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Association between interferon-gamma (IFN-γ) gene polymorphisms (+874A/T and +2109A/G), and susceptibility to hepatitis B viral infection (HBV)

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Abstract
Background: Interferon-gamma (IFN-γ) is a chief proinflammatory cytokine with a significant role in the immune response against viral infections. Today there is increasing evidence about the association between individual genetic polymorphisms and cytokines in predicting HBV infection susceptibility.
Aim: This study aimed to investigate the association between IFN-γ gene polymorphisms and susceptibility to hepatitis B viral infection (HBV), and the impact of these genetic polymorphisms on IFN-γ production. IFN-γ (+874A/T, rs2430561, and +2109A/G, rs1861494) was genotyped by single-stranded polymorphism-polymerase chain reaction (SSP-PCR) in 126 Egyptians with chronic HBV infection and in 100 healthy control subjects. The plasma levels of IFN-γ were measured by Enzyme-linked immunosorbent assay (ELISA).
Results: Compared to the control subjects there was a slight increase in +874TT genotype frequency in HBV patients. However, no statistical significance in IFN-γ (+874A/T and +2109A/G) genotype/allele distribution was demonstrated, indicating the lack of association between these SNPs and susceptibility to HBV infection. In +2109A/G, only AG genotype was observed with a complete abrogation of GG and AA genotypes. Haplotypes between different loci on selected genes showed insignificant changes in their frequency in patients and control subjects. HBV patients had a significantly higher level of IFN-γ ($P < 0.001$) compared to controls. The maximum significant increase in IFN-γ production was observed in subjects harboring the +874TA genotype.
Conclusions: As no association could be characterized between the polymorphism in IFN-γ (+874A/T and +2109A/G) and susceptibility to chronic HBV infection, our data support the concept that IFN-γ gene polymorphisms are not predictors of HBV susceptibility in this segment of the Egyptian population.

Keywords: Egyptian; HBV; IFN-γ; Polymorphism

Highlights:
- We studied IFN-γ production and gene polymorphism in HBV infected patients.
- We found a lack of association between selected SNPs and susceptibility to infection.
- HBV patients had a significantly higher level of IFN-γ compared to controls.
- The maximum increase in IFN-γ level was observed in subjects with +874TA genotype.
- Host genetic predisposition of IFN-γ SNPs has no effect on HBV infection.

Introduction

Hepatitis B viral infection (HBV) is one of the most critical liver diseases. Chronic liver diseases have approximately 240 million carriers worldwide and an estimated mortality rate of 887,000 deaths every year due to chronic liver diseases, including liver cirrhosis and hepatocellular carcinoma (HCC) (Tang et al., 2018; WHO, 2017). The number of Egyptians chronically afflicted by HBV is about 2–3 million (Habil et al., 2013). Despite the availability of a preventive vaccine and the use of effective and well-tolerated antiviral medications since 1998, almost 250 million people remain infected with HBV worldwide (Nguyen et al., 2020).

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The course of the infection is not consistently ranging from self-limited illness to chronic HBV infection who are unable to resolve the infection within six months. It is estimated that 5–10% of HBV patients will develop cirrhosis, end-stage liver disease, hepatocellular carcinoma (HCC) or require liver transplantation for several decades (Lee and Banini, 2019). The role of the immune response in HBV progression and its underlying mechanisms are still ambiguous. Although several host's genetic factors have been reported to play a significant role in an infection's resolution (Liu et al., 2006; Prasad et al., 2010; Ribeiro et al., 2007; Wu and Chang, 2015), more research is needed to clarify their role in HBV infection.

Cytokines play indispensable roles during HBV infection, which orchestrate the innate and adaptive immune response. They represent a regulatory molecule playing a fundamental role in the immunopathogenesis of HBV infection. Cytokines play an important role as a defense mechanism against hepatitis viruses. It is speculated that this is one of the determining factors of the HBV infection's outcome. Proinflammatory cytokines such as as Interleukin (IL-1β), IL-6, and tumor necrosis factor (TNF-α), were reported to be elevated among hepatitis patients. IL-6 interferes with regulating the immune response to HBV infection (Lan et al., 2015; Pan et al., 2012; Tunçbilek, 2014). T cells mediate viral clearance by killing infected hepatocytes or releasing proinflammatory cytokines, such as IFN-γ and TNF-α. The expression of various proinflammatory cytokines correlates with the degree of hepatic inflammation and liver fibrosis development (Huang et al., 2006; Hui and Lau, 2005; Koziel, 1999). Moreover, the cytokines secreted by Th1 T cells promote clearance of the infection (Trehanpati et al., 2013). IFN-γ is a proinflammatory cytokine produced by Th1 T cells, which has a great role in the immune response towards HBV infection by inhibiting the virus's replication (Srivastava et al., 2014; Stark, 2007; Trehanpati et al., 2013).

Single nucleotide polymorphism (SNPs) is considered one of the primary genetic variability sources, especially when they influence gene expression or function. SNPs might occur every 100–300 bp (Cheok et al., 2009). Many researchers have found that SNPs may help predict an individual's response to certain drugs, susceptibility to environmental agents such as toxins, risk of a particular disease development, or even progression of disease manifestations. SNPs can also be used to track the inheritance of disease genes. The presence of SNPs at a specific locus may lead to several disease conditions (Gisler et al., 2013; Masoodi et al., 2013).

The cytokines' genes were reported to be highly polymorphic, causing a diversity in the production capacity among different ethnic groups (Akcay et al., 2018; Dondeti et al., 2016; Hollegaard and Bidwell, 2006; Ollier, 2004). Moreover, polymorphisms in cytokine genes could affect different diseases' susceptibility (Abana et al., 2017; Hyvärinen et al., 2017; Manolova et al., 2018). Our previous studies found a strong correlation between the genetic polymorphism of (IL-10, TGF-β1, and other cytokines), which significantly affects the disease's progression (Dondeti et al., 2017; El-Maadawy et al., 2019; Talaat et al., 2013, 2014, 2017).

IFN-γ gene, located on chromosome 12, has four exons and three introns. Previous studies reported that IFN-γ +874A/T (rs2430561) of the first intron and IFN-γ +2109A/G (rs1861494) of the third intron to have a putative effect on IFN-γ levels. The effect arises from the binding of those SNPs to the Nuclear factor-kappa B (NF-κB) transcription factor, which is highly characteristic of the infection's course and progression (Pravica et al., 2000; Yu et al., 2006). Although several studies have been performed on the correlation between IFN-γ gene polymorphisms and HBV infection (Arababadi et al., 2011; Ghasemian and Shahbazi, 2016; Naghizadeh et al., 2018; Peng et al., 2007; Saxena et al., 2014; Sun et al., 2015a; Wei et al., 2016), non was performed to study such correlation among the Egyptian population. Thus the current study was tailored to investigate the role of IFN-γ +874T/A and +2109 A/G gene polymorphism in the susceptibility risk of Egyptians to HBV infection.

Materials and methods

Study population

One hundred and twenty-six patients (126) with HBV infection collected from the National Liver Institute, Menoufia University, Egypt, enrolled in this study. The males outnumbered the females (100 men and 26 women). The mean age was 33.4 ± 17.65 years (range: 70–20). The demographic and biochemical data are presented in Table 1. Hepatitis B surface antigen (HBsAg) was tested using commercially available kits (Sorin Biomedica, Milan, Italy). Standard polymerase reaction (Roche Diagnostics Corp., Indianapolis, IN) was used to confirm the presence of HBV-DNA in HBV-positive patients. HCV antibody was measured using third-generation enzyme-linked immunosorbent assay (ELISA) and verified by reverse transcriptase-polymerase chain reaction (RT-PCR) using TaqMan HCV assay (Roche instrument center AG, Switzerland). Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), albumin, direct and indirect Bilirubin, were all measured using Cobas 6000 (Roche Diagnostics GmbH, Germany) according to the manufacturers' instructions of the respective kits. Selected patients were positive for HBsAg, HBV-DNA, and negative for HCV antibodies. HCV or other virally infected patients were excluded from the study. One hundred healthy controls with no history of previous liver disease, and who had normal liver function tests, and negative HBV and HCV serology were involved in the study. All investigations were performed in accordance with the Menoufia University, Health, and Human Ethical Clearance Committee guidelines for Clinical Research. The local ethics committee approved the study protocol, and informed consent was received from all subjects.

Table 1. Biochemical characteristics of healthy controls and HBV patients

Parameter	Control group (N = 100)	HBV group (N = 126)	P
AST (IU/l)	20.97 ± 5.86	42.01 ± 23.50	$P < 0.001$
ALT (IU/l)	18.91 ± 5.05	44.13 ± 34.81	$P < 0.001$
Albumin (g/l)	4.26 ± 0.39	3.43 ± 0.76	$P < 0.001$
Total bilirubin (mg/dl)	0.67 ± 0.19	1.09 ± 0.60	$P < 0.001$
Direct bilirubin (mg/dl)	0.11 ± 0.07	0.31 ± 0.013	$P < 0.001$
Creatinine (mg/dl)	0.89 ± 0.15	1.09 ± 0.33	$P < 0.001$
Urea (mg/dl)	28.93 ± 7.04	33.41 ± 13.52	$P < 0.01$
HBV DNA (IU/l)	–	1641329.29 ± 958497.14	–

All data are presented as mean ± SD. Alanine aminotransferase (ALT), Aspartate aminotransferase (AST).

DNA isolation

Blood was collected by the withdrawal of 5 ml venous blood from each individual involved in this study into sterile

Ethylene diamine tetra-acetic acid (EDTA.K3)-tubes. It was then centrifuged at 1500 rpm for 10 min. Plasma was separated, aliquoted, and stored at –80 °C for cytokine secretion analysis. According to the manufacturer's instructions, genomic DNA was extracted from whole blood-EDTA samples by Wizard Genomic DNA Purification Kit (Gentra Puregene DNA extraction kit; Qiagen, Hilden, Germany).

Genotyping of IFN-γ +874T/A and +2109A/G

SNPs (IFN-γ +874T/A and +2109A/G) were genotyped by single-stranded polymorphism-polymerase chain reaction (SSP-PCR) using four primers mix (Table 2) according to Ghasemian and Shahbazi (2016) and Liu et al. (2006), respectively, with some modifications. PCR reaction components were 2× DreamTaq Green Master Mix (Fermentas, Thermo Fisher Scientific Inc), 10 pmoles of each primer (Metabion, Martinsried, Deutschland), and 0.1 µg DNA. The PCR cycling condition was as follows: one cycle of 95 °C for 1 min; followed by 10 cycles of 95 °C for 15 s; 62 °C for 50 s; 72 °C for 20 s; and 20 cycles of 95 °C for 20 s; 56 °C for 50 s, 72 °C for 50 s, and final cycle of 72 °C for 10 min. All PCR reactions were performed in Biometra thermal cycler (Biometra GmbH, Germany). Using 2% agarose gel electrophoresis, the PCR product's size for IFN-γ +874T/A was 261 bp, while it was 266 bp for IFN-γ +2109A/G and 429 bp for control primers.

Table 2. Primers used for genotyping the genetic polymorphisms of IFN-γ gene

SNP	Primer sequence
IFN-γ +874 T/A	**T F:** 5´-TTC TTA CAA CAC AAA ATC AAA TCT-3´ **A F:** 5´-TTC TTA CAA CAC AAA ATC AAA TCA-3´ **Reverse R:** 5´-TCA ACA AAG CTG ATA CTC CA-3 **Control F:** 5´-GCCTTCCCAACCATTCCCTTA-3´ **Control R:** 5´-TCACGGATTTCTGTTGTGTTTC-3´
IFN-γ +2109 A/G	**T F:** 5'-GAA GTA GGT GAG GAA GAA GCA-3' **A F:** 5'-GAA GTA GGT GAG GAA GAA GCC-3' **Reverse R:** 5'-CCT GGT ACC TAT TCA AAG ACT G-3' **Control F:** 5´-GCCTTCCCAACCATTCCCTTA-3´ **Control R:** 5´-TCACGGATTTCTGTTGTGTTTC-3´

Estimation of IFN-γ secretion level

Total IFN-γ plasma levels were measured in HBV patients and normal controls using sandwich enzyme-linked immunosorbent assay (ELISA) (R&D System, Inc., Minneapolis, USA) to the manufacturer's instructions. The digital data of raw absorbance value was readily processed into a standard curve using the ELISA reader-controlling software (Softmax), from which cytokine concentrations of unknown samples are derived directly and expressed as pg/ml.

Statistical analysis

The statistical analyses were performed by SPSS statistical package version 19 (SPSS, IBM Corporation, USA). Comparisons were made using an independent *t*-test, and results were presented as Mean ± SD. Chi-squared tests were performed to examine the allele frequency and genotype/haplotype distribution differences between different groups. The online tool SNPstats (https://www.snpstats.net/start.htm) was used to perform haplotype reconstruction from population genotype data and Linkage Disequilibrium (LD) parameters (D´ and r2). The odds ratio (OR) and 95% confidence intervals (CI) were calculated to assess the risk associated with a particular allele, genotype, or haplotype. A $P \leq 0.05$ was considered significant.

Results

Association between IFN-γ polymorphisms and HBV infection

Genotypes and allele frequencies of IFN-γ (+874T/A, and +2109A/G) in controls and patients are shown in Table 3. TA genotype of +874T/A was the most frequent genotype in HBV and controls. Both T and A alleles were more frequent in controls compared with HBV patients. Analysis of IFN-γ +874T/ASNP revealed an insignificant change in genotype distribution between HBV patients and controls. On the other hand, genotyping of IFN-γ +2109A/G showed complete disappearance of both homozygous genotypes (AA and GG) and the presence of heterozygous genotype (A/G) in both groups. Hence, this SNP may not have a significant role in the HBV

Table 3. Genotypes and allele frequencies of the IFN-γ (+874T/A and +2109A/G) SNPs in healthy controls and Hepatitis B patients

SNP	Control group (*N* = 100)	HBV group (*N* = 126)	*P*	95% Confidence interval (CI) OR (lower-upper)
IFN-γ +874 T/A (*N*, %)				
TT	28 (28%)	34 (26.9%)	NS	0.9503 (0.5280–1.7103)
TA	43 (43%)	59 (46.9%)	NS	1.2161 (0.7163–2.0645)
AA	29 (29%)	33 (26.2%)	NS	0.8687 (0.4831–1.5621)
T	99 (77%)	127 (50.3%)	NS	1.0365 (0.715–1.502)
A	101 (79%)	125 (49.7%)	NS	0.964 (0.665–1.398)
IFN-γ +2109 A/G (*N*, %)				
AA	0	0	–	–
AG	100 (100%)	126 (100%)	–	–
GG	0	0	–	–
A	50 (50%)	63 (50%)	–	–
G	50 (50%)	63 (50%)	–	–

NS: not significant.

infection among Egyptians. The haplotyping of IFN-γ (+874T/A, and +2109A/G) SNPs gives rise to four haplotypes (TA, TG, AA, and AG), IFN-γ haplotypes, and their frequencies shown in Table 4. There was no significant change in the distribution of the haplotypes among controls and HBV patients. This may be attributed to the absolute predominant presence of heterozygous genotype AG in both groups.

Differential expression of plasma IFN-γ in HBV patients and controls according to gene polymorphisms

Despite differences in genotype frequencies, the mean plasma level of IFN-γ is significantly higher ($p < 0.01$) in HBV infected patients (5419.63 ± 1571.47 pg/ml) than controls (539.77 ± 97.43 pg/ml). The differential IFN-γ plasma levels showed that the plasma level of IFN-γ varied according to the genotypes of IFN-γ SNPs, as shown in Table 5. In IFN-γ+874T/A, the highest level of IFN-γ synchronize with TA genotype (7635.50 ± 3276.79 and 640.59 ± 176.08 for HBV and controls, respectively), and the lowest level coincided with AA genotype (3423.69 ± 917.66 and 389.04 ± 118.81 for HBV and controls, respectively). In the control group, a significant increase in IFN-γ production in TA genotypes compared with TT ($P < 0.01$) and AA ($P < 0.001$) genotypes secretion. Moreover, the subjects' TT genotypes have more IFN-γ production than AA ($P < 0.05$). On the other hand, HBV patients with the TA genotype have the highest significant production of IFN-γ compared with TT and AA genotypes ($P < 0.001$).

Table 4. IFN-γ (+874T/A and +2109A/G) haplotype frequency in healthy controls and hepatitis B patients

Haplotype	Control group ($N = 100$)	HBV group ($N = 126$)	*P*	95% Confidence interval (CI) OR (lower-upper)
TA	24.7%	25.2%	NS	1.013 (0.5578–1.8699)
TG	24.7%	25.2%	NS	1.013 (0.5578–1.8699)
AA	25.3%	24.8%	NS	0.9789 (0.5332–1.7975)
AG	25.3%	24.8%	NS	0.9789 (0.5332–1.7975

Table 5. Mean plasma levels of IFN-γ in genotypes of +874T/A and +2109A/G SNP in healthy controls and hepatitis B patients

Genotype	Control group ($N = 100$)	HBV group ($N = 126$)	*P*
IFN-γ +874T/A (pg/ml)			
TT	531.15 ± 197.99	3648.58 ± 1345.83	$P < 0.001$
TA	640.59 ± 176.08	7635.50 ± 3276.79	$P < 0.001$
AA	389.04 ± 118.81	3423.69 ± 917.66	$P < 0.001$
IFN-γ +2109A/G (pg/ml)			
AA	–	–	–
AG	539.77 ± 97.43	5419.63 ± 1571.47	$P < 0.001$
GG	–	–	–

Discussion

IFN-γ is a potent proinflammatory cytokine produced by Th1 T cells and natural killer cells during the early phase of the immune response. It is the most potent antiviral cytokine as its main action is achieved through inhibiting viral replication (Chisari and Ferrari, 1995; Qi et al., 2005; Ribeiro et al., 2007). Hence, IFN-γ is reported as an active player in HBV clearance and resolution (Ben-Ari et al., 2003). The action of IFN-γ is directly proportional to its levels. Chronic HBV patients reported having lower IFN-γ levels than patients with acute infection (Chisari and Ferrari, 1995; Wai and Fontana, 2003). IFN-γ is one of the cytokines that participate in regulating the immune response, which effectively defends against viral infections such as HBV (Dorman and Holland, 2000; Qi et al., 2005). Cytokines' genetic polymorphisms affect cytokines' levels during HBV infection, thereby modulating the immune response, which can alter the natural course of the infection (Wai and Fontana, 2003).

The polymorphisms in cytokine genes have affected the expression levels of secreted cytokines. IFN-γ gene polymorphisms, such as +874T/A and +2019A/G, influence IFN-γ levels (Chevillard et al., 2003; Peresi et al., 2013; Pravica et al., 2000). Previous studies reported the correlations between IFN-γ genetic polymorphisms and chronic HBV in Chinese (Peng et al., 2007; Qi et al. 2005; Yu et al., 2006; Zhang et al., 2006), Indian (Srivastava et al., 2014), Iranian (Arababadi et al., 2011) and Asian (Sun et al., 2014) populations. SNPs in the IFN-γ gene were also investigated for their presence in chronic HCV infected German (Mihm et al., 1996), Taiwanese (Dai et al., 2006), Chinese (Gao et al., 2010), and Iranian (Sarvari et al., 2014) patients. The presence of IFN-γ genetic predisposition and development of HCC was investigated by Teixeira et al. (2013) and Saxena et al. (2014) in Brazilians and Indians, respectively. Thus, in the current study, we examined the correlation between IFN-γ gene polymorphisms and susceptibility to HBV infection, in addition to the effect of these polymorphisms on the IFN-γ secreted levels. According to the information we have, the role of IFN-γ gene polymorphisms in HBV infection among Egyptians has not been studied.

Our results showed that the intronic SNPs of +874T/A and +2109A/G might not significantly impact Egyptians' susceptibility to getting chronic hepatitis B infection. While there is an insignificant difference between the distribution of +874T/A, the TA genotype distribution may represent a slight elevation in its distribution. There are many studies performed on SNP and hepatitis B (Al-Kadi and Monem, 2017; Arababadi et al., 2011; Ghasemian and Shahbazi, 2016; Liu et al., 2006; Sun et al., 2014, 2015b; Yu et al., 2006; Zhang et al., 2006). Some of these studies reported the insignificant difference among alleles and genotypes of +874T/A, emphasizing the role of TA genotype and T allele, while others reported significant results. Our current work agrees with previous studies, which reported the insignificance in the distribution of the genotypes in Chinese (Sun et al., 2014; Yu et al., 2006) and Iranian (Arababadi et al., 2011; Ghasemian and Shahbazi, 2016) populations. In agreement with our results, Bahgat et al. (2015) estimated an insignificant elevation in TA genotype frequency in chronic

HCV-infected Egyptian patients. On the contrary, previously reported data suggested that AA genotype was an HBV chronic infection risk factor in Chinese (Liu et al., 2006; Sun et al., 2015b; Zhang et al., 2006) and Syrian populations (Al-Kadi and Monem, 2017). This may excuse the ethnic differences as those reports came from different populations.

On the other hand, few reports were found about the effect of +2109A/G on HBV infection susceptibility. The current results showed the impact of extensive migration in our population as – surprisingly – AG genotype was the exclusive genotype to appear in both groups, either controls or HBV patients. The study of Sun et al. (2015b) on the Chinese population revealed results contrary to our findings; the three genotypes appeared in both groups, with the GG genotype the least frequent genotype among the subjects. An older study on the Chinese population found an insignificant difference in the allelic frequencies of IFN-gamma +2109A/G between HBV cases and controls (Liu et al., 2006). The genotype GG was completely absent in our population, which indicates that GG may be an ancestry genotype in our people. Collectively, +2109 may not have an association with the HBV infection and does not appear to impact IFN-γ levels significantly.

Previous studies found that HBV infection correlated with elevated IFN-γ production levels (Li et al., 2016). The current results showed that IFN-γ plasma levels were significantly increased in HBV patients compared with healthy subjects. IFN-γ may affect the immune response of the HBV patients in light of its role in regulating and suppressing immune response (Ben-Ari et al., 2003; Dorman and Holland, 2000; Qi et al., 2005; Wai and Fontana, 2003). In chronic hepatitis B, the raised serum levels of interferon-α correlated with the presence of viral replication (Brunetto and Bonino, 2014; Hosseini Khorami et al., 2018).

Additionally, the levels of IFN-γ showed a strong association with the TA genotype with less high levels among TT and AA genotypes and T allele, which suggest the impact of both genotypes on the IFN-γ level. Intuitively, +874T/A may not substantially affect our population, but the TA genotype may have an indirect impact on the disease. The T allele of +874T/A was reported to be associated with high levels of IFN-γ, while the A allele is associated with low levels (Pravica et al., 2000). This effect might arise from a putative NF-κB, a functional factor in IFN-γ levels (Pfeffer, 2011). Additional large studies could confirm our preliminary data to get a stable result indicating the real role of this SNP.

Conclusions

Taking this preliminary data together, our observational prospective case-control study suggested that IFN-γ +874T/A and +2109A/G may not be associated with HBV infection. However, as AG genotype of +874 is considered a risk factor for the disease, some attention must be paid to individuals harboring that genotype. We also proved the production of higher levels of IFN-γ among patients than controls in addition to the association of increased IFN-γ levels with the TA genotype. Our study has some potential limitations, such as limited publications on IFN-γ polymorphism at +2109A/G; overall, there are 5 manuscripts, two of them on HBV, and none of them on the Egyptian population. Moreover, the absence of genotypes other than AG genotype +2109A/G shed some light on the deviation in genetic predisposition in our population. Larger prospective studies are needed to identify all the associations between these SNPs and HBV and confirm our findings. In the near future, another study will be designed to confirm our results, with a large sample size and different groups of participating subjects (HBV and HCC).

Ethical aspects and conflict of interests

The authors have no conflict of interests to declare.

Declaration of funding interests

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