


Figure 1. Methodology workflow of the current study.

4. Non-responders (NRs)—detectable levels of HCV in serum after 24 weeks of therapy.

2.4. Exclusion Criteria. Patients having an infection of the human immunodeficiency virus (HIV) or HBV were excluded from our study.

2.5. RNA Extraction and cDNA Synthesis. Total RNA was isolated from the extracted PBMCs using Wizol reagent M (Wizbiosolutions, Seongnam, South Korea). After RNA extraction, spectrophotometry (NanoDrop 2000c; Thermo Scientific) was used for the RNA quantification. RNA samples having ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}) of 1.8–2.2 nm were used for cDNA synthesis. cDNA synthesis was carried out using M-MLV reverse transcriptase (Invitrogen). The transcribed cDNA was quantified by spectrophotometry (NanoDrop 2000c; Thermo Scientific). After quantification, each sample was diluted to make final concentration up to 500 ng/ μL .

2.6. Primer Designing and Optimization. Primer3 software was utilized to design primers for the TP53 gene, and specificity process was carried out through NCBI Primer BLAST and UCSC in silico PCR. Primers used for housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were the same as used by Li et al.¹⁵ The sequences of primers are shown in Table 1.

2.7. Quantitative PCR. To quantify the transcript level of the TP53 gene, quantitative (q) PCR was performed. To perform this process, Rotor-Gene Q real-time PCR detection system (QIAGEN) was used. GAPDH was used as an endogenous control for normalization. Dilutions were made for each concentrated cDNA sample. For target and housekeeping gene, real-time PCR was performed using 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). PCR conditions were optimized as $95\text{ }^{\circ}\text{C}$ for 3 min, followed by 35 cycles at $95\text{ }^{\circ}\text{C}$ for 30 s, $59\text{ }^{\circ}\text{C}$ for 15 s, and $72\text{ }^{\circ}\text{C}$ for 15 s and further placed at $72\text{ }^{\circ}\text{C}$ for 10 min.

Table 1. List of Primers Used in the Current Study

gene	primer	sequence	product size	GC content (%)	T_m (°C)
TP53	forward	5'-ATTTGCGTGTGGAGTATTTGGATGA-3'	104-bp	42	58
	reverse	5'-GTAGTGGATGGTGGTACAGTCAGA-3'		58	58
GAPDH	forward	5'-CGGATTTGGTCGTATTGGG-3'	215-bp	52	56
	reverse	5'-CTCGCTCCTGGAAGATGG-3'		61	56

Quantitative PCR analysis was also carried out for positive and negative controls in each run to check the machine and all the reaction conditions.

GAPDH was also amplified for normalization and taken as an endogenous control. The Ct value was analyzed for each sample using the Livak method.¹⁵ The Livak method describes the fold change in gene expression after performing real-time PCR.

2.8. Statistical Analyses. All the data obtained through developed questionnaire and gene expression data were analyzed using GraphPad Prism (version 6; San Diego, CA). Relative gene expression of the TP53 gene was calculated through ordinary one-way analysis of variance (ANOVA) by comparing mean group values for each group. Graphical representation of analyzed results was also carried out through GraphPad Prism. In all analyses, $P < 0.05$ was considered statistically significant.

3. RESULTS

In the present study, 102 HCV patients were enrolled including 15 patients received double DAA, 58 received DAA, 11 received pegylated IFN + RBV, 8 untreated patients, 10 HCC patients, and 5 were healthy controls. Among 84 treated patients, 40 patients achieved SVR, 15 achieved EVR, 12 achieved RVR, and 17 were NRs (Table 2).

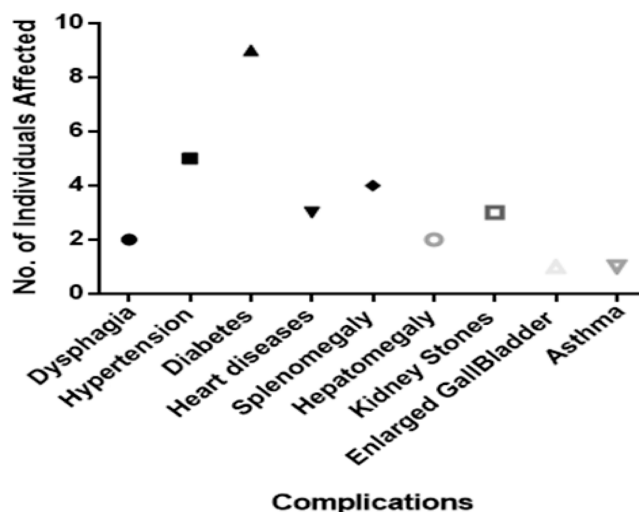
Table 2. Different Study Groups Selected in the Current Study

groups	study groups based on treatment				healthy controls	HCC patients
	double DAA	DAA	peg interferon + ribavirin	untreated		
no. of patients	15	58	11	8		
groups	study groups based on response				healthy controls	HCC patients
	SVR	EVR	RVR	NR		
no. of patients	40	15	12	17	5	10

HCV-related clinical factors and demographic factors like age, platelet count, total leucocyte count (TLC), hemoglobin (HB), bilirubin, ALT, AST, ALP, and viral load were recorded for each

group studied to examine an association between these factors and HCV infection. All individuals included in our study were found to be having age above 40 years. However, our data show that the response rate toward treatment decreases with age as HCC patients and NRs were found to be >45 years of age. A comparison of demographic and clinical factors of all the groups is given in Table 3. Liver function enzymes like ALT levels were found to be normal in each group but the AST value found to be high in all groups except HCC group patients. Elevated ALP value has been observed in each group, but the HCC group showed highest value of ALP enzyme. Other factors like platelet count, TLC, HB, and bilirubin levels were seen to be normal in each study group (Table 3).

3.1. Prevalence of HCV-Related Complications and Relapse Rate in Our Studied Population. HCV is associated with many complications like dysphagia, hypertension, diabetes, heart diseases, splenomegaly, hepatomegaly, enlarged gallbladder, and asthma. All these complications are systematically represented in Figure 2. Frequency of diabetic patients was

**Figure 2. Prevalence of HCV-related complications.****Table 3. Comparison of Clinical Factors among Different Studied Groups**

factors	healthy control	SVR	RVR	NR	HCC	naïve
age	28.7	42.7	45	48.1	56.8	42.1
^a PLT count (per μL) ^b	284196.4 \pm 78209.5	212391.5 \pm 77309	211612.5 \pm 80703.5	189665.1 \pm 82597.56	172990.8 \pm 62664.4	180636.5 \pm 64506.3
^a TLC (cmm) ^c	7912.4 \pm 1855.5	7223.3 \pm 1983.8	7100 \pm 2840.5	7845.5 \pm 1478.95	5523 \pm 843.246	6549.1 \pm 1487.5
^a hemoglobin (g/dL) ^d	13.8 \pm 4.25	12.6 \pm 2.0	12.72 \pm 4.45	12.9 \pm 1.2	13.4 \pm 0.9	12.5 \pm 1.4
^a BILIRUBIN (mg/dL) ^e	0.72 \pm 0.14	0.67 \pm 0.1	0.66 \pm 0.24	0.69 \pm 0.1	0.73 \pm 0.1	0.74 \pm 0.12
^a ALT (IU/L) ^f	49.4 \pm 9.2	55.0 \pm 22.3	44.3 \pm 21.5	55.7 \pm 9.9	42.1 \pm 8.2	55.3 \pm 8.9
^a AST (IU/L) ^g	36.5 \pm 5.9	50.1 \pm 7.6	44.1 \pm 5.34	55.75 \pm 9.2	32.8 \pm 4.7	52.5 \pm 6.2
^a ALP (IU/L) ^h	115.8 \pm 11.5	235.7 \pm 38.7	216.6 \pm 106	220.63 \pm 52	245.8 \pm 10.3	236.5 \pm 39.9

^aData are represented as mean \pm SEM. ^bNormal range 150,000–450,000 cells/ μL . ^cNormal range 4500–11,000 cells/ mm^3 . ^dNormal range 13.5–17.5 g/dL in men and 12.0–15.5 g/dL in women. ^eNormal range 0.1–1.2 mg/dL. ^fNormal range 7–56 IU/L. ^gNormal range 10–40 IU/L. ^hNormal range 44–147 IU/L.

(8.8%) found to be highest as compared to other complications as prevalence of dysphagia, hypertension, diabetes, heart diseases, splenomegaly, hepatomegaly, enlarged gallbladder, and asthma. Prevalence of other complication was not significantly high as 1.9% patients were found to be suffering from dysphagia, 4.9% from hypertension, 2.9% from heart diseases, 3.9% from splenomegaly, 1.9% from hepatomegaly, 2.9% from kidney stones, 0.9% from enlarged gallbladder, 0.9% from asthma, and 60.7% patients were not having any kind of complication or disease. HCV-infected patients included in our study were treated with three different drug combinations. Relapse rate of these treatments were compared, and figure shows that relapse was highest in the IFN-treated group as compared to DAA-treated and double DAA-treated groups. The relapse rates of IFN, DAA, and double DAAs were 11.9, 4.76, and 0%, respectively, that shows the IFN relapse rate is highest in comparison to the rest of the two groups (Figure 3).

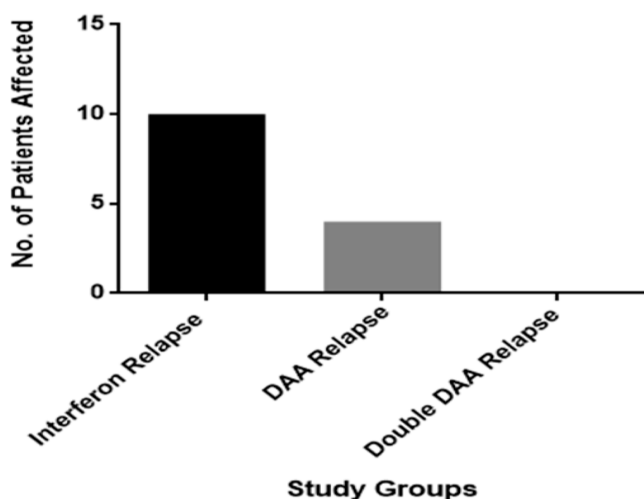


Figure 3. Comparison of the relapse rate after different treatments.

3.2. Association of Virologic Response with TP53 Expression. According to virologic responses toward HCV therapy, our study consisted of three groups RVR, EVR, and SVR. To control the group's wise error rate, we applied the Bonferroni correction. So, in this analysis, we have five groups and compared each group with the other group for five possible comparisons as seen from Figure 4. Our level of significance is 0.05 already discussed in the materials and methods section. For Bonferroni correction, we divided the level of significance 0.05 on the number of five possible comparisons (tests). After division we got the Bonferroni corrected p value is 0.01. In the RVR group, the TP53 expression was 0.22 folds higher as compared to EVR group ($p = <0.0001$) which is a minor difference but as compared to SVR grouped patients it was 0.42 folds higher ($p = <0.0001$). Comparison of naïve patients with RVR indicates 0.744 folds lower expression ($p = <0.0001$), with EVR 0.51 folds lower ($p = <0.0001$) and with SVR 0.32 folds lower expression ($p = <0.0001$). So, untreated patients showed the lowest level of TP53 gene expression and patients who rapidly clear the viral load in 4 weeks showed highest expression of TP53 gene. All the p values of each group comparison (test) with other group is less than Bonferroni corrected p value and highly significant.

3.3. NRs Show Least Expression of TP53. To control the group's wise error rate, we applied the Bonferroni correction. So,

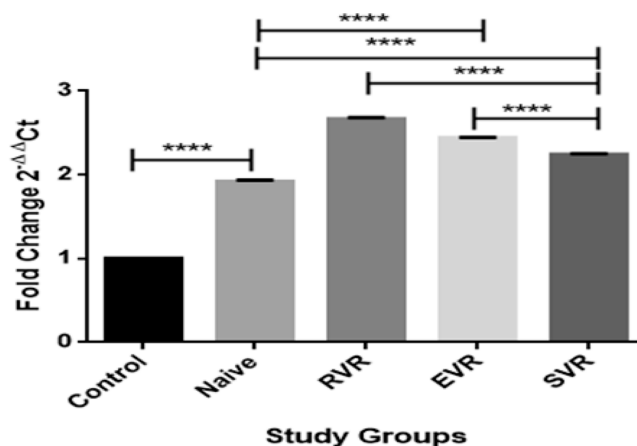


Figure 4. Comparison of TP53 gene fold change among naive patients, RVR group, EVR group, and SVR group. $2^{-\Delta\Delta CT}$ values for naive, RVR, EVR, and SVR were 1.932, 2.676, 2.445, and 2.25. Columns depict the mean fold change and error bars indicate the standard error of the mean for each group (**** $P < 0.0001$).

in this experiment, we have four groups and compared each group with the other group for possible comparisons (Figure 5).

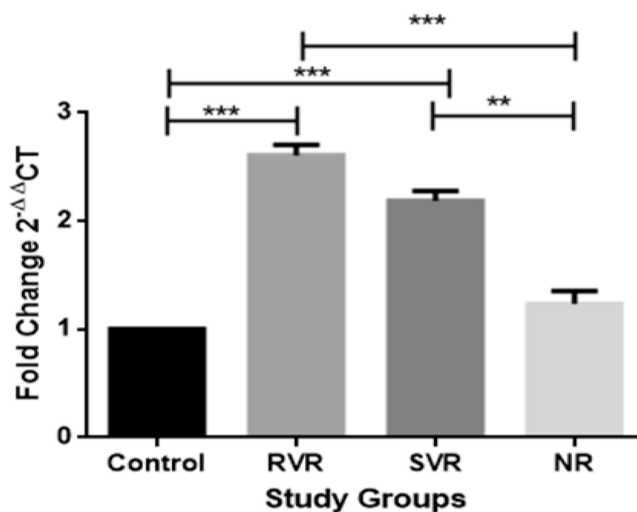


Figure 5. Comparison of TP53 gene expression levels among the RVR group, SVR group, and NR group. Relative expressions of p53 were 2.676, 2.250, and 1.320 in RVR, SVR, and NR groups, respectively. Columns depict the mean fold change, and error bars indicate the standard error of the mean for each group (** $P < 0.01$, *** $P < 0.001$).

For Bonferroni correction, we divided the level of significance 0.05 on the number of four possible comparisons (tests) and got the Bonferroni corrected p value was 0.125. Comparison of RVR and SVR with the NR group shows that very low TP53 gene expression is seen in the NR group. Fold change values for RVR, SVR, and NR were 2.676, 2.250, and 1.320, respectively. There was seen a difference of 0.95 between the SVR and NR group with p value of 0.0015. Comparison of the RVR group with NRs shows a significant fold change difference of 1.37 indicated by a p value of 0.0004. SVR group patients and RVR group patients have very less fold change difference of only 0.42 with p value of 0.0307. Non-significant fold change difference was seen among control and NRs ($p = 0.1806$) while comparing the Bonferroni corrected p value.

3.4. Comparison of TP53 Expression in NRs and HCC Patients. TP53 expression was analyzed specifically in NR and HCC patients, and it observed that there was only a difference of 0.05 folds in TP53 expression of both groups. To control the group's wise error rate, we applied the Bonferroni correction. So, in this analysis, we have three groups and compared each group with the other group for three possible comparisons, as seen from Figure 6. For Bonferroni correction, we divided the level of

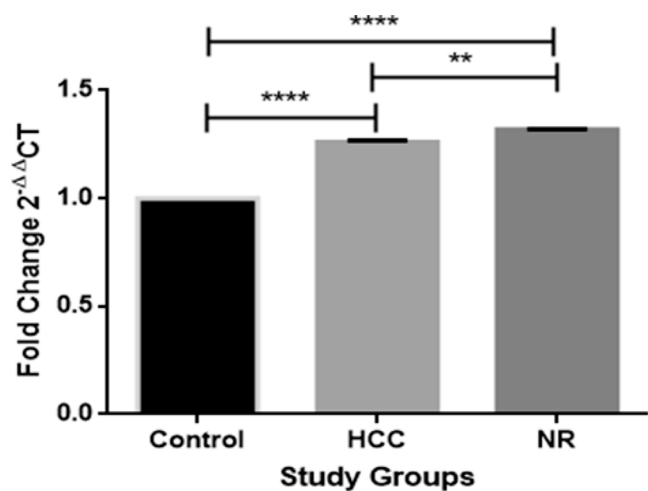


Figure 6. Comparison of TP53 gene expression levels among HCC and NR groups. Ordinary one-way ANOVA calculated the level of significance. The expression level for the NR group is 1.320 and for the HCC group is 1.266. Columns depict the mean fold change, and error bars indicate the standard error of the mean for each group (** $P < 0.01$ and *** $P < 0.001$).

significance 0.05 on the number of four possible comparisons (tests). After division, we got the Bonferroni corrected p value of 0.066. $2^{-\Delta\Delta CT}$ value for the NR group was 1.320 and for the HCC group was 1.266 ($p = 0.0024$). So, it was observed from the data that almost equal TP53 expression was observed in these two groups.

3.5. Sofosbuvir + Daclatasvir Treated Group Exhibited Highest TP53 Expression. TP53 expression was compared in three different treated groups. $2^{-\Delta\Delta CT}$ value was calculated for each group. After data analysis, TP53 expression was found to be highest 5.856 in the SOF + DCV treated group. Second highest TP53 expression 1.945 was observed in the sofosbuvir + RBV-treated group and least TP53 expression 1.079 was observed in the IFN-treated group. Untreated group showed the expression level of 1.932. To control the group's wise error rate, we applied the Bonferroni correction. So, in this analysis, we have five groups and compared each group with the other group for four possible comparisons as seen from Figure 7. For Bonferroni correction, we divided the level of significance 0.05 on the number of five possible comparisons (tests). After division, we got the Bonferroni corrected p value of 0.01. So, these data suggest that in the double DAAs (I)-treated group, TP53 expression was found to be 3.9 folds higher ($p < 0.0001$) as compared to the DAA group (II) and 4.7 folds higher ($p < 0.0001$) as compared to the IFN-treated group (III) (Figure 7). Naïve patients exhibit the second lowest expression of TP53 with the value of 1.932 that is 3.92 folds lower from group I ($p < 0.001$), 0.01 folds lower from group II ($p = ns$), and 0.8 folds higher from group III ($P < 0.0001$). Comparison to IFN-treated groups indicated by a significant p value of < 0.0001 . On the

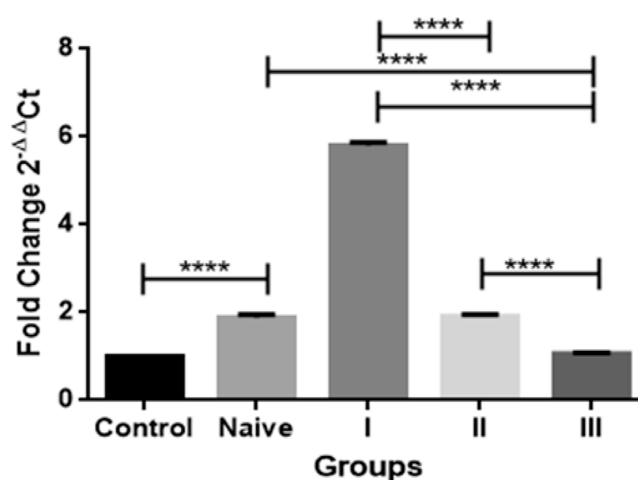


Figure 7. Comparison of TP53 expression in naïve, double DAA (I), DAA (II), and IFN (III)-treated groups. Error bars show the standard error of the mean. P -values were determined by ordinary one-way analysis. The highest TP53 expression, 5.85, was observed in group I, 1.94 in group II, and 1.07 in group III. Columns depict the mean fold change, and error bars indicate the standard error of the mean for each group (**** $P < 0.0001$).

other hand, a non-significant result was seen in comparison of naïve with DAA (sofosbuvir + RBV)-treated patients with a fold difference of -0.01 ($p = 0.98$). Non-significant fold change difference was seen between naïve and DAA-treated patients ($p = 0.98$), while comparing the Bonferroni corrected p value.

4. DISCUSSION

HCV may be called a silent killer pathogen because majority of hepatitis C-infected subjects are unaware of the HCV presence in their bodies, even a decade after infection.¹⁶ HCV infection not only results in chronic liver infection but it is also responsible for causing 27% of cirrhosis and 25% of HCC globally.⁶ HCV virus makes the liver cirrhotic that results in high oxidative stress. This oxidative damage leads to increased cell death and regeneration that damages cellular DNA, and consequently converting the normal hepatocytes into malignant cells.¹⁷ In many studies, HCV pathogenesis has been seen to be associated with TP53 gene. As in all types of human cancers, the most common genetic alterations have been found to be associated with the tumor suppressor gene p53.¹⁸ Mostly HCV proteins like HCV core protein,¹⁹ NS3,¹¹ or NS5¹⁰ suppress p53 gene expression and activation. HCV proteins, especially core protein, directly affect the p53 activity. At low viral load p53 activity gets upregulated by the HCV core protein but at higher viral load, it suppresses the p53 activity in hepatic cells.²⁰ This suppression of p53 expression causes the transformation of infected hepatocytes into cancerous cells. It has also been demonstrated that not only mutations but also polymorphism of the p53 gene increases the risk of HCC in HCV-infected Egyptian patients.²¹ The aim of this study is to observe the TP53 gene expression and its association with different combinations therapies administered to the studied infected population. If the administered drug is positively regulating the expression of TP53 gene might be a role to curb the chances of developing liver complications. Expression of the TP53 gene was checked through quantitative PCR.

The expression of TP53 mRNA and various clinical factors were compared among different studied groups. Comparison of age among different groups showed that the old age group

increases the risk of failure toward treatment that leads to HCC. As HCC was seen in the old age group followed by NR and many studies claim that age increases the risk of developing cirrhosis and HCC.²² All individuals included in our study were found to be having age above 40 years. However our data shows that the response rate toward treatment decreases with age as, HCC patients and NR were found to be >45 years of age that validates with the study of Wasley et al.,²³ who stated that patients of advanced age at time of infection are at increased risk of developing chronic HCV infection due to a weaker immune system. Denniston et al.²⁴ also correlates with our study by declaring that in the U.S., mostly HCV-infected people are about 50–70 years old. Liver function enzymes like ALT levels were seen normal in all groups but AST levels were high in each group except HCC patients, which coincides with the study of Pereira et al.,²⁵ who states that patients with chronic HCV infection can have normal levels of transaminases. Elevated ALP value has been observed in each study group, but the HCC group showed the highest value of ALP. This study suggests that the ALP enzyme was found significantly elevated in HCC patients, which agrees with the study by Ijaz et al.²⁶

Other factors like platelet count, TLC, HB, and bilirubin levels were found to be normal in each group that suggest available treatments for hepatitis C may be responsible in reducing hepatic inflammation or ameliorating liver function in HCC patients and are accountable for decreasing HCC complications²⁷ (Table 3).

Prevalence of HCV-related complications was analyzed to find out the highly prevalent complication associated with HCV because hepatitis C is linked with severe complications. Diabetes was seen in majority patients as compared to other complications and it correlates with study by Mehta et al.,²⁸ which observed HCV patients of ≥ 40 years of age had three times more chances of having type 2 diabetes than those without HCV infection. HCV is considered to be associated with diabetes and in agreements with many other previous studies²⁹ (Figure 2).

As our study was based on three different combinations of therapies for the treatment of HCV enrolled patients, these are (i) pegylated IFN + RBV, (ii) SOF + RBV, and (iii) SOF + DCV. The relapse rate of those treatments was analyzed and found that relapse rates of therapy (i–iii) were 11.9, 4.76, and 0%, respectively, that shows the IFN relapse rate is highest in comparison to the rest of the two groups (Figure 3). The IFN relapse rate in our study is parallel with the study of Raza et al.,³⁰ who studied and concluded that in Pakistan, the recurrence rate of HCV was high in patients treated with IFN as compared to DAAs. They suggested to select the best therapeutic options for the prevention and recurrence of HCV infection.

Next finding was to observe the TP53 gene expression in different characterized groups (SVR, RVR, EVR, NR, naïve, and healthy controls) in this study. $2^{-\Delta\Delta CT}$ values for naïve, RVR, EVR, and SVR were 1.932, 2.676, 2.445, and 2.250, respectively. Fold change difference of naïve patients with RVR is 0.744 folds lower ($p = <0.0001$), with EVR 0.51 folds lower ($p = <0.0001$), and with SVR 0.32 folds lower expression ($p = <0.0001$). So, in untreated patients, lowest level of TP53 expression was observed, while the highest expression of TP53 was seen in treated patients (Figure 4).

Next comparison was among the patients who cleared the virus after 6 months of treatment (SVR), after 4 weeks of treatment (RVR), and the patients who could not clear the virus after 6 months of advised treatment (NR). Fold change values

for SVR, RVR, and NR were 2.676, 2.250, and 1.320, respectively. We observed a difference of 1.37 between the RVR and NR group with a P value of $p = 0.0004$ and fold difference of 0.95 between the SVR and NR group ($p = 0.0015$), suggesting that patients who were NRs to all treatments had a very low expression of TP53 as compared to those who achieved SVR and RVR (Figure 5).

As the HCV infection progresses and the recommended treatments fail to clear the virus from the patients and progress the patients toward HCC. In this study, the TP53 gene expression was compared between patients who were NRs and others who have developed HCC. It was found that there was only a difference of 0.05 folds in TP53 expression of both groups. $2^{-\Delta\Delta CT}$ value for the NR group was 1.320 and for the HCC group was 1.266 ($p = 0.0024$) showing that both groups had almost same expression of TP53 (Figure 6). These results contradict with previous studies conducted on HCV patients of genotypes 1 and 2 that demonstrated upregulation of TP53 expression in chronic hepatitis C (CHC) patients.³¹ Our results like upregulation of TP53 mRNA expression in SVR and RVR compared with HCC and NR demonstrate that the TP53 gene plays a significant role in inhibiting HCV progression. These results correlates with the study by Dharel et al.,¹² who observed higher expression of HCV RNA as well as of viral protein in the Huh7 cells, where TP53 expressions were knocked down. In addition to that they found IFN was less effective in controlling HCV replication in cells in which the TP53 gene was knocked down and activation of the ISGs and ISRE are significantly attenuated by TP53 gene expression because this gene directly interacts with IRF9.

To the best of our knowledge, it is the first study to analyze p53 expression levels in response to IFN- α + RBV, DAA, and double DAAs treatment in HCV-infected patients. To find out the association of different treatments with TP53, patients were divided in three groups (i) double DAAs: SOF + DCV-treated, (ii) DAA: SOF + RBV-treated, and (iii) IFN + RBV-treated groups. It was analyzed that the TP53 expression was highly upregulated in patients treated with SOF and DCV (5.85), followed by SOF + RBV (1.94) and then the lowest expression (1.07) was seen in the IFN + RBV-treated group. In the untreated group, TP53 expression was 1.93 that was slightly 0.8 folds higher from group (III) ($p < 0.0001$) and 0.01 folds lower from group (II) that shows SOF and DCV significantly affect TP53 expression as compared to other treatments because this group exhibited a fold difference of 3.92 with naïve patients ($p < 0.0001$) (Figure 7). HCV proteins NS3 and NS5 inhibit TP53 gene functioning,¹¹ so higher expression of this gene after using SOF and DCV shows a role of these drugs in elimination of virus as well as in enhancing TP53 gene expression. On the basis of the obtained results, it can be predicted that SOF + DCV treatment not only protects HCV patients from progressing toward severe liver complications but also plays a significant role in preventing HCV relapse. TP53 gene can be used as a useful molecular marker toward different antiviral treatments.

5. CONCLUSIONS

HCV proteins like core proteins and NS3 proteins have been found to directly interact with the TP53 gene in various research studies but researchers could not find a clear understanding about their interactions. So, the focus of this study was to observe the expression levels of TP53 gene in HCV-infected patients. Our study indicates that the TP53 gene expression is highly up regulated in patients who achieved RVR and SVR as

compared to the HCC and NR group. Moreover, SOF + DCV therapy induces a significant rise in TP53 gene expression levels as compared to (SOF + RBV) and (IFN + RBV) treatment. According to these results, it can be predicted that SOF + DCV may play a significant part to prevent HCV patients from developing severe liver complications as compared to other two treatments. This study is novel as no such type of study has been conducted previously on the expression of TP53 in local HCV infected population treated with different combinations of therapies. This study is helpful for the development of new therapeutic strategies and to improve existing therapies.

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Notes

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