Genetic Study for the Role and Predictive Consequence of Certain Cytokines in Hepatitis B Iraqi Patients

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Abstract

Hepatitis B virus (HBV) infection is a serious and common infectious disease of the liver, HBV infection is endemic, particularly in developing countries, and is a serious public health problem. Cytokines and regulatory molecules play a fundamental role in the immuno pathogenesis of HBV infection. The gene loci for cytokines are defined, and polymorphisms of these genes are suggested to influence the outcome of HBV infection. In our study we revealed that there are elevated in Aspartate transaminase (AST), Alanine transaminase (ALT) and Bilirubin (Bil) among HBV patient than control group. IL–4 rs2070874 SNP Genotypes among patients with HBV was ONLY 68 positive to PCR results while in contrast it is only 9 within control group. The highest ratio of genotype in both groups was in CC as 36 (52.9%) in patients and 5 (55.6%) in control. Allele frequency on other hands describe as follow; in HBV and control group for C 72 (63.7%), 11 (61.1%) while for T 41 (26.3%) and 7 (38.9%) respectively. Genotypes of IL–10 rs1800872SNP among patients was in AA as 43 (62.3%) in patients and 13 (76.5%) in control. On other hands Allele frequency describe as follow; in HBV and control gro up for A 106 (76.8%), 29 (85.3%) while for G 32 (23.2%) and 5 (14.7%). IFN-γ (rs2069705 SNP) Genotypes among patients with HBV was in AG as 39 (28.8%) in patients and 10 (47.6%) in control with GG. Moreover, describe as follow; in HBV and control group for A 61 (33.3%), 28 (66.7%) while for G 122 (66.7%) and 28 (66.7%)

Keywords: Hepatitis, Cytokines, PCR, IFN, SNPs, IL-10, IL-2.

Introduction

Cytokines are proteinaceous moieties, produced chiefly by immune/non-immune cells. They are potent immune-modulatory molecules and major players in protection against viral infection, by either analyzing the host response pattern or by inhibiting viral replication. Since cytokine production is controlled genetically, variations caused due to single-nucleotide polymorphisms (SNPs) in cytokine genes' promoter region, affect the cytokine production to a great extent, thus affecting the immune balance response. Though, some previous studies have been carried out in this regard, which have reported variable results concerning association of cytokine polymorphism/expression with HBV-HCC has been appeared some risk in different ethnic groups, but till date, no substantial evidence has been yet obtained from the Indian population. Although, initial classification has divided the cytokines into four large groups, depend on of their biological functions: (1) Natural immunity mediators: such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), IL-6 (minor role), IL-5, IL-8 and the chemokine; (2) Lymphocyte activation, growth and differentiation regulators: for example IL-2, IL-4, transforming growth factor-β (TGF-β); (3) Regulators of Immune-mediated inflammation: IL-4, TGF-β, IL-10, IL-1, interferon-γ (IFN-γ), granulocyte macrophage-colony stimulating factor (GM-
CSF), macrophage activating factor; and (4) Stimulators of immature leucocyte growth and differentiation: IL-1, IL-3, IL-5, IL-6, granulocyte-CSF, macrophage-CSF, GM-CSF.

Hepatitis B virus (HBV) infection is a serious and common infectious disease of the liver, affecting 240 million people worldwide with an estimated 600000 deaths per year, and remains the major cause for chronic hepatitis, cirrhosis, and hepatocellular carcinoma5–6. HBV infection is endemic, particularly in developing countries, and is a serious public health problem6, 7. Following acute HBV infection, 1%-5% of adults develop chronic infection8. Rate of chronicity is inversely proportional to age, being higher in newborns and children than in adults. The prevalence of chronic HBV infection is also higher (over 8%) in areas where the disease is highly endemic than in those with intermediate and low endemcity9. The chronic diseases caused by HBV are chronic hepatitis, cirrhosis, and primary hepatocellular carcinoma10.

Chronic hepatitis can lead to end-stage liver disease in 15%-40% of patients11. A number of factors, including host-related factors (for example, genetic and immunological background), pathogen-related factors (for instance, viral load, genotype), and environmental factors (e.g., hygiene, nutrition, treatment, vaccination)12 affect the outcome of HBV infection. Gene polymorphisms such as the single nucleotide polymorphism (SNP; replacement of a nucleotide with another one) may change the structure and biological function of the protein coded by that gene. A SNP in the promoter region of a gene may cause increased or decreased production of the relevant protein. The presence of these types of inherited gene polymorphisms may make a person more susceptible or resistant to a certain disease13,14.

Cytokines and regulatory molecules play a fundamental role in the immunopathogenesis of HBV infection. The gene loci for cytokines are defined, and polymorphisms of these genes are suggested to influence the outcome of HBV infection15. Therefore, many recent studies have focused on the effect of gene polymorphisms of cytokines on disease outcome and response to vaccination and treatment10, 16. Understanding the genetic background of this common public health problem may give rise to new strategies for prevention, treatment, and control of HBV infection.

The host response to hepatitis viruses involves various components of the immune system, including T-lymphocyte immune-regulatory cytokines. Cytokines are a group of protein molecules involved in various biological processes including growth, differentiation, cell survival, haemato poiesis', immunological functions, inflammation, apoptosis, necrosis and fibrosis17.

The cytokine group is highly heterogeneous and consists of different types of molecules, such as the interleukins, the tumour necrosis factor family, the interferon’s, the chemokines, the transforming growth factor-b and others. The control of cytokine production is highly complex, while the effects of cytokines are widespread throughout multiple regulatory molecule networks. Cytokines are produced by a wide variety of cells, mainly the Th1 and Th2 cells18.

Th1 cells secretary-inflammatory cytokines, whereas the Th2 cells secrete anti-inflammatory cytokines. Th1 cytokines are involved in cell-mediated immunity, and play a crucial role in protection from intracellular pathogens and are associated with recovery. Th2 cytokines regulate humeral immune responses, and their rising levels are often associated with the development of persistent infections19, 20.

Materials and Methods

Patients

Healthy Control Group

This study has done on population consisted of 87 patients with chronic hepatitis B (46 men and 41 women), and 63 healthy controls (39 men and 24 women) aged between 22 and 67 years. Patients were recruited from the Main Hospital in Al-Hillah City, Educational Hospital, during 13 months. Patients with chronic hepatitis B were HBVDNA (+) by PCR, HBsAg (+), anti-HBc (+), anti-HBe (+). These samples have collected from Iraqi populations were arbitrarily.

Blood Sampling

Approximately five milliliters of venous blood have collected from each patient in the study.
The blood was divided into two parts: one part (about three milliliters) was collected into EDTA containing tubes. The second one of the blood was placed in gel tube for thirty minutes, then transferred to plain tube after that serum was obtained by centrifugation for 10 min at 3000×g the sera were separated, aliquot and then frozen at −40 °C. Avoiding Freeze/thaw cycles of the samples. The collected serum and kept in the freezer until it was used for immunoassay.

Serology

Serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT), total bilirubin.

Cytokines

Levels of IL-10, IFN-γ and IL-4 were measured with Elisa. The upper limit of ALT activity was set at 35 IU/L, of AST at 37 IU/L, of ALP at 104 IU/L, of GGT 38 IU/L and of total bilirubin in at 1.00 mg/dL.

HBV Detection

Real Time Kit for the Quantitative detection of Hepatitis B Virus in human plasma was used in this study for the Quantitative detection of Hepatitis B Virus in human plasma and simultaneous detection of a HBV-specific Internal Control (IC), by dual colour detection. HBV DNA is extracted from plasma, amplified using Real Time Amplification and detected using fluorescent reporter dye probes specific for HBV or HBV IC. Monitoring the fluorescence intensities during Real Time allows the detection and quantification of the accumulating product without having to reopen the reaction tube after the amplification.

Isolation of Genomic DNA

Genomic DNA was used for molecular study by sequestered from the fresh blood which collected in tubes of anticoagulant EDTA and for frozen blood samples we recommended using protease K were applied using for DNA purification; Promega Wizard genomic kits. The isolation of DNA depended on the 5 stage procedure utilizing salting out techniques.

Genetic Study

Genotyping for IL−4 and IL−10SNPs were achieved using a PCR (polymerase chain reaction) distinguished by grouping particular preliminary PCR system utilizing particular succession.

The PCR has done in a VERITI applied bios stem (USA) and response blends of aggregate volume of 20µl comprised 5 µl genomic DNA, 5 µl of every preliminary (Promega, USA) and 5 µl of 1X PCR mix (Taq PCR Master Mix Kit, QIAGEN, GmbH, Hilden, Germany) containing (200 5 µmol/l of each dNTP, 5 µl of 10 response cradle, and 1.25 U Taq Gold Polymerase, and 4 mmol/l MgCl2). All PCR items were settled using 2% agarose on electrophoresis in the wake of reclosing with ethidium bromide.

Results and Discussion

Chemical Parameters

In our study we revealed that there are elevated in Aspartame transaminase (AST), Almandine transaminase (ALT) and Bilirubin (Bil) among HBV patient than control group as showed in Table 1. Some study had watched that AST and ALT file were the best prescient factors for liver fibrosis 18, 21. There are many learns about serum HA and Bil as a fibrosis marker in ceaseless hepatitis C22,23. One of these studies has suggested cut-off estimations of HA and Bilirubin to segregate the diverse stages of liver fibrosis, yet it was restricted by the quantity of cirrhotic patients included adding up to just 5% of the study bunch 22, 24.

| Table 1: Laboratory data for and control including chemical and |
| --- | --- | --- | --- |
| Factor | Patients with HCV No. 87 (Mean±SD) | Control No. 63 (Mean±SD) | P value |
| Age (Yrs.) | 45.36±4.92 | 29.86±8.32 | 0.5 |
| AST (IU/L) | 67.03±11.21 | 30.95±7.64 | 0.5 |
| ALT (IU/L) | 64.54±17.84 | 32.61±5.91 | 0.5 |
| Bil (mg/dl) | 4.18±0.27 | 0.51±0.62 | 0.5 |
| Hb (mg/dl) | 16.52±1.39 | 11.41±0.96 | 0.5 |
| Plt (10^9/L) | 200.47±82.96 | 187.11±42.36 | 0.5 |
| TLC (10^3/L) | 7.82±0.61 | 8.34±1.22 | 0.5 |

AST= Aspartate transaminase, ALT= Alanine transaminase, Bil= Bilirubin, Hb= Hemoglobin, Plt= platelet, TLC= Total leucocyte count
Molecular Parameters

This study decided allele frequencies in the IL–4 (rs2070874 SNP), IL–10 (rs1800871 SNP), and IFN-γ(rs2069705 SNP) quality among patients with HBV and investigated the part each cytokines quality polymorphisms on the clinical attributes of HCV infection.

IL–4 (rs2070874 SNP)

We revealed that IL–4 rs2070874 SNP Genotypes among patients with HBV was ONLY 68 positives to PCR results while in contrast it is only 9 within control group. The highest ratio of genotype in both groups was in CC as 36 (52.9%) in patients and 5 (55.6%) in control. Allele frequency on the other hands describe as follow; in HBV and control group for C 72 (63.7%), 11 (61.1%) while for T 41 (26.3%) and 7 (38.9%) respectively as showed in table 2 and figure 1.

Table 2: Allele frequency and genotype polymorphisms for IL–4 (rs2070874 SNP) in patients with HBV and control

<table>
<thead>
<tr>
<th>Genotypes &amp; Alleles</th>
<th>Patients with HBV No. 87</th>
<th>Control No. 63</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2070874 SNPGenotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>36 (52.9%)</td>
<td>3 (33.3%)</td>
<td>0.1</td>
</tr>
<tr>
<td>CT</td>
<td>23 (33.8%)</td>
<td>5 (55.6%)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>9 (13.2%)</td>
<td>1 (11.1%)</td>
<td></td>
</tr>
<tr>
<td>rs2070874 SNPAlleles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>72 (63.7%)</td>
<td>11 (61.1%)</td>
<td>0.1</td>
</tr>
<tr>
<td>T</td>
<td>41 (26.3%)</td>
<td>7 (38.9%)</td>
<td></td>
</tr>
</tbody>
</table>

SNPs in many candidate genes have been linked to an increased risk of many diseases. In this study, we investigated the significance of the relationship between polymorphisms in several cytokine genes and susceptibility to HBV among Iraqi population with rs2070874 SNP is an iatrogenic SNP located at the IL-4 gene on chromosome 5.

IL-4 is a highly pleiotropic cytokine that acts as an important mediator and modulator of immune and inflammatory responses, including the ability to induce helper T (Th) cell differentiation. Early secretion of IL-4 induces the differentiation of Th cells into Th2-like cells, which secrete IL-4; moreover, anticrime production of IL-4 enhances cell proliferation27, 28 which is also demonstrated that rs2070874 (CC) may be a genetic risk factor for chronic hepatitis B in Chinese males, other study reported that individuals carrying the C allele of rs2070874 in the IL-4 gene are at a risk of disease compared to TT homozygotes29.

To rule out the possibility that IL-4 might be acting on cellular regulatory elements upstream of the HBV-integrated site in Hep3B, the effect of IL-4 was also studied in Hep3B cells transiently transfected with a plasmid pHBV3.6, which contained >1-U
length of the HBV genome and retains the ability to produce mature HBV virions. Thus, in pHBV3.6-transfected Hep3B cells, HB sAg can be encoded by both the integrated and the transected HBV DNA, whereas HB eAg is only produced by pHBV3.6. These results show that IL-4 suppressed the production of HBV proteins encoded by both the integrated and the transiently transected HBV genome.

It was evident that the transcripts with molecular sizes of 4.0/3.5 kb (HBsAg mRNA and precore/pregenomic RNA) and 2.4/2.1 kb (large HB sAg mRNA and middle/major HB sAg mRNA) were significantly suppressed by IL-4 over the various time periods studied. In addition, we measured endogenous DNA polymerase activity. The HBV core particles in the culture supernatant or in the cytoplasm of Hep3B transected with pHBV3.6 were isolated and reacted with [α-32P] Dctp30. Both the nicked circular and the linear forms of HBV DNA dramatically decreased after treatment with IL-4 for 12, 24, and 3h. These results clearly show that IL-4 suppressed HBV replication.

In addition, the transcriptional rate of HBV genes in Hep3B cells transected with pHBV3.6 was significantly reduced after IL-4 treatment. These results indicate that IL-4 may inhibit the expression of HBV genes at the transcriptional level.

**IL–10 (rs1800872SNP)**

We revealed that IL–10 rs1800872SNPGenotypes among patients with HBV was ONLY 69positives to PCR results while in contrast it is only 17 within control group. The highest ratio of genotype in both groups was in AA as 43 (62.3%) in patients and 13 (76.5%) in control. Allele frequency on other hands describe as follow; in HBV and control group for A106 (76.8%), 29 (85.3%) while for G32 (23.2%) and 5 (14.7%) respectively as showed in table 3 and fig2.

**Table 3: Allele frequency and genotype polymorphisms for IL–10 (rs2070872 SNP) in patients with HBV and control**

<table>
<thead>
<tr>
<th>Genotypes &amp; Alleles</th>
<th>Patients with HBV No. 87 ONLY 69+</th>
<th>Control No. 63 ONLY 17+</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rs1800872SNP Genotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>43 (62.3%)</td>
<td>13 (76.5%)</td>
<td>0.01</td>
</tr>
<tr>
<td>AG</td>
<td>20 (29.0%)</td>
<td>3 (17.6%)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>6 (8.7%)</td>
<td>1 (5.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs1800872SNP Alleles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>106 (76.8%)</td>
<td>29 (85.3%)</td>
<td>0.01</td>
</tr>
<tr>
<td>G</td>
<td>32 (23.2%)</td>
<td>5 (14.7%)</td>
<td></td>
</tr>
</tbody>
</table>

![Figure 2: Allele frequency and genotype polymorphisms for IL–10 (rs2070872 SNP) in patients with HBV and Control](image-url)
In this study, we identified the polymorphisms of IL-10 promoter region (rs1800872SNP) and the correlation of these polymorphisms with HBV susceptibility and recovery in Iraqi population. Genotype had been detected in the two groups A/A, A/G and G/G of IL-10 were found in patients and individuals with self-limited HBV infections, while genotype A/A was observed in very low ratio with normal controls.

After HBV infection, the immune response can be induced to eliminate the virus. As a crucial Th-2 cell cytokine, IL-10, whose expression can be affected by the polymorphisms in the regulatory regions, plays an important role in immune responses and inflammation in the liver. The levels of IL-10 might reflect the degree of inflammation, fibrosis, viral load, viral antigen load, and the occurrence of malignancy in the liver and such factors relating to the changes of individual cytokine levels differ with the disease phases.

It has been reported that the inter-individual differences in cytokine production appear to be related to allelic polymorphisms of cytokine genes. The cytokine levels are also influenced by various factors, such as inflammation, fibrosis, viral load, viral antigen load, and the occurrence of malignancy in the liver and such factors relating to the changes of individual cytokine levels differed with the disease phases.

It has been reported that when the patients with HCC are put in immunosuppressive conditions, the cancer cells themselves may contribute to the conditions by producing immunosuppressive agents, such as transforming growth factor beta 1 (TGF-β). Numerous other studies have also reported the elevated serum levels of IL-10 in HBV patients compared with controls. In conclusion, the polymorphisms of IL-10rs1800872SNP might be associated with the susceptibility to HBV in study population.

**IFN-γ (rs2069705 SNP)**

We revealed that IFN-γ (rs2069705 SNP) Genotypes among patients with HBV was ONLY 66positives to PCR results while in contrast it is only 21 within control group. The highest ratio of genotype in both groups was in AG as 39 (28.8%) in patients and 10 (47.6%) in control with GG. Allele frequency on other hands describe as follow; in HBV and control group for A 61 (33.3%), 28 (66.7%)while for G 122 (66.7%) and 28 (66.7%) respectively as showed in Table 4 and Fig 3.

**Table 4: Allele frequency and genotype polymorphisms for IFN-γ(rs2069705 SNP) in patients with HBV and control**

<table>
<thead>
<tr>
<th>Genotypes &amp; Alleles</th>
<th>Patients with HBV No. 87 ONLY 66+</th>
<th>Control No. 63 ONLY 21+</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2069705 SNP Genotypes</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>36 (54.5%)</td>
<td>10 (47.6%)</td>
<td>0.01</td>
</tr>
<tr>
<td>AG</td>
<td>39 (28.8%)</td>
<td>8 (38.1%)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>11 (16.7%)</td>
<td>3 (14.3%)</td>
<td></td>
</tr>
<tr>
<td>rs2069705 SNP Alleles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>122 (66.7%)</td>
<td>28 (66.7%)</td>
<td>0.01</td>
</tr>
<tr>
<td>A</td>
<td>61 (33.3%)</td>
<td>14 (33.3%)</td>
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</table>
IFN-γ was detected in HCC, and the authors concluded that IFN-γ may not play a large role in liver inflammation. Similar to that, no significant differences were found in IL-2-330 and IFN-γ-1615 genotype distribution in Guangxi people of China between HCC patients and controls in this study. Although IL-2-330 and IFN-γ-1615 are located on different genes, their positions are considerably near (rs2069762 and rs2069705).

Therefore, their locations may affect their biological functions through interactions. To date, SNP-SNP interactions between different cytokine genes and HCC risk have not yet been reported. Further studies with larger sample sizes are necessary to confirm our findings. Cytokine SNPs causing modulated expression of the encoded protein may play a role in influencing the immune response.

IFN-γ is an important Th1 cytokine, and the IFN-γ +874 polymorphisms have been associated with IFN-γ mRNA and IFN-γ levels. Moreover, the IFN-γ +2109A/G SNP located in intone 3 has been shown to be functional and it may eventually modify the effect exerted by the IFN-γ +874 SNP. In this study, we investigated the influence of these two SNPs on the susceptibility of HBV-LC in a Chinese population and identified the associations between the IFN-γ +2109A/G polymorphisms and HBV-LC risk. The results revealed that the GG genotype and G allele of +2109A/G were associated with a significantly decreased risk of HBV-LC. Moreover, we also found that the T874G+2109 heliotype between the +874 and +2109 loci of IFN-γ significantly decreased the HBV-LC risk, while A874A+2109 heliotype significantly increased the HBV-LC risk.

Studies have also reported the role of the IFN-γ gene in the response to drug treatment in cancer. Other paper indicated that the +2109 locus of the IFN-γ gene, which is also the specific binding site for NF-κB similar to the +874 locus, influenced HCV therapy.

Moreover, other study found that T alleles at the +874 locus of the IFN-γ gene were a risk factor in depressed patients with HCV who were treated with IFN-alpha. The IFN-γ gene polymorphism may further affect the development of HCC and the response to cancer drugs by increasing the risk of hepatitis virus-related diseases, although we did not observe a significant association between the (+874T/A) polymorphism and HCC risk in this study, which is worthy of further investigation in the future.

References


