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Research Article

Distribution of IFNG and IFNGR1 Genotypes among HCV-Infected Pakistani Patients: A Case–Control Study

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Abstract

Hepatitis C virus infection is a significant health concern that leads to serious liver-related complications such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC). The immunoregulatory cytokines, interferon gamma (*INFG*) and interferon gamma receptor-1 (*IFNGR1*), genes play vital role in viral infections outcomes. The current study aimed to determine the genetic distribution of single nucleotide polymorphisms (SNP), in *IFNG* and *IFNGR1* genes in HCV positive patients. A cross-sectional study was carried on total of 190 subjects, including 30 Sustained Virological Responder (SVR), 80 HCV positive patients and 80 healthy controls. The RNA and DNA were extracted; PCR was carried out for HCV detection and genotyping. The in-house PCR was used for amplification of Intron-1 and Promoter of INF-γ, and IFN-γR1, while sequencing was performed through Sanger's method. The prevalence of TT, AT and AA genotypes of *IFNG* at +874 was (0.40), (0.33) and (0.27) in SVR, (0.20), (0.55) and (0.25) in HCV positive patients, while (0.45), (0.39) and (0.15) in healthy control respectively ($p < 0.001$).The frequency of the AT genotype was high in HCV positive patients (0.55) while the TT was significantly high in SVR (0.40) and in healthy control (0.45) ($p < 0.0001$). The frequency of TT, TC, and CC genotypes of *IFNGR1* gene at -56 was (0.27), (0.43), and (0.30) in SVR, (0.29), (0.48) and (0.24) in HCV positive patients, while (0.39), (0.20) and (0.40) in healthy individuals respectively ($p = 0.008$). The TC genotype was significantly high in SVR (0.43), and (0.48) in HCV positive patients while the CC was more prevalent in healthy control (0.41). It is concluded that the AT and AA genotypes of the *IFNG* and the TC genotype of the *IFNGR1* were more prevalent in HCV-positive patients, while the TT genotype of the *IFNG* and the CC genotype of the *IFNGR1* were highly prevalent in SVR and in healthy individuals. This suggests that certain genetic variations may influence susceptibility to HCV infection and affect treatment outcomes.

Keywords: Interferon gamma, Interferon gamma Receptor1, Hepatitis C, HCV

1. Introduction

Hepatitis C virus (HCV) infection is a major health issue worldwide, impacting around 180 million individuals globally (Sun et al. 2015). HCV is an RNA virus that consists of a single strand, with increasing infection rates and signifying serious health concern. The infection of HCV greatly impacts mortality and morbidity associated with LC and HCC (Habeeb et al. 2021). The reported prevalence of HCV infection from Pakistan is 11.55% and LC from 20% to 25%. In Pakistan, genotypes 1 and 3 of the HCV are most common, and the annual mortality rate as result of HCV infection is 1.6% (Nasir, Maham, and Sakhawat 2024). Chronic HCV infection can cause liver inflammation, increasing the risk of liver complications. The genetic makeup of an individual can influence the outcomes of HCV infection and the inadequate inflammatory response promotes fibrogenesis and contributes to cirrhosis and other manifestations (Seeff 2002).

Table 1: Antibiogram of Targeted SNPs in the *IFNG and IFNGR1* genes.

Gene	Chromosome region	Site	Part	Amplicon size bp	Methods for sequencing
IFNG	12q24	+874	Intron1	324	Sanger Method
		-764	Promoter	1080	Sanger Method
IFNGR1	6q23	-56	Promoter	275	Sanger Method

Immune cells (CD4+ T helper 1 and T helper 2) secrete antiviral cytokines, especially IFN-γ protein. This protein targets HCV by activating B cells and CD8+ T cells specific to the virus while inhibiting viral RNA replication and protein synthesis, thereby enhancing immune responses (Kaplan et al. 2007). Genetic variations in the *IFNG* gene may influence disease outcomes (Sun et al. 2015). IFN-γ protein binds to its specific receptor, IFN-γ receptor-1 (IFN-γR1). Genetic variations in the *IFNGR1* gene can affect this binding and may progress to different complications (Khanizadeh et al. 2011). Polymorphism in the *IFNGR1* ge ne may reduce receptor function, potentially increasing disease severity by reducing IFN-γ binding. Genetic variations in different parts of the *IFNG* gene can affect the expression of gene (Alvarez et al. 2023). Mutations in intron-1 have been linked to hepatitis B, tuberculosis, arthritis, cancer, malaria and multiple sclerosis (Alspach et al. 2019). Variations in the promoter region of the *IFNGR1* gene are linked to the development of chronic Hepatitis B and Hepatitis C infections, as well as increased the risk of hepatic fibrosis (Khanizadeh et al. 2011).

Several SNPs, have been identified such as +874 A/T, -764 C/G, and +2109 A/G, and have linked with HCV outcomes. The -764 C/G promoter polymorphism related to spontaneous viral clearance (McHutchison 2011). The +874 A/T SNP of the *IFNG* gene influences IFN-γ production, the TT genotype at +874 associated with increased level of IFN-γ and antiviral defense. While the AA and AT genotypes associated with lower and intermediate level of IFN-γ, possibly increasing infection risk that may lead to chronic HCV complications (Pravica et al. 2000). These genetic variations may shape immune responses and susceptibility to HCV infection (El-Bendary et al. 2017). HCV infection triggers a strong innate immune response, activating interferon-stimulated genes (ISGs) (Thomas et al. 2012). The polymorphism at +874 in intron-1 of *IFNG* gene may influence immune response and vulnerability to viral infections. The IFN-γ protein and its receptor IFN-γR1 are essential in preventing HCV infection. Variations in these genes can affect infection outcomes, particularly in the intron part of the *IFNG* and the promoter region of the *IFNGR1* genes. Studies worldwide have shown a link between these genes and different infections, including HCV (Habeeb et al. 2021). This study aims to analyse genetic diversity in *IFNG* (+874A/T), Promoter G/A and *IFNGR1* (-56T/C) SNPs and to assess their relationship with HCV infection in patients from Peshawar, Pakistan.

2. Methodology

2.1 Study Design and Study Population

This study was carried out on clinically confirmed HCV positive patients from Khyber Pakhtunkhwa, Pakistan. The study populations involved one hundred and ten (n=110) participants and were divided into three main groups. Group I included Sustained Virological Responder (SVR) comprised 30 subjects, Group II included HCV positive patients consisting of 80 individuals. While group III included 80 healthy control individuals.

2.2 Inclusion/Exclusion Criteria

HCV antibodies and RNA- positive patients were included in the study. Patients with Hepatitis B surface antigen (HBsAg) or Hepatitis B virus (HBV) DNA positivity were excluded from study.

Table 2: Analysis of the demographic and clinical features of patients with HCV infection.

Parameters	SVR	HCV patients	Control	P value
Lab Assessment (U/L)	n=30	n=80	n=80	0.0001
SGPT	30.50±3.2	152±62.5	18.50± 3.5	0.0001
AST	32.35±4.5	172.50±105.7	22.60±4.5	0.0001
ALK	105.50±35.2	318±115.9	60.50±17.2	0.0001
Bilirubin mg/dL	1.153± 1.2	4.11±1.3	0.35±0.11	0.0001
Mean Age± SD (Y)	30.20±7.5	45.80±15.5	25.50±8.5	0.0001
Age range (Y)	20-50	25-60	25-65	0.0001
Lab Assessment (U/L)	n=30	n=80	n=80	0.0001

Note: SVR= Sustained Virological Responder

2.3 Data and Samples Collection

Data were collected using a pre-designed questionnaire that was administered to each participant after obtaining their written informed consent. A 5 mL blood sample was collected from each participant using a disposable syringe and transferred into EDTA tubes. The samples were then transported to the Laboratory of Virology and Immunology at the Institute of Zoological Sciences, University of Peshawar, Khyber Pakhtunkhwa, Pakistan.

2.4 Ethical Approval of the Study

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Research Ethics Board (REB), University of Peshawar, Pakistan, under the registration number 04/03 on 16 June-2024.

2.5 HCV Antibody Screening

Serum samples were analyzed for antibodies against HCV using the Bioline™ HCV kit, following the manufacturer's instructions.

2.6 Molecular Detection of Hepatitis C Virus

The PCR detection of HCV was carried out by performing the following procedures.

2.6.1 Nucleic Acid Extraction

HCV RNA was isolated from anti-HCV positive serum samples using a commercial RNA extraction kit (Favorgen, USA) following the manufacturer's protocol. Genomic DNA was then extracted from the HCV-positive blood samples by using the Gene JET Genomic DNA Purification Kit (catalog number K0721, Thermo Scientific™, USA), according to the manufacturer's instructions. The quality of the extracted DNA was assessed by Nano-drop quantification with a Multi Scan micro-plate reader (Thermo Fisher Scientific). The purified DNA was stored at -20°C for subsequent analyses.

2.6.2 cDNA Synthesis, Amplification and HCV Genotyping

Complementary DNA (cDNA) was generated from the untranslated region (5'UTR) of the HCV genome using an antisense primer, according to the instructions provided with the RT-PCR kit (Thermo Fisher, USA).

Subsequently, this cDNA was subjected to nested PCR amplification with two sets of primers specific to the 5' UTR sequence, utilizing Taq DNA polymerase (Thermo Fisher, USA). To amplify and differentiate various HCV genotypes, multiplex PCR was performed following the methodology described by (Shemis et al. 2012).

Table 3: Distribution of genotype and Allele frequency of *IFNG* in HCV-positive patients and healthy controls.

IFNG genotypes	HCV-infected patients						
	SVR 30		HCV patients 80		Control 80		
+874A/T	Number	Frequency	Number	Frequency	Number	Frequency	P value
TT	12	0.40	16	0.20	36	0.45	< 0.011
AT	10	0.33	44	0.55	31	0.40	
AA	8	0.27	20	0.25	13	0.15	
Allele							
T	34	0.57	76	0.48	103	0.64	0.01
A	26	0.43	84	0.52	57	0.36	
IFNG-764							
AA	22	0.73	71	0. 89	55	0.69	0.008
AG	8	0.27	9	0.11	25	0.31	
Allele							
A	52	0.87	151	0.94	135	0.84	0.014
G	8	0.13	9	0.06	25	0.16	

Note: $p < 0.05$ was significant, SVR= Sustained Virological Responder

Sequence of the Primers for Regular PCR

5`-CCCTGTGAGGAACTACTGTCTTCACGC-3`

5`-ACTCGCAAGCACCCTATCAGGCAGTAC-3`

Sequence of the Primers for Nested PCR

5`-GAAAGCGTCTAGCCATGGCG-3`

5`-CACAAGGCCTTTCGCGACC-3`

2.7 Detection and Amplification of the *IFNG* and *IFNGR*1 genes

The *IFNG* intron-1 (+874 A/T) microsatellite CA genes were carried out in a volume of 25µL. This volume included 12µL of Master Mix (Ampli Taq was amplified according to the protocol of (Azam et al. 2015). The primers used for *IFNG* gene (+874A/T) were; forward 5`-TCGTTGCTCACTGGGATTTTG-3`and reverse: 5`-CATCTACTGTGCCTTCCTGT-3` and for promoter region the primers were; forward 5`-CCCACTTCGCCCTGGTAAAA-3` and reverse: 5`- GGAGGGCTTTTTGTGCCATC-3`. The *IFNGR1* gene was amplified according to the methodology of (Juliger et al. 2003) and the set of primers to be used were: forward: 5`-CCTCCCACACCCAGAAGTCC-3` and reverse: 5`-TGCATGACAAGGGGTAGGAG-3`. A single , reaction for amplifying the *IFNG* and *IFNGR1* Gold, Catalogue number: 4390939), 2.5µL of the targeted DNA, 0.5µL of each primer, and 10µL of distilled water. The PCR reaction conditions for the *IFNG* gene (+874A/T) included a single cycle at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 64°C for 30 seconds, 72°C for 1 minute, and a final round at 72 °C for 5 minutes. The Thermal cycle for promoter region consisted single cycle at 95°C for 3 minutes, followed by 38 cycles at 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 1 minute, and a final single round at 72°C for 5 minutes. While the reaction conditions for the *IFNGR1* gene consisted of a single cycle at 94°C for 5 minutes, 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, and a final single round at 72°C for 7 minutes.

2.8 Gel Electrophoresis

PCR-amplified Products of the *IFNG* and *IFNGR1* genes were separated on 2% agarose gel by electrophoresis stained with ethidium bromide and visualized using a UV trans-illuminator. A 100 bp DNA ladder (Thermo Fisher, USA) was used as molecular size marker **(Figure 1)**.

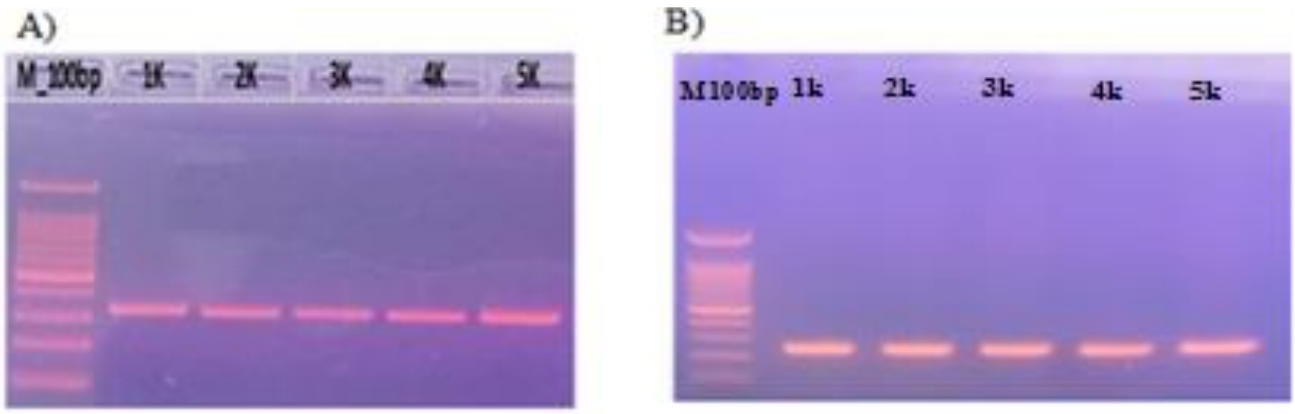

Figure 1: Gel Electrophoresis images showing PCR products size of the *IFNG* and *IFNGRI* genes: (A) IFNG Intron-1 +874A/T polymorphism product size: 324bp. DNA ladder marker 100bp used as size marker. (B) PCR amplification of the *IFNGR1* gene promoter region -56T/C polymorphism, product size of 275bp, with a 100bp DNA ladder marker used for size estimation.

2.9 *IFNG* and *IFNGR1* Genes Sequencing and Analysis

The PCR-amplified products of the *IFNG* and *IFNGR1* genes were analyzed using the Sanger sequencing method. Sequence confirmation was carried out by using the BLAST. The latest version of BioEdit software for Windows was applied to assess multiple sequence alignments and detect genetic polymorphisms within the sequences.

2.9.1 Targeted SNPs in the *IFNG* and *IFNGR1* genes

Table 1 represents the Cytogenetic region, Position, Location, Amplicon size and method of sequencing for the *IFNG* +874 A/T, -764 G/A, and for *IFNGR1* -56 T/C.

2.10 Data analysis

Data were statistically analyzed by using IBM SPSS, latest version 24. One-way analysis of variance (ANOVA) was employed to compare means between the groups. The χ^2 test was applied to assess differences in genotypes and allele frequencies. Statistical significance was defined as a P-value less than 0.05.

3. Results

3.1 Analysis of the demographic and clinical features of patients with HCV infection

The demographic and clinical features of the participants, including 30 sustained virological responders (SVR), 80 HCV- positive patients and 80 healthy control individuals are described in **Table 2**. The HCV patients exhibited significantly elevated liver enzyme levels, as evidenced by higher mean values of "SGPT", "AST" and "ALK" as compared to the SVR and healthy control group (P = 0.0001). Similarly, serum bilirubin levels were significantly increased in HCV patients (4.11 ± 1.3 mg/dL) relative to those with SVR (1.153 ± 1.2 mg/dL) and healthy controls (0.35 ± 0.11 mg/dL), indicating greater hepatic dysfunction in the infected cohort.

In terms of demographic characteristics, the mean age of HCV patients (45.80 ± 15.5 years) was higher than that of the SVR group (30.20 ± 7.5 years) and controls (25.50 ± 8.5 years), with the age range also extending higher among HCV patients (25-60 years) compared to the other groups. The current findings highlight the substantial alterations in liver function and demographic disparities among individuals with active HCV infection compared to those who achieved SVR and in healthy controls.

3.2 Distribution of genotype and allele frequency of the *IFNG and IFNGR1* in HCV-positive patients and healthy controls

The frequency of TT genotype was most common in controls individuals (0.45), with lower

Table 4: A Distribution of different genotypes of *IFNGR1* in HCV-infected patients and healthy controls.

IFNGR1 genotypes	SVR 30		HCV-infected patients HCV patients 80		Control 80		P value
-56T/C	Number	Frequency	Number	Frequency	Number	Frequency	
TT	8	0.27	23	0.29	30	0.38	0.009
TC	13	0.43	38	0.48	17	0.21	
CC	9	0.30	19	0.24	33	0.41	
Allele							
T	29	0.48	84	0.53	77	0.48	0.707
C	31	0.52	76	0.47	83	0.52	

Note: $p < 0.05$ was significant, SVR= Sustained Virological Responder

frequencies observed in SVR (0.40) and HCV patients (0.20). The genotype AT was highly detected in patients group (0.55) than the SVR (0.33) and controls subjects (0.39), whereas the AA genotype had similar frequencies across the groups. The T allele was highest in controls (0.64), whereas the A allele was more common in HCV patients (0.52). Chi-square analysis revealed considerable variations in both genotypes ($p < 0.011$), and alleles ($p = 0.01$) distributions among groups. For the *IFNG* -764, polymorphism, the AA genotype predominantly detected in all groups, especially in HCV positive patients (0.89), followed by SVR (0.73) and controls (0.69). The AG genotype was less frequent. The allele A was most common across all groups, while the G allele was relatively rare. The significant differences were observed in genotype ($p = 0.008$) of SVR, HCV positive patients and control group and differences were also noted between the allele frequencies ($p = 0.014$) of the *IFNG* at -764 (**Table 3**).

The distribution of IFNGR1 -56T/C genotypes varied significantly among SVR, CHC, and control groups ($p = 0.009$). The TT genotype was most frequent in controls (0.38), while the TC genotype predominated in HCV positive patients (0.48). The CC genotype was observed at similar frequencies in controls (0.41) and SVR (0.30), but less so in HCV positive patients (0.24). Conversely, there was no significant difference in allele frequencies between the groups ($p = 0.707$) (**Table 4**).

4. Discussion

IFNG is a modulatory multifunctional cytokine that plays a crucial role in the body's defense mechanisms against viral infections, in addition to its fibro-genic properties (Falleti et al. 2007). *IFNG* attaches to a specific receptor located on the immune cells (*IFNGR1*), playing important role in many cancer types and triggering JAK-STAT pathways. The IFNG +874 T/A variant may impair IFNGR1 function, potentially raising the risk of chronic liver diseases (Sun et al. 2015). The +874T allele sequence provides attachment site for the transcription factor (NF-κB) (Rossouw et al. 2003). Alteration of the NF-κB signaling pathway can result in oxidative stress, potentially raising the risk of developing cancer, LC, and HCC (Pravica et al. 2000). The transcription factor regulates the expression of *IFNG* and the +874 T allele linked to elevated *IFN-γ* levels, whereas the allele A is related to reduced expression (Rossouw et al. 2003). The synthesis of cytokines varies between individuals and is linked to specific mutations found in both the coding and regulatory regions (Ollier, 2004). The current study aimed to investigate the diversity of different genotypes of *IFNG* and *IFNGR1*, in HCV positive patients and also analyse which genotype is more susceptible to infection. The analysis of SNPs in IFNG at +874A/T and *IFNGR1* at -56T/C genotypes in this study associated with chronic HCV infection. The current study supports previously reported data

and shows that the IFNG +874 A/T and –56C/T SNPs in the promoter region of *IFNGR1* act as indicators for chronic HBV and HCV infection (Korachi et al. 2005).

Genetic variations in *IFNG* and *IFNGR1*, pathway may influence chronic HCV infection progression. *IFNG* is a key regulatory cytokine defending against viral infections and possesses fibro-genic properties (Falleti et al. 2007). The variation at locus +874A/T may impair *IFN-γR1* function, raising the risk of liver diseases complications). Data reported that genetic variation in cytokine genes contributes to the development and progression of chronic Hepatitis C (Sun et al. 2015). The current work identified statistically significant variance in the frequencies of *IFNG* and *IFNGR1* SNPs in HCV infected patients across different groups. The higher frequencies were detected for AT, AA, as compared to TT of the IFNG (+874). The TC, and CC genotypes of IFNGR1 (-56) were also highly frequent than the TT genotype. The current study finding findings are consistent with the previously reported data, which suggested that the TT genotype at +874 in *IFNG* is associated with cirrhosis, indicating a link between +874A/T polymorphism and cirrhosis in CHC patients (Dai et al. 2006).

IFNG promotes immune responses especially Th1-type cellular responses, which plays a role in inflammation and the development of liver fibrosis in patients with chronic Hepatitis C. It is the main predictor of recurrence and survival in hepatocellular carcinoma (Bouzgarrou et al. 2009).The results confirm that the IFNG +874A/T and -56C/T *IFNGR1* polymorphisms are indicators of chronic HBV and HCV complications (Korachi et al. 2013). Participants with the IFNG +874TT genotype generate increased amounts of IFN-γ, leading to a stronger immune response against viral infections and influencing the progression of HCV infection. Those with the TT genotype of *IFNG* have stronger antiviral defenses, while individuals with the TA and AA genotypes have lower *IFN-γ* levels, increasing their risk of HCV infection and related complications (Pravica et al. 2000). The current study supports the aforementioned study, that the genotype TT was observed high in SVR and healthy controls as compared to HCV infected individuals, while the AT genotype at +874 of the *IFNG* is common in HCV infected individuals than the SVR and control. The current study aligns with the data reported from Egypt and they found higher prevalence of the TT genotype in healthy and SVR individuals. A meta-analysis involving Asian and Caucasian populations found that individuals with the TT genotype and T allele are less susceptible to liver diseases. On the contrary, the presence of the AA genotype and A allele at the +874 position is associated with a 1.4-fold increased risk of developing complications related to hepatitis C virus infection (Sun et al. 2015), the current research study was in agreement with previous reported study.

The current study detected *IFNG* promoter polymorphism G/A in HCV infected patients and two genotypes were identified i.e., GA and AA. The genotype AA was examined high in HCV positive patients as compared to the GA genotype. Several authors reported *IFNG* promoter -764 C/G and identified that the mentioned SNP may play role in clearance of HCV and also assist in the treatment (Azam et al. 2015). Several other studies reported that -764 GC SNP plays role in clearance of HCV infection and treatment responses (McHutchison 2011). The AA genotype at the promoter was more common in SVR in current study, while the GA genotype was less common because it is less frequently present in study population, so it is lower than AA.

Several studies have investigated the -56T/C promoter SNP of the IFNGR1 gene, finding significant associations with susceptibility to HCV infection. The TT and TC genotypes appear more frequently in patients with HCV. Study conducted by Sun et al. (2015) suggests that genetic variations in *IFNG* and *IFNGR1* may contribute to an increased risk of chronic liver diseases, such as

fibrosis and HCC. (Aref et al. 2021) also reported that polymorphisms in *IFNG* and its receptor *IFNGR1* can influence IFN-γ production in acute HBV patients, potentially heightening the risk of hepatitis-related complications. Moreover, genetic variations in the promoter region of *IFNGR1* have been linked to a range of diseases (Juliger, 2003). The AA genotype of IFNG may increase the likelihood of HCC in cirrhotic patients by inhibiting the IFN-γ signaling pathway, thereby diminishing its anticancer properties. Additional studies confirm that genetic polymorphisms in the promoter region of IFNGR1 are linked to various diseases (Juliger, 2003). In the Chinese population, the –56C/T variants have been found to influence HBV infection outcomes, with the -56C allele playing a role in recovery from acute HBV infections (Azam et al. 2015). The present study's findings are consistent with these previously reported results.

5. Conclusion

It is concluded that the AT genotype and A allele at +874 in *IFNG*, and TC genotype and T allele at -56 promoter in *IFNGR1*, were significantly high in HCV positive patients. In contrast, the TT genotype and T allele at +874 in *IFNG*, and CC genotype and C allele at -56 in *IFNGR1*, were more common in SVR and healthy individuals.

Conflict of Interest

The authors declare no conflicts of interest.

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Ethical Approval

The study was approved by the Research Ethics Board (REB), University of Peshawar, Pakistan, under the registration number 04/03.

Consent Forms

Every participant signed a consent form before participating in the research.

Author Contributions

KR conducted original draft preparation, methodology, and investigation. SK contributed to conceptualization and supervised the study. MBK performed data curation and formal analysis. MY contributed to visualization. All authors have read and agreed to the published version of the manuscript.

Data Availability

All data generated or analyzed in this study are fully presented within this article.

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