Impact of IL-12B Genetic Variants on Antiviral Treatment Response among Hepatitis B Patients in Pakistan

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Abstract: HBV is a continuous major global health concern. Genetic factors of hosts are known to play a role in HBV infection outcomes. This study aimed to reveal the association of IL-12b 3′ UTR variant rs3212227 in HBV patients. Genotyping was performed using ARMS-PCR to detect IL-12b rs3212227 polymorphism. The patients were categorized into groups based on their response to the antiviral therapy. Group I: non-sustained virological response (NSR); Group II: sustained virological responders (SVR); and Group III: HBV-positive fresh cases. ALT levels were measured to evaluate liver function, and viral load was determined to evaluate viral infectivity among the study groups. The variant genotype CC was found to be significantly associated with the non-sustained virological response to the antiviral therapy (with a $p$-value of 0.0117; OR = 2.914; RR = 1.556). It was also determined that the genotype CC was the most prevalent genotype among both genders in the NSR group. Viral load was found to be 6-fold higher in Group III compared to Group I and Group II. The results suggest that genotype CC is the most prevalent genotype in the NSR groups, and it is associated with a poor response to antiviral therapy in Pakistani patients with HBV infection.

Keywords: hepatitis B virus; viral load; single nucleotide polymorphism; genotyping; ARMS-PCR

1. Introduction

Hepatitis B Virus infection is still one of the deadliest diseases around the globe, particularly in the areas where HBV is endemic. According to an estimate, 296 million people are living with chronic HBV infection [1]. In 2019, 1.5 million new cases of HBV and 0.82 million HBV-related mortalities were reported [2]. There are several vaccines available for the prevention of HBV; nevertheless, millions of new cases of HBV are reported every year [3]. There are types of HBV genotypes that are prevalent in various geographical areas of the world. Among these genotypes, HBV genotype D is the most prevalent and widely distributed globally [4]. HBV infection can be acute with spontaneous clearance of the virus or can be chronic HBV infection. Chronic HBV infection can be categorized into asymptomatic carrier disease or chronic hepatitis B infection (CHBI) [5]. As an estimate, 25% of CHBI progresses to liver cirrhosis and subsequent hepatocellular carcinoma (HCC) [6]. It has been reported that up to 20% of patients with chronic hepatitis B who do not receive any treatment can develop HCC within 5 years [7–9].

The intricate interplay of numerous contributing elements, including host variables, viral variables, and external variables, can be ascribed to the distinct outcomes reported in HBV infection. Genetic variants, age, sex, and specific comorbidities such as obesity and diabetes are among the host factors that are well-known for affecting the clinical manifestations of HBV infection [7,10]. Genetic variation in host genes influences the immune system’s reaction and predisposition to HBV infection. Variations in specific
genes may impact a person’s ability to limit viral replication and, as a result, disease outcomes [11]. Gender disparities in HBV infection have been found, which could be attributed to hormonal factors, genetic predispositions, or variations in immunological responses [12]. HBV genotypes, viral load, and mutations all have a substantial impact on disease development. The viral load, which represents the quantity of circulating HBV DNA, is an important predictor of illness severity [13]. Higher viral loads are linked to an increased risk of chronic HBV infection and the development of serious liver disorders [14,15]. Furthermore, various comorbidities, such as diabetes and obesity, as well as certain environmental factors, also play a critical role in the clinical outcomes of HBV infection [16].

There are therapeutics available for the treatment of HBV, including Peg-IFN, lamivudine, telbivudine, entecavir, adefovir, and tenofovir. However, several reports have revealed that lamivudine is the most frequently used nucleoside reverse transcriptase inhibitor for the treatment of HBV [17]. A study reported that patients treated with lamivudine developed a sustained virological response, which was defined as a permanent normalization of ALT levels, and HBV-DNA copies lower than 200 copies/mL, while those patients who showed no sustained response were regarded to have a non-sustained response (NSR) [18,19].

Interleukins are essential biomolecules that play a crucial role in limiting infection and viral clearance [20]. These molecules differentiate, mature, and activate the cells of the immune system to fight off the pathogen. Interleukin 12 is a pro-inflammatory cytokine primarily produced by the dendritic cell in response to IFN stimulation [21]. IL-12 is a disulfide-bonded hetero-dimer and consists of a 35 kDa alpha subunit and a 40 kDa beta subunit [22]. IL-12 also plays a critical role in the initiation of the immune response and, therefore, has a critical role in the differentiation of Th1 and Th2 helper cells. As a result, the role of IL-12 becomes essential in determining the progression of HBV infection as well as regulating the response of antiviral therapy by regulating the balance in Th1/Th2 responses [23,24].

Studies have reported that genetic polymorphism in the 3′ untranslated region (UTR) of the IL-12 p40 (IL-12b) gene resulted in the substitution of A to C at position 1188 (rs3212227). Studies have shown that a variant allele is responsible for elevated levels of IL-12 [25]. Previously, studies have reported that altered allele C in IL-12b is associated with an increased risk of chronic hepatitis B disease after HCV infection in the Tunisian population [26]. Another study also depicted the IL-12b 1188A/C variant to be associated with HCV infection and HCC in the Han Chinese population [27]. Based on the experimental evidence that the IL-12b 3′ UTR variant is associated with the risk of HBV infection, this study investigated the association of IL-12b rs3212227 with response to antiviral therapy in HBV-infected patients.

2. Materials and Methods
2.1. Selection of Samples
Approval for this project was attained from the Institutional Review Board of Atta-Ur-Rahman Scholl of Applied Biosciences (ASAB), NUST. Prior to the collection of samples, written and informed consent was attained from each participant that was selected for this study. The blood samples were taken from the NUST diagnostic lab and Combined Military Hospital Rawalpindi, Pakistan. The total number of clinical samples was 332, which were collected from both male and female subjects. The samples were age and sex-matched. Those patients who were positive for HIV (immunocompromised), have HCV co-infection and other liver diseases such as hepatocellular carcinoma (HCC) were excluded from this study.

2.2. Categorization of Hepatitis B Virus Positive Samples
The participants who were positive for HBV-induced hepatitis (HBsAg S/C > 5.00; HBV DNA level > 2000 IU/mL; ALT ≥ 80 IU/mL) received antiviral treatment with
lamivudine (100 mg/day) for a period of twelve months. The threshold level for HBV DNA and ALT levels was determined based on the guidelines proposed by the American Association for the Study of Liver Diseases (AASLD) [17]. Those patients who, at the end of the therapy, were HBV PCR-positive were regarded as non-sustained virological responders (NSR). Only those participants who were PCR-negative for HBV at the end of antiviral therapy were regarded as sustained virological responders (SVR). Another group of HBV-positive patients was also included in this study, which involved those patients who were newly diagnosed with HBV and received no antiviral therapy. The control samples were attained from healthy individuals without any known serious liver and health conditions.

2.3. Determination of Alanine Aminotransferase Levels

To evaluate the levels of alanine aminotransferase in the samples, an ALT level test was performed on the serum samples of both disease and control groups. For this purpose, serum from the blood samples was separated immediately to prevent hemolysis. The ALT assay kit by AMP Diagnostics (Graz, Austria) was used according to the manufacturer’s protocol. Serum from all samples was taken for the ALT level analysis. Lactate was generated as the end product due to the chemical reaction between kit reagents and the serum sample, and its absorbance was measured at 340 nm through a MicroLab300 Biochemistry Analyzer (Q-line Biotech Private Limited, Lucknow, India). The ALT levels were calculated using a formula: concentration = ∆E/min × kit factor, where the kit factor is 1746 for absorbance at 340 nm. The normal value for the ALT level is 40 IU/L.

2.4. Detection of Hepatitis B Virus DNA

HBV DNA was extracted from HBs Ag-positive serum samples by using the QIAamp Viral DNA kit (QIAGEN Inc., Hilden, Germany) and amplified using Nested PCR. A 20 µL PCR reaction mixture contained DNA, PCR Master Mix (50 units/mL of Taq DNA polymerase supplied in a proprietary reaction buffer pH 8.5, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM MgCl2) (Promega, Southampton, UK, Cat.# M7502), 10 mM of forward primer P55′CCCGAATTCCGCCACCATGCATCCTGCTGCTATGCCTCATCT3′, and 10 mM of reverse primer P56′CCCGAATTCCGCCACCATGGGCACTAGTAAACTGAGCCA3′. The primers were annealed at 64 °C for 45 s. The PCR product was further amplified with the inner nested primer set, 10 mM forward primer P75′CCCGAATTGCCACCATGGGTATGTTGCCCGTTTGTCCTCT3′, and 10 mM of reverse primer P85′CCCGAATTCCGCCACCATGGGCACTAGTAAACTGAGCCA3′ for an additional 30 cycles under the same reaction conditions. Nested PCR products were analyzed by electrophoresis on 2% w/v agarose gels and visualized under UV-transilluminator. HBV DNA was detected and quantified through real-time PCR using an HBV quantification kit (RoboGene HBV DNA quantification kit 3.0, Roboscreen, Leipzig, Germany) according to the manufacturer’s protocol. The viral load was determined in HBV-positive samples for comparison and to associate the genetic variant with disease severity.

2.5. Genotype Analysis

The genomic DNA from the samples was extracted using a GeneJET Whole Blood Genomic Extraction Mini Kit by ThermoFisher Scientific (Waltham, MA, USA). The quantification of DNA was performed using NanoDrop8000 (ThermoFisher Scientific). A 280/260 ratio of around 1.8–2.0 was considered pure for the extracted DNA samples.

For the genotype analysis of polymorphism in the IL-12b gene, ARMS-PCR was used. ARMS-PCR primers were designed with Primer3 software, and they were validated using NCBI primer blasts. Forward inner primer (for allele A) 5′AATGAGCATTTAGCATCT3′, reverse inner primer (for allele C) 5′AATGAGCATTTAGCATCG3′, forward outer primer 5′GACACAACGGGAAATAGCCA3′, and reverse outer primer 5′TGCCAAGTGGAGCACCCAA3′. The reaction mixture was prepared by adding 12.5 µL of GoTaq® Green Master Mix (Promega, Southampton, UK), 4 µL of DNA sample, and 2 µL of each primer. The PCR
reaction involved initial denaturation for 5 min at 95 °C, followed by 35 cycles for 45 s, each involving denaturation at 95 °C, annealing at 57 °C, and extension at 72 °C followed by final elongation at 72 °C for 7 min. 2% (w/v) agarose gel electrophoresis was used to analyze amplicons of ARMS PCR.

2.6. Statistical Analysis

GraphPad Prism 9 (GraphPad Software Incorporated, San Diego, CA, USA) was utilized for performing the statistical analysis, and graphs were made using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). In order to compare the quantitative variables among the groups, such as age, ALT levels, and viral load, one-way ANOVA was conducted. Genotype association, distribution frequencies of genotypes, relative risk, and odds ratios were determined at 95% CI by applying the Fisher Exact Test. A p-value of less than 0.05 was regarded as significant.

3. Results

3.1. Participants Demographics

Among the cohorts investigated in this study, 109 were identified as males, while 223 were identified as females. Among 109 male subjects, 46 exhibited NSR, 24 exhibited SVR, and 17 had freshly diagnosed HBV at the initial stages. Of 223 female participants, 75 females were categorized under NSR, 103 displayed SVR, and 26 were diagnosed with initial stages of HBV. In addition to the HBV-positive samples, 41 age and sex-matched healthy individuals volunteered as study control (Table 1). Matching for age and gender is critical in a case-control study to reduce the impact of confounding variables, improve comparability among HBV-positive subjects and controls, and enhance association accuracy. It also reduced biases due to participant’s demographics and increased the likelihood that the observed differences were attained due to the factor that was being investigated. The ratio of female patients was higher in comparison to that of male patients in all cases, including SVR, NSR, and fresh cases. It was also observed that the number of cases reported in the age group 1–20 years was infrequent. Many of the experimental subjects fell in the age groups of 21–40 and 41–60 years. The illness burden was found to be significantly higher amongst young adults (≤40 years), while in the elderly population, the disease burden decreased progressively, with the least number of cases being reported in the age of 61–80 years.

Table 1. Comparison of primary characters between controls and HBV-infected patients.

<table>
<thead>
<tr>
<th></th>
<th>Total Samples (N = 332)</th>
<th>NSR (N = 121)</th>
<th>SVR (N = 127)</th>
<th>Fresh Cases (N = 43)</th>
<th>Healthy Controls (N = 41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>46/75</td>
<td>24/103</td>
<td>17/26</td>
<td>22/19</td>
<td></td>
</tr>
<tr>
<td>Age (Mean ± S.D)</td>
<td>(38.58 ± 12.18)</td>
<td>(37.50 ± 13.32)</td>
<td>(33.81 ± 12.86)</td>
<td>(38.41 ± 13.76)</td>
<td></td>
</tr>
</tbody>
</table>

3.2. Alanine Aminotransferase Levels

ALT is a crucial test performed to assess the liver’s function and detect inflammation or liver damage caused by the hepatitis B virus. The analysis revealed that ALT levels in HBV-infected patients were higher in comparison to the controls. The average values of ALT levels in HBV-positive groups versus controls are demonstrated in Figure 1. Average ALT values were highest for the fresh cases and NSR group (96 ± 12 IU/mL and 66 ± 68 IU/mL) compared to the control group (39 ± 56 IU/mL). However, for the SVR group, the average ALT levels were the lowest (32 ± 59 IU/mL), which is an indicator of a successful response to the antiviral therapy.
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Figure 1. Mean ALT values in study groups. ALT levels are highest for Group III, comprising freshly diagnosed HBV patients, followed by the NSR group (Group I). There is a significant difference in the ALT levels among the groups with NSR, SVR, fresh cases, and controls. Statistical significance was measured by one-way ANOVA (**** \( p < 0.0001 \)). ns: Non significant.

3.3. Viral DNA Analysis

Nested PCR is a highly sensitive and selective technique for the identification of the exact nucleotide sequence of the HBV DNA. Nested PCR was performed on viral DNA extracted from the fresh samples. The size of HBV amplicons was 234 bp, and the amplicons were visualized on 2% agarose gel (Figure 2).

Figure 2. Nested PCR-amplified products of HBV DNA on 2% agarose gel. Lane 1 contains 100 bp Ladder, Lane 2 contains negative control, Lane 3 HBV positive patient, Lane 4 contains negative control, and Lane 5 has positive control.
The viral load was evaluated by qRT-PCR analysis of the plasma samples of HBV-positive subjects. Viral loads of more than 800,000 IU/mL are regarded as high. In the current study, the mean value of viral load in all the HBV-positive samples was observed to be 237,910 ± 5483 IU/mL. For the NSR group, the mean viral load was noted to be 141,108 ± 876 IU/mL, while in the SVR group, the mean viral load was lowered to the value of 440 ± 321 IU/mL. It was also noted that the viral loads were the highest in fresh cases of HBV with the mean value of 1,206,147 ± 535, which was around six folds more than of NSR patients, as shown in Figure 3a.

![Figure 3](image-url)

**Figure 3.** Viral load in HBV patients plasma samples. (a) Viral load in various study groups. There is a significant difference in viral load among the study groups except for between NSR-Control and SVR-Control. (b) Viral load is plotted against the three genotype variants of IL-12 SNP rs3212227. The graph shows that genotype CA is most prevalent in patients with HBV infection. Statistical significance was measured by one-way ANOVA (**p < 0.01, ****p < 0.0001), ns: Non significant.

The viral load has also been evaluated against the three genotypes of the selected IL-12b loci 1188 C/A, as depicted in Figure 3b. Notably, the heterozygous genotype CA exhibited a trend toward elevated levels of viral load in HBV-infected patients compared to the other two genotypes. However, this distinction could not attain statistical significance.

3.4. Genetic Analysis

DNA was extracted from the HBV-positive samples and controls, and then they were genotypes for the detection of variant 1188C/A in the IL-12b gene in the 3'UTR region. Genotype analysis was performed using tetra ARMS-PCR, and this technique utilized four primers to amplify the targeted sequence of the gene. Among the study population of positive subjects. Viral loads of more than 800,000 IU/mL are regarded as high. In the current study, the mean value of viral load in all the HBV-positive samples was observed to be 237,910 ± 5483 IU/mL. For the NSR group, the mean viral load was noted to be 141,108 ± 876 IU/mL, while in the SVR group, the mean viral load was lowered to the value of 440 ± 321 IU/mL. It was also noted that the viral loads were the highest in fresh cases of HBV with the mean value of 1,206,147 ± 535, which was around six folds more than of NSR patients, as shown in Figure 3a.

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### Table 3.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NSR (%)</th>
<th>SVR (%)</th>
<th>Fresh Cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>74.23%</td>
<td>14.09%</td>
<td>11.68%</td>
</tr>
<tr>
<td>CA</td>
<td>9.09%</td>
<td>16.54%</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>18.18%</td>
<td>7.09%</td>
<td></td>
</tr>
</tbody>
</table>

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respondents and had high values of odds ratio (2.914) and relative risk (1.556), indicating its association with poor therapeutic response of HBV patients to therapy.

Table 2. Genotype and allele frequencies of IL-12b+1188 A/C in HBV-infected patients.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>HBV-Infected Frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>A/A</td>
<td>216</td>
</tr>
<tr>
<td>C/A</td>
<td>41</td>
</tr>
<tr>
<td>C/C</td>
<td>34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>236</td>
</tr>
<tr>
<td></td>
<td>81.10%</td>
</tr>
<tr>
<td>C</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>18.90%</td>
</tr>
</tbody>
</table>

Table 3. Comparison of IL-12B+1188 (A/C) genotypes in treatment outcome between HBV-infected patients (responders and non-responders) receiving antiviral therapy.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NSR</th>
<th>SVR</th>
<th>OR</th>
<th>95% CI</th>
<th>RR</th>
<th>95% CI</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>88</td>
<td>97</td>
<td>0.8247</td>
<td>0.4691–1.437</td>
<td>0.9081</td>
<td>0.6979–1.224</td>
<td>0.5606</td>
</tr>
<tr>
<td>CA</td>
<td>11</td>
<td>21</td>
<td>0.504</td>
<td>0.2239–1.057</td>
<td>0.6750</td>
<td>0.3950–1.041</td>
<td>0.0904</td>
</tr>
<tr>
<td>CC</td>
<td>22</td>
<td>9</td>
<td>2.914</td>
<td>1.320–6.836</td>
<td>1.556</td>
<td>1.137–1.969</td>
<td>0.0117</td>
</tr>
</tbody>
</table>

The results of our study indicated that both female and male patients carrying the variant genotype CC were more likely to be non-respondents compared to the patients carrying AA and CA genotypes. Among male patients carrying genotype CC, 81.82% were NSR compared to 60.78% and 62.50% AA and CA carriers, respectively. Similarly, in female patients, the altered genotype CC was most prevalent among the NSR group in comparison to genotypes AA (42.54%) and CA (25%). However, the genotype frequencies in both female and male participants within the NSR and SVR groups did not exhibit significant differences (Table 4).

Table 4. Comparison of IL-12B+1188 (A/C) genotypes in treatment outcome between HBV-infected patients according to gender parameters.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NSR (%) Distribution</th>
<th>SVR (%) Distribution</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA-Male</td>
<td>60.78%</td>
<td>39.22%</td>
<td>0.405</td>
</tr>
<tr>
<td>AA-Female</td>
<td>42.54%</td>
<td>57.46%</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>CA-Male</td>
<td>62.50%</td>
<td>37.50%</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>CA-Female</td>
<td>25.00%</td>
<td>75.00%</td>
<td>0.07</td>
</tr>
<tr>
<td>CC-Male</td>
<td>81.82%</td>
<td>18.18%</td>
<td>0.3</td>
</tr>
<tr>
<td>CC-Female</td>
<td>65.00%</td>
<td>35.00%</td>
<td>0.05</td>
</tr>
</tbody>
</table>

4. Discussion

Hepatitis B virus (HBV) infection is a major global public health issue, with over 2.57 billion individuals worldwide thought to be afflicted. Chronic HBV infection increases the chance of developing HBV-related liver disorders such as hepatic cirrhosis and hepatocellular carcinoma (HCC). Without a more comprehensive preventative strategy, the number of people infected with hepatitis B is projected to stay stable. Hepatitis B is expected to kill about 780,000 people worldwide each year. The prevalence of HBV infection
varies around the globe. High prevalence areas are defined as having more than 8% of the population positive for the HBV surface-antigen; higher intermediate prevalence areas are defined as having 5–7.99% of the population positive for HBsAg; lower intermediate prevalence areas are defined as having 2–4.99% of the population positive for HBsAg; and low prevalence areas are defined as having less than 2% of the population positive for HBsAg [28].

In Pakistan, the rate of HBV infection is increasing progressively. Several studies have reported that Afghan refugees in Pakistan, intravenous drug users, blood donors, health care professionals, multiple transplant patients, prisoners patients, psychiatric patients, and the general population of certain regions, such as Interior Sindh, Southern Punjab, Kurram agency, Baltistan, and areas of Lahore, have an extremely high prevalence of HBV, i.e., more than 5% (>5%) [29]. The reason may be the lack of proper health facilities, poor economic conditions, and lack of public health awareness about the transmission of major infectious diseases [30].

A variety of factors play a critical role in the progression of HBV [31]. The external factors include viral co-infections, alcoholism, use of drugs, smoking, and aflatoxin [32]. Several studies have established a link between alcohol consumption and progression of HBV infection. Alcohol consumption was reported to increase the viral replication and HBV surface antigen in the serum of transgenic HBV C.B-17 SCID mice model [33]. The possible explanation for this association can include the role of alcohol in elevating the levels of oxidative stress, increase in hepatic inflammation, and weakened immune response, which together contribute towards the progression of this disease [34].

There are several host-related factors, such as the age of the host (at the time of infection), gender, host genetic factors (SNPs, CNVs), immune system (immune competent or immune compromised), viral load, and viral genotype [35] that also tend to play a critical role. HLA class II locus genetic variants were found to be substantially linked with susceptibility to chronic HBV infection. Other polymorphic variants in the genes, such as EHMT2, TCF19, and HLA-C, as well as near the HLA class II loci and UBE2L3, were also found to be linked to chronic hepatitis B infection. Meanwhile, KIF1B, GRIK1, and STAT4 polymorphisms were linked to HBV-associated hepatocellular carcinoma. HLA class II genetic variants were found to be strongly linked not only to persisting HBV infection but also to the progression of disease and HBV-induced HCC in chronic HBV patients [36].

It was also reported that the IL-28b polymorphic variant, rs8099917, was significantly associated with the risk of HBV-induced HCC. It was also reported that homozygous genotype TT of variant IL-28b variant, rs12979860, was significantly associated with the risk of progression of chronic HBV infection into HCC in Chinese patients [37]. A study found that genetic differences in the IL-28b gene were related to interferon therapy responses in HCV-infected patients. The investigation was carried out to see if these variances influenced interferon treatment success rates in patients with persistent hepatitis B infection. They analyzed 512 individuals with chronic hepatitis B who were being treated with interferon therapy and observed that a specific polymorphic variant in IL-28b (rs8099917 genotype GG) was more frequently present among the responders (8.3%) compared to the non-responders (3.9%), indicating a possible link with the efficacy of the therapy. This indicates that certain genetic variants are associated with improved therapy outcome rates in patients with chronic HBV receiving interferon therapy [38].

Studies have reported that IL-12b is a pro-inflammatory cytokine that is produced by APCs that play a critical role in regulating the clearance of HBV during natural infection [39]. IL-12 is regarded as the most crucial determining factor for Th1 and Th2 activation. It activates T cells and NK cells, resulting in the production of cytokines, primarily interferon-gamma, and confers tumor resistance by increasing Th1 adaptive immune response and cytotoxic T cell responses [21]. IL-12 is important in regulating the immunological response to HBV antigens during spontaneous infection [40]. Several studies have reported that a genetic variant in the 3′UTR of IL-12b1188 A/C is associated with various inflammatory diseases such as HCV infection, Rheumatoid arthritis, liver cirrhosis, and
cancers [41–43]. Genetic association of HBV infection in the Han Chinese population with 3’UTR of IL-12B, IL-10, and TNF-α genetic variants was determined, and it was revealed that the polymorphism in these genes was significantly associated with increased susceptibility and persistent HBV infection [44].

In the current study, we investigated the association of IL-12b 3’ UTR genetic variant in response to antiviral therapy for the treatment of HBV among the Pakistani population. It was observed that the ratio of female patients was higher in both respondent and non-respondent groups of the HBV antiviral therapy, though this observation could not result in any significant difference. Several studies have reported that females have two to four times lower incidence rates of HBV infection compared to that in males [12]. However, unfortunately, in Pakistan, the diagnosis of HBV-related hepatic diseases is delayed, leading to poor patient outcomes [45].

It was also observed that the younger patients demonstrated better response to the antiviral therapy. Patients in the age group of ≤40 years showed better response to the treatment, while the patients with a mean age of 41.4 did not respond well to the antiviral treatment. Data has shown that the elderly population has a higher risk of developing HBV infection compared to young adults. Furthermore, the younger population has a higher chance of achieving a sustained response to the treatment and overall lower mortality rates than the older patients [46]. This finding is also in accordance with several other studies that have reported that age was a major defining factor in the success rate of antiviral therapeutic outcomes [47].

HBV DNA was isolated from the serum of the patients, amplified through Nested PCR, and then subsequently quantified. Viral load in the NSR group was notably higher compared to the SVR group due to the poor response to the antiviral therapy. However, when the viral loads in the fresh cases were evaluated and compared with the other experimental groups, they were six-fold higher compared to the NSR group, as the fresh cases did not receive any kind of antiviral treatment prior to the isolation of serum. The differences in the viral loads among various study groups were found to be statistically significant. However, when the levels of HBV viral load were compared among various genotypes, heterozygous genotype CA exhibited elevated levels of viral load compared to homozygous genotypes AA and CC, but the difference in the quantities of viral load among the three genotype groups was not found to be significant statistically. Therefore, in order to further investigate if there is any association between the viral load and IL-12b genotypes, future studies with large cohort sizes should be conducted.

Moreover, concentrations of alanine transaminase (ALT) were also evaluated. In this study, the normal value of ALT level was determined to be 40 IU/L. The ALT test is commonly used to screen, detect, and monitor the progression of liver disorders. Many scientists believe that the ALT cut-off value should be around 30 IU/mL. However, the upper limit of normal (ULN) for ALT varies widely amongst testing facilities, with ULN ranging from 31–55 IU/L in women and 35–79 IU/L in men. Although no uniform ALT ULN standard has been created, the usual threshold value of ALT in both women and men is 40 IU/L [48]. Furthermore, the cut-off value used in this study was also selected because of the previous studies that were conducted on the Pakistani population, and they reported that for both males and females, the upper limit of normal for measurement of ALT was 40 IU/mL [49].

The levels of ALT were highest in fresh cases (Group III), followed by NSR. The higher ALT value is also an indication of the disease’s presence, and it devalues only with the course of treatment. Whereas ALT levels in the SVR group and control groups were lower, indicating that the liver function in the responders of antiviral therapy is remarkably improved. An interesting observation that was attained from this study was that the ALT levels were lower in the SVR group compared to the controls. This can be explained by the fact that the antiviral drug suppressed the replication of the virus, which led to the improved function of the liver with diminished hepatic inflammation and ALT levels to the normal threshold or even below the baseline value (<40 IU/mL). Furthermore, there are several external factors that also play a significant influence on the levels of ALT.
These factors involve diet, exercise, medication, and alcohol consumption. Studies have reported that high-fat and high-carbohydrate diets can lead to higher ALT levels \[50,51\]. Furthermore, scientists have also confirmed that a sedentary lifestyle and obesity are also responsible for higher ALT levels. Improving lifestyles \[52\], such as consuming a high-fiber diet, reducing the consumption of processed food, saturated fats, alcohol consumption, and smoking, can lower the levels of ALT up to 10 IU/mL \[53\]. Since the consumption of high-caloric and high-fat diets and obesity are higher among the Pakistani population \[54\], these can be the potential reasons for the elevated ALT levels in the healthy population \[55\].

In this study, the IL-12b variant, rs3212227, was genotyped in a total of 291 HBV-infected patients. The distribution of genotypes in the NSR group was 72.73%, 9.09%, and 18.18%, while in the SVR group, it was 76.38%, 16.54%, and 7.09%, for genotypes AA, CA, and CC, respectively. Among these genotypes, only genotype CC attained a statistically significant value, and it was associated with a poor response to antiviral treatment in hepatitis B patients. A previous study has reported that the altered genotype CC in IL-12b+1188 A/C variant is associated with chronic hepatitis B disease \[56\]. It has been demonstrated that the presence of genetic variants in the IFN-\(\gamma\) signaling pathway, including IL-12b, was significantly associated with HBV infections among children and young individuals in the Chinese population \[57\]. Another study also reported that the IL-12 variant was involved in the development of HBV-induced liver cirrhosis among male patients in the Caucasian population \[58\].

The allele frequency of wild-type allele A in the HBV-infected patients was 81.10%, while in the control group, it was 87.80%. The distribution frequency for variant allele C was 18.90% in HBV-positive patients, while in control samples, it was 12.20%. However, these allele frequencies did not show any statistical significance. A study has also reported similar results as their study reported no significant association between the allele frequencies and incidences of HBV infections in the Han Chinese population \[59\].

Previous studies reported that C alleles of IL-12b+1188 A/C are associated with altered expression of IL-12 by immune cells \[60,61\]. Another study finds a clear relationship between IL-12 CA, AA, and IL-12 CC genotypes and HBV infection in the Korean population \[62\] (Park et al., 2007). Results indicated in this study suggest that IL-12b genotype CC is significantly associated with poor response to antiviral therapy. This data coincides with previous studies reported on IL-12B+1188 C/A polymorphism and its association with HBV infection.

An important delimitation of the current study is that there is a lack of information regarding the available data on the expression as well as functional analyses in the hepatic tissues and the analysis of the association of this variant with poor antiviral response, which might have a significant impact on confirmation of the results. Therefore, underlying mechanisms and pathways for how this SNP in IL-12b is involved in poor therapy response need to be elucidated. Lastly, this study reported, for the first time, antiviral treatment responses in HBV patients with a genetic variant in the IL-12b gene in the Pakistani population. Altered genotype CC of IL-12b was found to be associated with poor therapy outcomes. Moreover, this study also highlights the importance of genetic predilection for the personalized therapy of hepatitis B infection. Determination of these variants can help the patients to have alternative or tailor-made treatment that can lead to improved therapeutic outcomes. However, there is a need for a multi-ethnic and large cohort size HBV population study to have a deep insight into the exact molecular mechanisms through both in-vitro and in-vivo analysis to evaluate the impact of this variant in determining the response to antiviral treatment.

5. Conclusions

In conclusion, this study investigated the association of IL-12b+1188 A/C polymorphism with antiviral therapeutic response among HBV-infected patients from the Pakistani population. The finding of this elucidated that the variant genotype CC is associated with nonresponsiveness to the antiviral treatment. Thus, this variant has the potential to be
further explored as the biomarker for treatment response in HBV-infected patients by using extensive in vitro and in vivo assays. A more comprehensive investigation with a larger sample size, combined with a thorough examination of the transcriptome and expression of IL-12b+1188 A/C polymorphism, will allow for a more in-depth understanding of its role in the treatment response in hepatitis B virus-infected patients.

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