Role of Proinflammatory Cytokines (Interferon Gamma) and Anti-Inflammatory Cytokine (Interleukin-10) Gene Polymorphisms in Chronic Hepatitis B Infection: An Indian Scenario

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Immune-mediated mechanisms have been found to play an important role in the progression of hepatitis B virus (HBV) infection. The outcomes of infection do not appear to be determined by viral strains. Instead, allelic variants in human genome are likely to affect the disease progression. Allelic variation of proinflammatory cytokines such as interferon gamma (IFN- γ) participates in the elimination of HBV, and interleukin-10 (IL-10) helps in inhibition of Th1 effector mechanisms for host defense. The aim of this study was to determine the influence of host genetic factors in chronic HBV infection and gene promoter polymorphism or single-nucleotide polymorphism analysis of *IFN- γ + 874* and *IL-10* (–1082, –592, and –819) on disease progression and persistence. A total of 232 patients along with 76 healthy controls were included. Allele-specific primers for IFN- γ and restriction fragment length polymorphism for IL-10 were used. The study indicated that low IFN- γ expression probably impairs host immune response to HBV, rendering these subjects more prone to HBV infection. No significant differences were detected between the 2 groups in the distributions of IL-10 genotype at the –1082, –819, and –592 positions. Odds ratio indicated that heterozygosity of genotypes –819 CT and –592 AC was more strongly associated with liver chronicity. Significantly, AA homozygous genotype was dominant in chronic hepatitis B cases in *IFN- γ + 874* and *IL-10* (–1082 and –592) and is associated with increased risk of persistent infection.

CHRONIC HEPATITIS B VIRUS (HBV) infection affects an estimated 400 million people worldwide with a million deaths annually (Lee 1997; WHO factsheet 2009). About one third of chronically infected subjects have liver-related morbidity, namely, cirrhosis and hepatocellular carcinoma (HCC), and the remaining two thirds, although asymptomatic, are at an increased risk of developing liver damage (Hadziyannis and others 2003; Liu and others 2006). The outcomes of HBV infection do not appear to be determined by viral strains. Instead, allelic variants in human genome are likely to affect the viral hepatitis progression after infection. The hepatocellular injuries caused by HBV infection are predominantly immune mediated. Immune attacks by the host against HBV are mainly mediated by a cellular response to HBV proteins. The immunopathogenic mechanisms of HBV, by which a portion of chronic cases evolve into progressive liver disease, are under investigation. Several proinflammatory cytokines such as Th1 cytokines including interferon gamma (IFN- γ) are believed to participate in the elimination of HBV. IFN- γ is localized at the region of the HBV infection and can eliminate viruses from

infected cells by its functional upregulation of viral antigen processing and presenting (Kakimi and others 2001). It is a secretory protein exclusively produced by T lymphocytes and natural killer cells when activated by antigens, alloantigens, or mitogens. It is the most important cytokine for cell-mediated immunity. As with other interferons, IFN- γ inhibits viral replication. A functional study done by Chisari and Ferrari (1995) demonstrated that 2 single-nucleotide polymorphisms (SNPs) located in the *IFN- γ* gene intron (at positions +874 and +2109) were involved in its transcriptional regulation (Gao and others 2009).

On the contrary, interleukin-10 (IL-10), a Th2 cytokine, acts as a potent inhibitor of Th1 effector mechanisms. The promoter region of *IL-10* gene contains 3 SNPs at positions –1082 (A/G), –819 (T/C), and –592 (A/C), which may assort into 3 different haplotypes. By analyzing the distributions of IL-10 promoter SNPs, it was revealed that –819T and –592A wild-type alleles in the IL-10 gene promoter were significantly more common in asymptomatic carriers than in patients with chronic progressive liver diseases (Miyazoe and others 2002). In efforts to discover

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polymorphism(s) in genes of which variant(s) have been implicated in HBV progression, we scrutinized the SNPs in IL-10 gene. The aim of this study was to determine the influence of host genetic factors in chronic HBV infection and gene promoter polymorphism or SNP analysis of *IL-10* (–1082, –592, and –819) and *IFN- γ* +874 on disease progression and persistence.

Naive patients with HBV-related chronic hepatitis or cirrhosis, with or without decompensation, were selected and taken consent for this prospective study from gastroenterology outpatient and wards of the University Hospital, during May 2008 to March 2011. The study was approved by the ethical committee of the institute.

Inclusion criteria:

- (1) Age > 16 years
- (2) Alanine aminotransferase (ALT) levels > 2 times upper limit normal and/or grade II or more fibrosis on histology
- (3) HBV DNA > 10⁵ copies/mL in HBeAg-positive cases and > 10⁴ copies/mL HBeAg-negative cases.

Exclusion criteria:

- (1) Coinfection with hepatitis C virus (HCV), hepatitis E virus (HEV), hepatitis A virus (HAV) or human immunodeficiency virus (HIV).
- (2) Presence of sepsis or hepatorenal syndrome. Patient could be included once the above complications are treated.
- (3) Presence of HCC
- (4) Active alcohol abuse over last 3 months.

Blood samples from 232 subjects with chronic liver disease along with 76 healthy controls were collected and stored at –80°C. Detailed clinical examination, routine hematologic and biochemical tests, and serology for the samples were investigated. Viral markers like HBsAg (hepatitis B surface antigen), HBeAg (hepatitis B envelope antigen), and its antibody (anti-HBe; DIA.PRO), anti-HCV, anti-HEV IgM, anti-HAV IgM (Organics), and HIV I and II (SD Elisa kits) were detected using enzyme linked immunosorbent assay. Model for end stage liver disease (MELD) score was calculated using the United Network for Organ Sharing formula, that is: $9.6 \times \log_e(\text{creatinine mg/dL}) + 3.8 \times \log_e(\text{bilirubin mg/dL}) + 11.2 \times \log_e(\text{INR}) + 6.4$ (etiology: biliary or alcoholic 0; others 1). Subjects underwent quantitative HBV DNA estimation.

Real-time quantitative polymerase chain reaction (PCR) was done for HBV DNA estimation. It followed the following steps:

DNA extraction: HBV DNA was extracted from 200 μ L serum using high-pure viral nucleic acid kit as instructed by the manufacturer (Roche Diagnostics). Extracted DNA was stored at –20°C till the assay was done.

HBV DNA quantification: Serum HBV DNA quantitative levels were evaluated using real-time PCR assay (Miniopticon™; Biorad). This diagnostic PCR was carried out on 10 μ L of extracted template DNA using HBV geno-Sense kit (Genome Diagnostics) by TaqMan probe analysis as per the manufacturer's instruction.

Genomic DNA was extracted from whole blood by the salting out method using following reagents: Reagent A known as RBC lysis solution [HiMedia; 0.01 M Tris-HCl (pH 7.4), 320 mM sucrose, and 5 mM magnesium chloride] and 1% Triton X-100 (Sigma). Reagent B known as cell lysis solution [1 M Tris-HCl (pH 7.4), 0.5 M ethylenediaminetetraacetic acid

(pH 8), and sodium chloride]. Reagent C, that is, 5 M sodium perchlorate (Sigma) was used for cell lysis.

The anti-inflammatory cytokine, *IFN- γ* +874 (T versus A) polymorphism was detected by allele-specific PCR using primers (Rad and others 2004), with modified cycling conditions: initial denaturation at 94°C for 2 min, 10 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 40 s, extension at 72°C for 40 s, 25 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 40 s, extension at 72°C for 50 s followed by final extension at 72°C for 7 min.

The 3 biallelic IL-10 promoter polymorphisms were detected by PCR using specific primers for *IL-10* (–592, –819, and –1082). The primers used for SNP analysis are mentioned in Table 1.

PCR protocol followed for *IL-10* (–1082): initial denaturation at 94°C for 8 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62.5°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 1 min. For –592 and –819, the similar protocol was followed except annealing temperature, which was 53°C for –592 and 61°C for –819, respectively.

This step was followed by restriction fragment length polymorphism where the amplified product was subjected to restriction using specific enzymes (Table 1).

Mean variation between 2 samples was compared with a Mann–Whitney *U*-test, and Kruskal–Wallis analysis of variance was used in group comparisons. The results were reported as mean $\pm$ standard deviation or median (range). A *P* value of <0.05 was considered statistically significant. χ^2 test was used to apply for Hardy–Weinberg equilibrium (HWE) and to compare allele and genotype frequencies between the chronic hepatitis B (CHB) and control group. Odds ratio for genotypes was calculated using univariate analysis. Quantitative clinical data were compared between subjects with CHB, and liver cirrhosis was compared by the unpaired Student's *t*-test, whereas the rank sum test was performed to compare the mean concentrations.

Observation revealed that 232 subjects [82.9% (104) men and 17.1% (22) women] included in the study underwent various serological tests having serum ALT level of 102.47 ± 121.6 , mean serum bilirubin of 3.7 ± 4.02 , and albumin of 2.98 ± 0.75 . Of these, 126 subjects were diagnosed with decompensated cirrhosis (mean age 41.65 ± 14.5 years).

TABLE 1. PRIMERS AND RESTRICTION ENZYMES FOR INTERLEUKIN-10 (–1082, –819, AND –592)

Cytokine	PCR primers	Restriction enzyme
–1082 (G/A)	FP 5'-TCGCTGCAACC CAACTGCC-3' RP 5'-GGTCCCTTACTTTGC TCTTAC-3'	<i>Mnl I</i>
–819 (T/C)	FP 5'-GACTCCAGCCACAG AAGCTTAC-3' RP 5'-AGCTCTCTGGGCC TTAGT-3'	<i>Rsa I</i>
–592 (A/C)	FP 5'-ATCCAAGACAACAC TACTAA-3' RP 5'-TAAATATCCTCAAA GTTCC-3'	<i>MaeIII</i>

See reference Rad and others (2004).
PCR, polymerase chain reaction.

TABLE 2. GENOTYPE FREQUENCY OF THE CYTOKINES INTERFERON GAMMA +874, IL-10 (-1082), IL-10 (-819), AND IL-10 (-592) GENES

Cytokine and clinical status	No. (%) of patients (frequency)		
<i>IFN-γ</i> +874	AA	TT	AT
CHB patients (106)	32 (30)	13 (12.5)	61 (57.5)
LC patients (126)	44 (34.9)	29 (23.0)	53 (42.1)
Healthy controls (76)	14 (19)	7 (9)	55 (72)
	$\chi^2 = 4.95, p = 0.084$		
<i>IL-10</i> (-1082)	AA	GG	AG
CHB patients (106)	50 (47.5)	29 (27.5)	27 (25)
LC patients (126)	46 (36.5)	34 (26.9)	46 (36.5)
Healthy controls (76)	32 (42.6)	1 (1.4)	43 (56.0)
	$\chi^2 = 1.162, p = 0.446$		
<i>IL-10</i> (-819)	CC	TT	CT
CHB patients (106)	27 (25)	34 (32.5)	45 (42.5)
LC patients (126)	41 (32.7)	19 (15.4)	66 (51.9)
Healthy controls (76)	9 (12)	38 (50)	29 (39)
	$\chi^2 = 3.77, p = 0.151$		
<i>IL-10</i> (-592)	AA	CC	AC
CHB patients (106)	32 (30)	32 (30)	42 (40)
LC patients (126)	20 (15.4)	36 (28.5)	70 (55.5)
Healthy controls (76)	35 (45.9)	9 (12.2)	32 (41.9)
	$\chi^2 = 3.38, p = 0.184$		

IFN- γ , interferon gamma; CHB, chronic hepatitis B; LC, liver cirrhosis; IL-10, interleukin-10.

Among them, 67% were HBeAg positive. Mean HBV DNA was $6.68 \pm 1.62 \log_{10}$ copies/mL, and the mean MELD score was found to be 17.08 ± 5.58 . The remaining 106 were classified as CHB patients (mean age 34 ± 12.86 years). Almost 46% of them were HBeAg positive. The mean HBV DNA and the mean MELD score were $6.26 \pm 1.73 \log_{10}$ copies/mL and 11.34 ± 3.7 , respectively.

IFN- γ polymorphism was analyzed in both CHB and decompensated cirrhosis patients. *IFN- γ* +874 polymorphism study was done using allele-specific primers (Table 2). The distribution of different genotypes and alleles were consistent with HWE. The frequency of the A allele was higher than T allele in both the group of patients. The difference of allele frequency between CHB and decompensated cirrhosis groups was not statistically significant ($P=0.08$) (Table 2). At the *IFN- γ* +874 locus, AT heterozygous genotype was dominant in CHB cases compared to cirrhosis (OR 1.87, $P<0.05$). The subjects included under healthy controls revealed the dominance of heterozygosity.

Similarly for *IL-10* (-1082) promoter gene polymorphism, it was found that frequency of A allele was higher than G allele and there was no significant difference between the CHB and cirrhosis cases ($P=0.44$) (Table 2). At the *IL-10* (-1082) locus, AA homozygous genotype dominated CHB group than cirrhosis group (OR 1.55, $P<0.05$). However, in case of healthy controls, heterozygous AG genotype was observed to be dominant.

In case of *IL-10* (-819), C allele was more frequent than T allele (53% versus 47%, $P>0.05$). Unlike *IL-10* (-1082),

heterozygous CT genotype was dominant over both the homozygous genotype CC and TT both in the CHB and decompensated cirrhosis patients (i.e., 42.5% and 52%, $P=0.15$). At the *IL-10* (-819) locus, greater expression of heterozygous CT genotype was revealed, which indicates increased risk of disease (OR 2.65, $P<0.05$), while in healthy controls distribution was not significant between CT and TT genotypes whereas very few individuals were found to have CC homozygous genotype.

Among *IL-10* (-592), C allele was more frequent than A allele in the diseased population (54% versus 46%, $P>0.05$). More than half of the decompensated cirrhosis patients had heterozygous genotype AC (55.5%), which was also dominant in CHB patients but occur less frequent (40%) and was nonsignificant. At the *IL-10* (-592) locus, AA homozygous genotype was dominant in CHB cases compared to cirrhosis (OR 2.29, $P<0.05$). It was also found that genotypes AA and AC were almost equally distributed in healthy controls, and very few individuals had CC homozygous genotype with nonsignificant outcome (Table 2).

Discussing the results, it can be stated that the host genetic factors are believed to be responsible for clinical outcome of infectious diseases involving genetic polymorphisms (Miyazoe and others 2002) because differences in the propensity to infection or severity of disease cannot solely be attributed to the virulence of an organism. For chronic viral hepatitis, genetic associations are likely to provide some clues to viral persistence and disease progression toward cirrhosis and might lead to a new

therapeutic approach. It is well known that the elimination of HBV by the infected host attributes to a coordinated innate and adaptive humoral and cell-mediated immune response. During this immune process, cytokines play a significant role in modulating almost all phases of the host immune response. Recent studies have shown that several immunoregulatory cytokines such as IFN- γ inhibit HBV replication through the noncytolytic process (Guidotti and others 1996; Guidotti and Chisari 2001). In contrast, IL-10 counteracts their effector mechanisms. Because the capacity for cytokine production in individuals largely depends on genetic polymorphisms (Fiorentino and others 1991; Westendorp and others 1997), heterogeneity of the candidate gene in patients with HBV infection emerges as a probable biomarker for determining the disease phenotypes.

In the present study, allelic *IFN- γ +874* intron and 3 biallelic polymorphisms in the *IL-10* gene promoter, which are known to alter their gene expression were analyzed. IFN- γ is one of the most representative cytokines, which shows impact in causing acute inflammation. We found that IFN- γ +874A allele was associated with susceptibility to HBV infection. The risk of HBV infection in participants with a +874A allele was 2.5-fold higher compared to those with the T wild allele. It has also been reported earlier that the possession of +874T and A alleles should be associated with high and low IFN- γ expression, respectively (Rossouw and others 2003). Therefore, it is meaningful that low IFN- γ expression probably impairs host immune response to HBV, rendering these subjects more prone to HBV infection. In concordance to the present study, it was reported that IFN- γ +874 AA genotype was associated with an increased risk and IFN- γ +874 AT genotype with a reduced risk of persistent HBV infection (Gao and others 2009).

IL-10 is an important anti-inflammatory cytokine secreted from Th2 cells. The levels of IL-10 production determine immune regulation and the balance between the inflammatory and humoral response. High level of IL-10 production may increase viral replication in chronic HBV infection. Even there are contradictory reports about the exact effect of *IL-10* promoter polymorphisms on the natural outcome of HBV infection. The (–1082) GG is associated with high IL-10 production, (–1082) AG with intermediate production, and (–1082) AA with low production. It was earlier studied that polymorphisms at positions (–819) and (–592) have no independent influence on IL-10 production (Turner and others 1997). The results indicated that IL-10 (–1082) AA genotype was associated with risk, which leads to lower IL-10 production and persistent infection and shows positive correlation with clinical outcome, but (–1082) AG was not significantly associated with disease persistence. Similarly, a study showed that IL-10 (–1082) AA had the similar effect as in the present study, but they also noted that –1082 AG was associated with reduced risk of persistent HBV infection (Gao and others 2009). On the contrary, it was suggested that the polymorphism (–1082) GG and (–1082) AG, respectively, leads to persistent HBV infection susceptibility (Zhang and others 2006, 2011).

No significant differences were detected between the 2 groups in the distributions of IL-10 genotype at the –1082, –819, and –592 positions, which were consistent with the results revealed by one more study (Gao and others 2009). Our findings indicated that heterozygosity of genotypes (–819) CT and (–592) AC were more strongly associated with liver

chronicity compared to homozygosity, and (–592) AA was associated to easily clear the HBV infection, which concurs several findings (Yan and others 2009; Chen and others 2010; Zhang and others 2011). In contrast, another study showed that the carriers of (–592) A allele have higher risk of persistent HBV infection and found that the frequency of CC genotype at position (–592) was higher in the HBV clearance group than in persistent group (Cheong and others 2006).

In summary, overall no significant differences were detected between CHB/liver cirrhosis and healthy control groups in the distributions of *IFN- γ +874* and *IL-10* genotype at the –1082, –819, and –592 positions, but the odds ratio reveals beneficial role of certain allele/genotype. In a recent study, there was no significant difference in the frequencies of alleles or genotype/haplotype in the *IL-10* gene promoter between HBV carriers and healthy volunteers (Wang and other 2011). But our findings indicated IFN- γ +874 AA genotype was associated with an increased risk of disease progression, and heterozygosity of *IL-10* genotypes –819 CT and –592 AC was more strongly associated with liver chronicity compared to homozygosity. Significantly, AA homozygous genotype was dominant in CHB cases in *IFN- γ +874* and *IL-10* (–1082 and –592) and is associated with increased risk of persistent infection, which should be considered for the therapeutic approach.

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Author Disclosure Statement

No competing financial interests exist.

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