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An association study of functional polymorphic genes *IRF*-1, *IFNGR*-1, and *IFN*-γ with disease progression, aspartate aminotransferase, alanine aminotransferase, and viral load in chronic hepatitis B and C

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SUMMARY

Background: Investigational approaches based on genome-wide association studies have proven useful in identifying genetic predictors for many diseases, including susceptibility to chronic hepatitis B and C. In these studies, the majority of genetic variants that have shown a positive association have been identified in genes involved in the immune response. In this study *IFN*-γ, *IFNGR*-1, and *IRF*-1 genes were analyzed for their role in susceptibility to the development of chronic hepatitis B and chronic hepatitis C in a Turkish population.

Methods: Polymorphic genes *IRF*-1 (−410, −388), *IFNGR*-1 (−56, −611), and *IFN*-γ (+874) were analyzed in a total of 400 individuals: 100 chronic hepatitis B patients, 100 hepatitis B carriers, 100 chronic hepatitis C patients, and 100 healthy controls. A single base primer extension assay was used. Correlations between genes and gender, viral load, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were also investigated.

Results: The *IRF*-1 gene at positions −388 and −410 were observed to be candidate gene markers for susceptibility to the development of chronic hepatitis B and C ($p < 0.05$). *IFN*-γ +874 and *IFNGR*-1 (−56 and −611) correlated with chronic hepatitis B but not chronic hepatitis C. Correlation of functional genotype with viral load and AST and ALT levels revealed an association of *IFN*-γ +874 and *IFNGR*-1 −611 with chronic hepatitis C and *IFN*-γ +874 with viral load and chronic hepatitis B ($p < 0.05$).

Conclusions: Findings suggest that *IFN*-γ (+874), *IRF*-1 (−410, −388), and *IFNGR*-1 (−56, −611) are candidate gene markers for determining patient susceptibility to the development of chronic hepatitis B and C.

1. Introduction

Upon invasion of hepatocytes with the hepatitis B virus (HBV) or hepatitis C virus (HCV), a host defense-mediated response is triggered, which involves the induction of the interferon family of pleiotropic cytokines.[1] Interferons constitute a heterogeneous group of proteins and are best known for their ability to induce cellular resistance to viral infections. Interferon gamma (IFN-γ) is the only type II interferon and is a markedly different cytokine to the type I interferons. Its classification into the interferon family is due to its role in host antiviral mechanisms.[2]

IFN-γ is an acid-labile protein produced by CD4 T cells and other cell types, including natural killer cells, CD8 T cells, and macrophages. It acts as a regulator of gene expression through activation of a receptor complex comprising two subunits, each encoded by a different gene: IFN-γ receptor *IFNGR*-1 on chromosome 6q23–4 and *IFNGR*-2 on chromosome 21q22.1–22.2. Homodimers of IFN-γ interact with both receptor proteins leading to receptor dimerization, and each of the three molecules plays a non-redundant role in ligand-activated receptor signaling.[3,4]

The intracellular antiviral responses induced by IFN-γ play a critical role in the pathogenesis of HBV and HCV infection.[1] Upon induction of the signaling pathway, IFN-γ functions in an autocrine manner to up-regulate the transcription factor interferon regulatory factor 1 (IRF-1) and its target genes.[5,6] Subsequently, a feedback loop in which IFN-γ and IRF-1 are up-regulated results in the amplification of the IFN-γ-dependent

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response to lipopolysaccharides (LPS).[7] The importance of IFN-γ for the clearance of HBV is well known.[1,7] It has been shown that IFN-γ can inhibit the replication of HBV-infected cells, directly reducing viral load by mediating the antiviral effect of the cytotoxic lymphocytes (CTLs).[8]

These cellular responses mediated by interferon are primarily due to the modulation of gene expression, which in turn can be modulated by certain gene polymorphisms. Genetic polymorphisms of these cytokines and their receptors can occur in multiple allelic states. The presence/absence of certain polymorphisms has been shown to affect the expression and the production of various cytokines, both in vitro and in vivo,[9] thereby playing a role in susceptibility to a number of clinical conditions, such as autoimmune diseases, infectious diseases, and graft rejection.[9] Several polymorphisms within the IFN-γ non-coding regions have been implicated in autoimmune, chronic inflammatory conditions[10] and HBV infection.[11] Furthermore, cytokine polymorphisms involved in the IFN-γ signaling pathway have previously been found to play a role in other viral/host-mediated immune responses, including *IFNGR*-1, which has been shown as a strong candidate gene for the complex phenotype of tuberculosis,[12] while polymorphisms in the *IRF*-1 region have been linked to altered susceptibility to numerous infections and immunopathologies including schistosomiasis and allergy/atopy.[13–15] IRFs are known to be important for the initiation and fine-tuning of immune responses. They comprise a tight regulatory network of genes that have shown importance in promoting antibacterial immunity.[16,17] Previous studies have suggested that human hepatoma cell lines show different levels of *IRF*-1 transcription in response to interferons, thereby affecting the regulation of hepatic inflammation and viral eradication by interferon treatment for chronic viral hepatitis.[18]

This study investigated whether genetic variation(s) in cytokines and receptors (IFN-γ, IFNGR-1, and IRF-1) involved in the interferon type II pathway, are associated with susceptibility to the development of chronic HBV and HCV infections in a Turkish population. Allele and genotype distributions were compared between chronic hepatitis B (CHBV) and chronic hepatitis C (CHCV) subjects, asymptomatic hepatitis B carriers, and healthy controls. Furthermore, correlations between polymorphisms in these genes were analyzed with respect to gender distribution, viral load, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Any association with these genes will provide insight into the clinically important immune mechanisms influencing these infectious diseases.

2. Materials and methods

2.1. Study subjects

This was a case–control study including a total of 400 Turkish adults (205 males and 195 females). Subjects were enrolled from the outpatient clinics of the gastroenterology departments of Haydarpaşa Numune Teaching Hospital and Umraniye Teaching and Research Hospital in Istanbul, Turkey, between March 2009 and May 2011.

Patients were classified into the following groups: (1) Chronic HBV infection: 100 individuals (mean ± SD age, 52.74 ± 13.17 years) who were hepatitis B surface antigen (HBsAg)-positive, antibody to hepatitis B core antigen (anti-HBc) IgG-positive, antibody to hepatitis B e antigen (anti-HBe)-positive, had an HBV-DNA level >2000 IU/ml,[19] without anti-HCV antibodies, manifested by elevated ALT (more than two times the upper limit of normal) during the follow-up period of 6 months. (2) HBV carriers: 100 individuals (mean ± SD age, 43.26 ± 11.42 years) who were considered inactive HBsAg carriers based on a sustained normal ALT level, with positivity for anti-HBe and an undetectable level of HBV-DNA; (3) Chronic HCV infection: 100 individuals (mean ± SD age, 44.13 ± 12.11 years) who were positive for HCV antibodies but negative for HBV antigens, and had an HCV-RNA level >800 000 IU/ml and ALT values more than two times the upper limit of normal. (4) Healthy controls: 100 individuals (mean ± SD age, 46.32 ± 11.29 years) who were negative for HBV and HCV, with normal liver transaminase levels and ultrasonography.

All subjects were regularly followed with blood tests for serum transaminase and with ultrasonography at 6-month intervals for more than 18 months. No patients were diagnosed with hepatocellular carcinoma. Patients with other types of chronic liver disease were excluded from the study. Informed consent was obtained from each patient included in this study and the study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki, as reflected in the prior approval of the institutional human research committees.

2.2. Determination of AST and ALT levels

Plasma ALT and AST activity was routinely evaluated by spectrophotometric method, in 100 μl of plasma.[20] Following a 10-min incubation at room temperature, the absorbance was measured at λ = 340 nm by spectrophotometer (Thermoscientific, UK).

2.3. HBV DNA and HCV RNA quantification

HBV and HCV serologic markers were tested using commercially available enzyme immunoassays (Abbott Laboratories, Chicago, IL, USA). HBV and HCV nucleic acids were extracted from blood using Trizol-LS (Life Technologies, Paisley, UK), or by Roche DNA/RNA extraction kit (Roche, Germany), and were resuspended in nuclease-free water (Promega, Madison, WI, USA). HBV-DNA and HCV-RNA were amplified using Cobas Taqman kits, as per the manufacturer's instructions (Roche, Germany), and was quantified using a Roche Cobas Taqman 48 Analyzer with a lower detection limit of 25 IU/ml in serum.

All samples were analyzed in duplicate and the mean value reported as the viremic level in the serum.

2.4. Single nucleotide polymorphism genotyping

Genomic DNA was extracted from 200 μl of a peripheral whole blood sample using a commercially available DNA isolation kit (Qiagen, UK) in accordance with the manufacturer's instructions. Five single nucleotide polymorphisms (SNPs) of three polymorphic genes were assessed in all study subjects: *IRF*-1 gene promoter positions −410 and −388, *IFN*-γ gene at position +874, and *IFNGR*-1 at −56 and −611. The sequences of the primers and probes used in the PCR assays are shown in Table 1; the sequencing primers were designed from the National Center for Biotechnology Information (NCBI) sequence data.[21]

PCR products were purified by a Qiagen PCR purification kit, and the polymorphisms detected by single base primer extension assay (SNP-IT assay) using a previously described method.[22] The thermocycling parameters for interferon genes were as follows: an initial activation step of 95 °C for 10 min preceded the cycling program, followed by 35 cycles of denaturation at 95 °C, annealing at 72 °C for 1 min, and final extension at 72 °C for 7 min.

2.5. Statistical analysis

Results were analyzed using SPSS version 11.0 software (SPSS, Inc., Chicago, IL, USA). The Hardy–Weinberg equilibrium (HWE) of the polymorphic frequency for alleles and genotypes were compared by Chi-square test. An independent sample *t*-test was