Association between the *IFNA1* (–2C→T) Polymorphism and Increased *IFNAR1* Gene Expression Levels in Chronic Hepatitis B Infection

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Key Words

Gene expression · Hepatitis B virus · *IFNAR1* · Interferon- α · Single nucleotide polymorphisms

Abstract

Background: Single nucleotide polymorphisms and variant expression of some interferon (IFN) genes in individuals with chronic hepatitis B virus (HBV) infection might be related to higher viral load and disease complications. Thereby, whole blood samples of 208 patients (94 chronic HBV-infected patients and 114 HBV immune subjects) were analyzed to investigate the association between *IFNG* (–5A→G), *IFNA1* (–2C→T) and *IFNAR1* (–97T→C) genes with their expression levels and HBV viral load. **Methods:** Genotyping was performed by high-resolution melting analysis with quantitative PCR (qPCR). Viral load quantification and gene expression were also carried out using qPCR. **Results:** Chronic HBV-infected subjects with *IFNA1* CT genotype and T allele were more likely to develop protection against HBV when compared to immune subjects with wild-type genotype (*IFNA1* CT/CC: OR = 0.45, $p = 0.01$, and T/C allele: OR = 0.55; $p < 0.01$). In patients with *IFNAR1* wild-type TT genotype, the expression levels of this receptor may explain the lower viral load

($r^2 = 0.40$; $p = 0.04$) and protection against chronic infection.

Conclusions: These findings suggest that the polymorphic variant of *IFNA1* (–2) gene is associated with chronic HBV infection, and high expression levels of the *IFNAR1* gene and low levels of *IFNA1* might contribute to the pathogenesis of chronic infection in these subjects.

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Introduction

Hepatitis B virus (HBV) infection is a serious health problem worldwide [1]. It is estimated that 350 million people suffer from chronic HBV infection globally, which may eventually evolve into cirrhosis and hepatocellular carcinoma [2]. HBV-related end-stage liver disease or hepatocellular carcinoma is responsible for over 0.5–1 million deaths per year and 5–10% of liver transplantations currently [3, 4].

The prevalence of HBV infection varies markedly in different geographic areas worldwide [5]. In Brazil, this prevalence is approximately 10% in nonvaccinated and 4.3% in vaccinated individuals [6]. Host and viral risk factors are associated with HBV-related liver disease pro-

gression and increased cirrhosis rates, e.g. older age, HBV genotype, high levels of HBV DNA, habitual alcohol consumption and coinfection with hepatitis C or D virus or human immunodeficiency virus (HIV) [2, 7].

Single nucleotide polymorphisms (SNPs) are known to affect disease progression following chronic HBV infection. The cytokine production-induced cell-mediated antiviral immune response plays a crucial role in the control of viral infection. Genetic variants of cytokines involving interferon (IFN)- γ and interleukin (IL)-10 genes, for example, have been associated with the outcome of HBV infection [8–10].

Several studies reported that human leukocyte antigen (HLA) class I and II loci, many cytokines and cytokine receptor genes were associated with HBV susceptibility and/or HBV persistence or disease severity [11, 12]. In addition to the identification of SNPs, IFN- γ and IFN- α cytokine expression patterns and genetic variations in the IFN- α receptor subunit 1 (IFN- α r1) might affect clearance rates or contribute to the development of chronicity of early-stage HBV. It may also persistently influence HBV pathogenesis during the chronic long-term infectious process [13].

Recent Asian population studies [14, 15] have analyzed the relationship between the presence of gene polymorphisms and serum levels of different cytokines related to chronic HBV infection. However, there are discrepancies concerning host susceptibility to chronic HBV infection.

IFN- α has been widely used in the treatment of chronic HBV infection [16]. Furthermore, several studies found that *IFNAR1* gene polymorphisms in promoter, intron and exon regions were associated with HBV infection [13, 17, 18]. However, data on genes polymorphisms in *IFNG* (–5A→G), *IFNA1* (–2C→T) and *IFNAR1* (–97T→C) regions are lacking.

Controversy exists whether or not these genes are significantly associated with chronic HBV infection and other diseases [19, 20], or their location near promoter genes. Thus, polymorphisms in these promoter region genes might influence the gene expression pattern associated with the clinical outcome of persistent HBV infection [16].

The identification of SNPs in addition to cytokine expression associated with viral load might be useful in clinical practice to be able to stratify into higher- or lower-risk groups, which may result in more effective therapy and provide a better understanding of immune responses to HBV.

The aim of the present study was to investigate the relationship between genotypic and allelic frequencies of *IFNG* (–5A→G), *IFNA1* (–2C→T) and *IFNAR1* (–97T→C)

gene polymorphisms with cytokine expression levels and HBV viral load in individuals with chronic HBV infection.

Patients and Methods

Subjects

The case-control study included 208 Brazilian adults: 94 individuals with chronic HBV infection not receiving any antiviral treatment (64 men and 30 women) and 114 immune subjects (84 men and 30 women). Both groups attend two outpatient referral centers for hepatitis (Clinical Hospital of the Federal University of Pernambuco and Helvio Auto Hospital School, Alagoas State University of Health Sciences) from October 2010 to August 2013.

The diagnostic criteria of chronic HBV infection were HBsAg positivity (>125 mIU/ml) and presence of anti-HBc (>1.5 U/ml) or IgG for more than 6 months. In addition, the immune controls had seropositivity for anti-HBs (>10 mIU/ml) and anti-HBc, and were negative to HBsAg.

Individuals receiving any antiviral treatment or undergoing immunosuppressive therapy during the last 6 months before study entry, or presenting HIV and/or hepatitis C virus coinfection, were excluded from the study.

SNP Genotyping

Genomic DNA was isolated from white blood cells using the phenol (pH $\pm$ 8.0)/chloroform-based method [21]. Genomic DNA quantification measured by NanoDrop 2000 (ThermoScientific®) was approximately 500 ng/ μ l (100–1,000). Artificially constructed sequences were used as positive controls for the wild-type and polymorphic alleles with 75-bp amplicons related to each genotype (table 1). Heterozygous genotypes were obtained by an equimolar mixture from the sequences of the two alleles.

Real-time quantitative PCR (qPCR) and the high-resolution melting (HRM) method were carried out on Rotor-Gene (Qiagen) to identify *IFNG* (A→G, rs202055606), *IFNA1* (C→T, rs181407537) and *IFNAR1* (T→C, rs2850015) polymorphisms. HRM-PCR contained 2 μ l of fluorophore master mix EvaGreen 1 $\times$ with BSA (Solis BioDyne®), 0.7 pmol/ μ l of each primer (forward and reverse) at 10 pmol/ μ l and 2 μ l of isolated genomic DNA; the following reaction conditions were employed: denaturalization at 94°C for 15 s, followed by 45 cycles at 95°C for 15 s, annealing at 60°C for 15 s and a final extension step at 72°C for 60 s. Raw data obtained were analyzed using Rotor-Gene Q software (version 2.3; Qiagen).

The melting conditions were 50–90°C of gradual warming, with step increases of 0.7°C every 7 s for dissociation curve formation and a 90-second resting period for premelt conditions. Using the saturating dyes, it was possible to identify heteroduplex DNA without redistribution during melting, which allows HRM to detect SNPs [22]. HRM analysis is a rapid, powerful and accurate method, which is suitable for this study type [23]. The percent confidence level accepted for genotyping accuracy was above 99%.

HBV-DNA Detection and Quantification

HBV-DNA was isolated from 400 μ l of plasma using the phenol (pH $\pm$ 8.0)/chloroform-based method [21] and eluted in 100 μ l of rehydration solution (1 $\times$ buffer TE), obtaining 1–1,500 ng/ μ l by NanoDrop 2000 measurements.

Table 1. Polymorphism position, NCBI number, PCR primer sequences, annealing temperature and product size in *IFNG* (–5), *IFNA1* (–2), *IFNAR* (–97) genes, *GAPDH* and β -*actin* housekeeping genes and in HBV

SNP position	Polymorphism	NCBI number	PCR primer sequences (forward/reverse)	Annealing T, °C	bp
<i>IFNG</i> (–5) (rs181407537)	A→G	NC_000012.12	(F): 5'-AACTACTGATTTCAACTTCTTTG-3' (R): 5'-TGAAAAGCCAAGATATAACTTGT-3'	68	75
Pos. control <i>IFNG</i> (–5)	Allele G		5'-AAC TAC TGA TTT CAA CTT CTT TGG CTT AAT TCT CTC GGA AAC GAT GAA ATA TAC AAG TTA TAT CTT GGC TTT TCA-3'		
Pos. control <i>IFNG</i> (–5)	Allele A		5'-AAC TAC TGA TTT CAA CTT CTT TGG CTT AAT TCT CTC GAA AAC GAT GAA ATA TAC AAG TTA TAT CTT GGC TTT TCA-3'		
<i>IFNA1</i> (–2) (rs202055606)	C→T	BC112302.1	(F): 5'-CCCATCTCAGCAAGCCCAGAA-3' (R): 5'-CACCAGGACCATCAGTAAAGCA-3'	62	75
Pos. control <i>IFNA1</i> (–2)	Allele T		5'-CCC ATC TCA GCA AGC CCA GAA GTA TCT GCA ATA TCT ATG ATG GCC TCG CCC TTT GCT TTA CTG ATG GTC CTG GTG-3'		
Pos. control <i>IFNA1</i> (–2)	Allele C		5'-CCC ATC TCA GCA AGC CCA GAA GTA TCT GCA ATA TCT ACG ATG GCC TCG CCC TTT GCT TTA CTG ATG GTC CTG GTG-3'		
<i>IFNAR1</i> (–97) (rs2850015)	T→C	AP000044.1	(F): 5'-GTAGAGGGGCGGTGAGAGCTA-3' (R): 5'-CGCCATCGCCCCGTCCTAAG-3'	66	75
Pos. control <i>IFNAR1</i> (–97)	Allele T		5'-GTA GAG GGG CGG TGA GAG CTA AGA GGG GCA GCG CGT GTG CAG AGG GGC GGT GTG ACT TAG GAC GGG GCG ATG GCG-3'		
Pos. control <i>IFNAR1</i> (–97)	Allele C		5'-GTA GAG GGG CGG TGA GAG CTA AGA GGG GCA GCG CGT GCG CAG AGG GGC GGT GTG ACT TAG GAC GGG GCG ATG GCG-3'		
<i>GAPDH</i>	Constitutive gene	NG_007073.2	(F): 5'-CCAAATCAAGTGGGGCGATG-3' (R): 5'-AAAGGTGGAGGAGTGGGTGTGCG-3'	50	200
β - <i>Actin</i>	Constitutive gene	NW_005179884.1	(F): 5'-ATTAAGGAGAAGCTGTGCTACG-3' (R): 5'-TCATTGCCAATGGTGATGACCT-3'	50	125
HBV	Viral load	JQ000009.1	(F): 5'-GGACCCCTGCTCGTGTTACA-3' (R): 5'-GAGAGAAGTCCACCACGAGTCTAGA-3'	60	90

bp = Amplicon size in base pairs; T = temperature.

The PCR mixture contained 2 μ l of HBV-DNA template (1–1,500 ng/ μ l), 2 μ l of 5 $\times$ master mix EvaGreen without BSA and 0.7 μ l of each primer at 10 pmol/ μ l in a final volume of 10 μ l of PCR (table 1). PCR conditions were the following: denaturalization at 94°C for 15 min, followed by 45 cycles at 95°C for 15 s, annealing at 60°C for 15 s and a final extension step at 72°C for 60 s. The melting conditions were 50–90°C of gradual warming, with step increases of 0.7°C every 7 s for dissociation curve formation and a resting period of 90 s for premelt conditions. The melting temperature was between 80.1 and 80.6°C. A standard curve ($R^2 = 0.99$; slope = –3.31; efficiency = 1.00) consisted of 5 serial dilutions referring to international standards (NIBSC; code 97/746; in IU/ml and converted in $\log_{10}$).

HBV-DNA quantification results were stratified according to viral load levels: a low viral load was defined as <2,000 IU/ml or <3.3 $\log_{10}$ and a high viral load as $\geq 3.3 \log_{10}$ or $\geq 2,000$ IU/ml. The cutoff level of viral load was according to the EASL Clinical Prac-

tice Guidelines for chronic hepatitis B infection [24] and was one of the criteria for antiviral therapy initiation.

Absolute Gene Expression of Cytokines

Peripheral blood mononuclear cells (PBMC) were harvested by Ficoll-Paque™ Plus (GE Healthcare®) separation and stored in a guanidine isothiocyanate solution (4 M) at –80°C. mRNA was extracted from the PBMC solution using the acid phenol (pH ± 5.0)/chloroform-based method [21] and stored in a 0.1% (v/v) DEPC aqueous solution at –80°C. cDNA was synthesized using the Im-Prom-II™ reverse transcription system (Promega®) and random primers.

Absolute cytokine gene expression levels were evaluated using RT-PCR containing 5 μ l of master mix SYBR Green-I, 0.7 μ l of each primer (table 1) at 10 pmol/ μ l and 2 μ l of cDNA synthesized with qPCR cycles at 94°C for 15 min, 45 cycles at 95°C for 15 s, 60°C for 20 s followed by a final extension of 25 s at 72°C. The

Table 2. Laboratory parameters of the 94 chronic HBV-infected patients according to viral load levels

Laboratory parameters	Mean values	Low viral load (<3.3 log ₁₀) (37: 26 males/11 females)		High viral load (≥3.3 log ₁₀) (57: 38 males/19 females)		p
		median	range	median	range	
Serum levels						
ALT, IU/l	28.6 (0.55)	25	13–102	25	9.9–70.0	0.1773
AST, IU/l	30.1 (0.77)	24	12–104	24	10.9–81.3	0.3256
GGT, IU/l	57.5 (0.90)	33	13–171	25	10–426	0.1911
Total bilirubin, mg/dl	0.64	0.60	0.20–1.50	0.52	0.20–2.20	0.4106
Indirect bilirubin, mg/dl	0.42	0.30	0.1–1.0	0.30	0.10–1.70	0.7622
Gene expression						
<i>IFNG</i> , ng/μl	2.56×10 ⁻¹	13.0	9.6×10 ⁻¹⁰ –6.9×10 ⁶	3.0	1.5×10 ⁻⁸ –1.2×10 ⁷	0.8400
<i>IFNA1</i> , ng/μl	4.54×10 ⁵	6.7×10 ⁻⁷	2.8×10 ⁻¹² –5.4	1.7×10 ⁻⁷	1.7×10 ⁻¹⁰ –11.4	0.8730
<i>IFNAR1</i> , ng/μl	6.92×10 ⁹	11.7	0–1.1×10 ¹¹	168.0	3.5×10 ⁻⁸ –1.2×10 ⁷	0.1784

The Mann-Whitney test was performed. Normalized values for ALT, AST and GGT are shown in parentheses.

melting conditions were the same for SNP genotyping and viral load, with melting temperatures of 62°C for *IFNA1*, 68°C for *IFNG* and 66°C for *IFNAR1*.

Absolute expression (AE) was quantified using *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) and *β-actin* (table 1) as constitutive (housekeeping) genes, in which: AE = (reporter gene)/(*GAPDH* × *β-actin*) [25].

Gene expression levels were analyzed only for the chronic HBV-infected group and they were stratified into low (values below the median expression level) and high expression levels (values equal to or above the median expression level) for each gene analyzed. The specificity of expression primers was confirmed by the melting curve for each gene.

Ethics, Consent and Permissions

This study has been approved by the Center for Health Sciences of the Federal University of Pernambuco Ethics Committee (reference No. 96.288). All subjects gave informed consent to participate in the study.

Statistical Analysis

Allele and genotype frequencies among the groups were tested by the Hardy-Weinberg equilibrium compared to observed and expected genotype values.

The χ^2 test was performed to identify associations between age, sex, viral load, gene expression, and allelic and genotypic frequencies. Odds ratios (OR) with 95% confidence intervals (CI) were calculated. The Mann-Whitney test and the Kruskal-Wallis non-parametric test were applied to compare genotype frequencies with gene expression levels and viral load.

Nonlinear associations between the variables (gene expression and viral load) and the degree of association (significant p value) were verified using the determination coefficient (r^2) based on Spearman's rank correlation coefficient (ρ). p values <0.05 were considered to indicate statistical significance throughout the study using BioEstat 5.0 and GraphPad Prism 5.0 software.

Results

Subject Characterization and Laboratory Parameters

The clinical and laboratory characteristics were compared between HBV-infected subjects and controls, and no significant difference ($p > 0.05$) with respect to gender (M/F = 64:30 and 84:30, respectively) was found. However, there was a significant difference in age between the control group and the HBV-infected group ($p = 0.01$). The mean age was 44.54 (± 13.52) years for HBV-infected subjects and 37.74 (± 11.40) years for the control group.

In relation to genetic markers, median expression levels were lower for *IFNA1* (2.56×10^{-1} ng/μl) than *IFNG* (4.54×10^5 ng/μl) and *IFNAR1* genes (6.92×10^9 ng/μl).

There was no significant difference ($p > 0.05$) with respect to viral load between ALT, AST and GGT enzymes, cytokine expression levels, and total and indirect bilirubin (table 2).

The frequencies fit the criteria set out by Manly [26] in 2005. The genotype distribution in our study did not significantly differ from the distribution expected according to the Hardy-Weinberg equilibrium for the three genes analyzed ($p > 0.05$). This suggests that these populations had a confirmed genetic background and were suitable for genetic statistical analysis.

Genotype and Allele Distribution of *IFNG* (–5A→G), *IFNA1* (–2C→T) and *IFNAR1* (–97T→C) in Chronic HBV-Infected Patients and Immune Controls

The melting temperatures were 88.4–89.4°C for *IFNA1* genotypes, 88.4–89.7°C for *IFNG* and 75.9–76.9°C for

Fig. 1. Real-time qPCR and HRM genotyping of *IFNG* (-5A→G), *IFNA1* (-2C→T) and *IFNAR1* (-97T→C) carried out on Rotor-Gene (Qiagen). Differences between the three genotypes in normalized plots (**a**, **c**, **e**) and melting curve plots (**b**, **d**, **f**).

Fig. 2. *IFNA1* (–2C→T), *IFNAR1* (–97T→C) and *IFNG* (–5A→G) gene expression according to the genotypes of chronic HBV-infected patients.

Fig. 3. *IFNAR1* gene expression according to *IFNA1* (CC, CT and TT) and *IFNAR1* (TT, TC and CC) genotype combination in patients with chronic HBV infection.

IFNAR1. The accepted level of confidence for genotyping accuracy was >99% (fig. 1).

The *IFNA1* (–2) CT heterozygous genotype showed an association with protection from HBV chronicity compared to the wild-type *IFNA1* (–2) CC genotype (OR =

Table 3. Genotypic and allelic frequencies of *IFNA1* (–5A→G), *IFNG* (–2C→T) and *IFNAR1* (–97T→C) in 94 chronic HBV-infected patients and 114 immune controls

Genotypic and allelic frequencies of polymorphisms	Chronic HBV infection, n (%)	Immune controls, n (%)	OR	95% CI	p value
<i>IFNG</i>					
AA	42 (44.7)	56 (49.1)	1	–	
AG	40 (42.5)	48 (42.1)	1.11	0.62–1.98	0.8350
GG	12 (12.8)	10 (8.8)	1.60	0.63–4.05	0.5995
A	124 (66.0)	160 (70.2)	1	–	
G	64 (34.0)	68 (29.8)	1.21	0.80–1.84	0.4156
<i>IFNA1</i>					
CC	53 (56.4)	41 (36.0)	1	–	
CT	35 (37.2)	60 (52.6)	0.45	0.25–0.81	0.0109
TT	6 (6.4)	13 (11.4)	0.36	0.13–1.02	0.0850
C	141 (75.0)	142 (62.3)	1	–	
T	47 (24.0)	86 (37.7)	0.55	0.36–0.84	0.0077
<i>IFNAR1</i>					
TT	44 (46.8)	53 (46.5)	1	–	
TC	45 (47.9)	43 (37.7)	1.26	0.71–2.25	0.5236
CC	5 (5.3)	18 (15.8)	0.34	0.12–0.98	0.0663
T	133 (70.7)	149 (65.4)	1	–	
C	55 (29.3)	79 (34.6)	0.78	0.52–1.18	0.2863

0.45; 95% CI = 0.25–0.81). Regarding allele frequencies, the *IFNA1* (–2) T polymorphism was also associated with an increased protection against HBV chronicity (OR = 0.55; 95% CI = 0.36–0.84) compared with the *IFNA1* (–2) C allele ($p = 0.0077$). However, *IFNA1* (–2) CC/TT polymorphisms did not significantly differ between the groups (table 3).

The *IFNAR1* (–97) and the *IFNG* (–5) gene polymorphism did not reach statistical significance, neither in genotype nor in allele distribution, between any of the groups analyzed (table 3).

Genotype and Allele Distribution according to Viral Load and Gene Expression of Cytokines

Patients with *IFNAR1* (–97) TC genotype showed higher expression levels (mean = 1.38×10^{10} ng/μl, $p = 0.0166$) compared to *IFNAR1* (–97) TT and CC genotypes. Additionally, patients with *IFNA1* (–2) CC genotype showed higher expression levels (mean = 3.84×10^{-1} ng/μl) compared to *IFNA1* (–2) CT and TT genotypes, although they did not significantly differ ($p = 0.7488$). There was no statistical significance between *IFNG* (–5) genotypes and expression levels (fig. 2).

Table 4. Genotype and allele distribution of *IFNA1* (–5A→G), *IFNG* (–2C→T) and *IFNAR1* (–97T→C) according to viral load and gene expression of the 94 chronic HBV-infected patients

Genotypic and allelic frequencies of polymorphisms	Viral load levels, log ₁₀		Gene expression levels, ng/μl		P value ¹
	<3.3 log ₁₀ (n = 37)	≥3.3 log ₁₀ (n = 57)	low (n = 47)	high (n = 47)	
<i>IFNG</i>					
AA	17 (45.95)	25 (43.86)	20 (42.55)	22 (46.81)	0.66
AG	16 (43.24)	24 (42.11)	22 (46.81)	18 (38.29)	0.26
GG	4 (10.81)	8 (14.03)	5 (10.64)	7 (14.90)	0.50
A	50 (67.57)	74 (64.91)	62 (65.96)	62 (65.96)	0.16
G	24 (32.43)	40 (35.09)	32 (34.04)	32 (34.04)	0.03
<i>IFNA1</i>					
CC	19 (51.35)	34 (64.82)	29 (61.70)	25 (53.19)	0.09
CT	17 (45.95)	18 (31.48)	15 (31.92)	19 (40.43)	0.89
TT	1 (2.70)	5 (3.70)	3 (6.38)	3 (6.38)	0.54
C	55 (74.32)	86 (80.56)	73 (77.66)	69 (73.40)	0.04
T	19 (25.68)	28 (19.44)	21 (22.34)	25 (25.60)	0.49
<i>IFNAR1</i>					
TT	16 (43.24)	28 (59.58)	28 (59.57)	16 (34.04)	0.02
TC	18 (48.65)	27 (57.45)	16 (36.17)	29 (59.58)	0.83
CC	3 (8.11)	2 (4.26)	2 (4.26)	3 (6.38)	0.53
T	50 (67.57)	85 (7.28)	72 (77.66)	61 (63.83)	0.01
C	24 (32.43)	31 (26.72)	22 (22.34)	33 (36.17)	0.70

Low gene expression levels are values below the median expression levels. The *IFNG* median corresponds to 3.70 ng/μl, *IFNA1* equals 4.83×10^{-7} ng/μl and *IFNAR1* corresponds to 5.02×10^1 ng/μl. High gene expression levels are values greater than the median of all samples according to the expression levels. ¹ p value for χ^2 regarding viral load and gene expression according to genotypes and alleles.

Table 4 shows a comparison of genotype and allele frequencies with viral load levels. The G allele of the *IFNG* (–5) polymorphism showed higher frequency ($p = 0.03$) with high viral load quantification, independent of the *IFNG* expression levels in these subjects. However, *IFNG* (–5) AA/GG genotypes and A alleles did not differ significantly between any of the groups analyzed.

The C allele of the *IFNA1* (–2) gene polymorphism showed higher frequency ($p = 0.04$) with high viral load and low *IFNA1* expression. However, no statistically significant association with the *IFNA1* (–2) T allele was observed (table 4).

A genotypic and allelic association was observed between high viral load and low *IFNAR1* (–97) levels in subjects expressing the TT genotype gene ($p = 0.02$) and wild-type T allele ($p = 0.01$). The *IFNAR1* (–97) TC/CC genotypes and the *IFNAR1* C allele did not reach statistical significance (table 4).

When analyzing *IFNAR1* expression levels with respect to *IFNAR1* (–97) and *IFNA1* (–2) genotypes, subjects with *IFNA1* (A/G) and *IFNAR1* (T/C) heterozygous

genotype had higher transcript expression of *IFNAR1* compared to individuals with a polymorphic allele in one of the genes [*IFNA1* CT and *IFNAR1* TT ($n = 13$) vs. *IFNA1* CT and *IFNAR1* TC ($n = 18$): $p = 0.0124$; *IFNA1* CT and *IFNAR1* TC ($n = 18$) vs. *IFNA1* TT and *IFNAR1* TC ($n = 13$): $p = 0.0124$; fig. 3]. None of the patients had both *IFNA1* TT and *IFNAR1* CC genotypes.

Correlation between Gene Expression and Viral Load

Spearman's rank correlation was applied between gene expression and viral load in patients with chronic HBV infection. The p value obtained was >0.60 and the correlation was considered strong and substantial.

Regarding *IFNA1* polymorphism, subjects with *IFNA1* (–2) CC and wild-type *IFNG* (–5) AA genotype presented a Spearman's rank correlation coefficient of -0.60 , indicating a negative correlation among the genotypes analyzed ($p = 0.043$). Thus, increased expression of *IFNA1* in *IFNA1* (–2) CC genotype subjects explains 36% of the decrease in *IFNG* gene expression in subjects with *IFNG* (–5) wild-type AA genotype.

Subjects with *IFNAR1* (-97) TC and *IFNG* (-5) AG heterozygous genotypes presented a positive statistically significant correlation ($p = 0.025$; $p = 0.62$). Thus, increased expression of *IFNAR1* in subjects with TC heterozygous genotype is associated in 38% with *IFNG* (-5) GA genotype lower expression. Likewise, a high viral load in patients with wild-type *IFNAR1* (-97) TT genotype may also to be associated in 40% ($p = 0.63$) with low expression of this gene ($p = 0.040$).

Regarding the *IFNG* gene, low gene expression levels in wild-type *IFNG* (-5) AA genotype patients explain in 60% ($p = 0.77$) the viral load elevation detected in these patients ($p = 0.005$).

Discussion

The outcome of HBV infection and the severity of HBV-induced liver disease vary widely among people. Approximately 95% of HBV-infected adults have acute infection, which is rapidly resolved without long-term consequences, whereas 5% fail to control viral infection and progress to chronicity [27]. Some patients with chronic HBV infection are at risk of developing complications such as cirrhosis, liver failure and hepatocellular carcinoma [28]. Persistent HBV infection is considered a multifactorial disorder, and various factors such as the genetic background of the host, and viral and environmental factors are involved in its pathogenesis [2].

Some cytokines play a crucial role in regulating HBV immune and inflammatory responses in the early stages of HBV exposure and also influence chronic HBV infection pathogenesis in the long term [14]. SNPs are well known to affect disease progression following HBV infection. Cytokine production-inducing cell-mediated antiviral immune responses play a crucial role in the control of viral infection. Genetic variants of cytokines involving IFN- γ and IL-10 genes have been associated with the outcome of HBV infection [8–10]. Therefore, regulatory regions of SNPs and cytokine-related gene expression are important genetic predictors of disease susceptibility or clinical outcome of chronic HBV infection.

In some genes, polymorphic variants influence serum levels of cytokines and clinical outcome of HBV, which can also be influenced by the age during infection and immune responses [9]. Chronic HBV individuals are assumed to have a Th2 response which predominates over Th1, which results in increases in anti-inflammatory cytokine production such as IL-10 and IFN- α [29]. As polymorphisms affect the expression of cytokines that initiate

and regulate the immune system, response to viral infections may probably be reduced.

In 2005, Uno et al. [30] showed individual variations in IFN- α production levels and found that the capacity of IFN- α production is determined by both the genetic background and physical conditions, such as the presence/absence of infection, metabolic disorders and cancer. These differences in expression could be based on polymorphic changes in *IFNA* gene promoter regions, which influence the course of viral infection.

In a study by Wu et al. [31] in 2013, intrahepatic expression of IFN- α was higher in HBV-infected patients than in the normal group, and significantly higher in severe and chronic hepatitis. Thus, intrahepatic IFN- α expression might correlate with liver inflammation after HBV infection and play an important role in the progression to severe hepatitis B.

In our study, we identified that the *IFNA1* (-2) CT genotype is associated with HBV clearance, which may lead to a better protection against HBV infection. Thus, the presence of the *IFNA1* (-2) T allele can also be associated with HBV clearance. When data were stratified by viral load and *IFNG*, *IFNA1* and *IFNAR1* expression levels, it was confirmed that the *IFNA1* (-2) C polymorphic allele is a risk factor for chronicity.

Therefore, in chronic HBV-infected subjects presenting the wild-type *IFNAR1* (-97) TT genotype, this gene expression (mean $8.24 \pm 3.0 \times 10^9$ ng/ μ l) explains in 40% the lower viral load in this group ($p = 0.63$; $p = 0.04$). It might suggest a better outcome of chronic HBV infection regarding the *IFNAR1* expression levels and the *IFNAR1* (-97) CC polymorphic genotype (OR = 0.34).

In subjects with the *IFNAR1* (-97) TC and *IFNG* (-5) AG polymorphic genotypes, a significant positive correlation was observed ($p = 0.62$; $p = 0.03$). Increased expression of both *IFNAR1* and *IFNG* suggests that high expression of the *IFNAR1* (-97) gene (median $409 \pm 4.5 \times 10^{10}$ ng/ μ l) is associated with chronicity of HBV infection. Similarly, increased expression of the *IFNAR1* TC heterozygous genotype is assigned in 62% with low *IFNG* (-5) expression in GA genotype subjects. In a study by Li et al. [32] in 2014, IFN- γ and TNF- α expression were remarkably downregulated in natural killer cells from chronic HBV subjects in immune-activated stage compared to those in the immune-tolerant stage and healthy individuals.

However, studies correlating the position 97 *IFNAR1* (-97) polymorphism in the promoter region and susceptibility to HBV infection are lacking. Nevertheless, when the same region was analyzed regarding progression and

susceptibility to HIV-I in a French cohort study [19] and the IFN- α response in chronic myeloid leukemia cases [20], no significant differences were identified. The present study, though, confirms the allelic distribution identified by Diop et al. [20] in 2006 in both case and control groups.

Regarding the *IFNG* (-5) gene polymorphism, the G polymorphic allele showed a higher frequency ($p = 0.03$) in patients with high viral load considering the *IFNG* (-5) expression levels analyzed. It was also found that *IFNG* (-5) expression levels (median $3.7 \pm 1.7 \times 10^6$ ng/ μ l) in *IFNG* (-5) AA genotype patients are considered a protective factor of chronic HBV infection, since they explain 60% of the viral load reduction.

According to Bream et al. [33], the regulation of *IFNG* expression is stringently controlled at transcriptional level, and this polymorphism seems to modify *IFNG* gene expression and the intracellular pathogen association. Consequently, this gene expression may play an important role in immune responses and inflammatory diseases, including chronic active hepatitis [34].

IFNG gene regulation is complex and several transcription factors have been shown to interact with the proximal promoter region either constitutively or following induction. Aberrant *IFNG* expression in transgenic mice resulted in pronounced B cell lineage reduction, T cells and myeloid progenitors in the bone marrow, whereas the interruption of *IFNG* production led to susceptibility to intracellular pathogens [35].

The IFN gene polymorphisms analyzed in this study were not associated with viral load or ALT, AST or GGT serum levels. In effect, in 2013 Kimkong et al. [16] also did not find any association between *IFNA1* (-2529) and *IFNA5* (-1823) gene polymorphisms and HBV-DNA and serum levels ALT and AST.

Reports on these three polymorphisms in the promoter gene in association with HBV infection have not been published previously. In Brazil, there are only three studies evaluating cytokine gene polymorphisms in HBV infection [9, 36]. One of them was performed in Recife [37] and determined the association between class II HLA alleles from HBV-infected blood samples; in this study, HBV infection was associated with HLA-DRB1*09 and DRB1*08 alleles, respectively [37].

Ribeiro et al. [36] analyzed cytokine genes polymorphisms in TNF- α (-308 G/A), TGF β 1 (+869 T/C, +915 C/G), IL-10 (-1082 A/G, -819 T/C, -592 A/C), IL-6 (-174 C/G) and *IFNG* (+874 T/A) and found no differences between chronic HBV individuals and self-limited infection. Conde et al. [9] also analyzed TNF- α 308 G/A, IFN- γ

+874 T/A, TGF- β 1 509 C/T and IL-10 1082 A/G gene polymorphisms in chronic HBV-infected patients in order to find specific immunogenetic markers. They detected a 2.6-fold higher risk of developing chronic disease in individuals with the TNF- α (-308) A allele polymorphism suggesting the risk of disease progression.

Therefore, this is the first study in a Brazilian population concerning the *IFNG* (A $\rightarrow$ G, rs202055606), *IFNA1* (C $\rightarrow$ T, rs181407537) and *IFNAR1* (T $\rightarrow$ C, rs2850015) polymorphisms in chronic HBV-infected individuals and *IFNG*, *IFNA1* and *IFNAR1* gene expression. These SNP identifications play an important role in the prognosis and treatment of chronic HBV infection; they help to elucidate the host's immune response profile and predict the course of chronic HBV, and therefore help to choose the appropriate therapy.

Conclusions

Our findings revealed that the presence of the *IFNA1* (-2) polymorphic variant gene and high gene expression of *IFNAR1* and low *IFNA1* levels are associated with the pathogenesis of chronic HBV infection. However, the individuals who presented *IFNA1* (-2) CT and T allele polymorphic genotypes were likely to show increased HBV chronicity protection.

The HBV group stratification according to the SNP identification, cytokine expression pattern and viral load can play a major role in the prediction of the course of chronic HBV infection.

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References

- 1 Lavanchy D: Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004;11:97–107.
- 2 Fattovich G: Natural history and prognosis of hepatitis B. *Semin Liver Dis* 2003;23:47–58.
- 3 Ganem D, Prince AM: Hepatitis B virus infection – natural history and clinical consequences. *N Engl J Med* 2004;350:1118–1129.

- 4 Lok ASF, McMahon BJ: Chronic hepatitis B. *Hepatology* 2007;45:507–539.
- 5 Hou J, Liu Z, Gu F: Epidemiology and prevention of hepatitis B virus infection. *Int J Med Sci* 2005;2:50–57.
- 6 Pereira L, Martelli C, Merchán-Hamann E, Montarroyos U, Braga M, de Lima M, et al: Population-based multicentric survey of hepatitis B infection and risk factor differences among three regions in Brazil. *Am J Trop Med Hyg* 2009;81:240–247.
- 7 Yim HJ, Lok AS-F: Natural history of chronic hepatitis B virus infection: what we knew in 1981 and what we know in 2005. *Hepatology* 2006;43:S173–S181.
- 8 Wang S, Huang D, Sun S, Ma W, Zhen Q: Interleukin-10 promoter polymorphism predicts initial response of chronic hepatitis B to interferon alfa. *Viral J* 2011;8:28.
- 9 Conde SRS, Feitosa RNM, Freitas FB, Hermes RB, Demachki S, Araújo MTF, et al: Association of cytokine gene polymorphisms and serum concentrations with the outcome of chronic hepatitis B. *Cytokine* 2013;61:940–944.
- 10 Komatsu H, Murakami J, Inui A, Tsunoda T, Sogo T, Fujisawa T: Association between single-nucleotide polymorphisms and early spontaneous hepatitis B virus e antigen seroconversion in children. *BMC Res Notes* 2014;7:1–9.
- 11 Almajhdi FN, Al-ahdal M, Abdo AA, Sanai FM, Al-anazi M: Single nucleotide polymorphisms in CXCR1 gene and its association with hepatitis B infected patients in Saudi Arabia. *Ann Hepatol* 2013;12:220–227.
- 12 Chang S-W, Fann CS-J, Su W-H, Wang YC, Weng CC, Yu C-J, et al: A genome-wide association study on chronic HBV infection and its clinical progression in male Han-Taiwanese. *PLoS One* 2014;9:1–10.
- 13 He X-X, Chang Y, Jiang H-J, Tang F, Meng F-Y, Xie Q-H, et al: Persistent effect of IFNAR-1 genetic polymorphism on the long-term pathogenesis of chronic HBV infection. *Viral Immunol* 2010;23:251–257.
- 14 Gao Q-J, Liu D-W, Zhang S-Y, Li-Min W, Wu L-H, Wang S-Y, et al: Polymorphisms of some cytokines and chronic hepatitis B and C virus infection. *World J Gastroenterol* 2009;15:5610–5619.
- 15 Kim SS, Cheong JY, Lee D, Lee SK, Kim M-H, Kwack K, et al: Interleukin-1 β and interleukin-1 receptor accessory protein gene polymorphisms are associated with persistent hepatitis B virus infection. *Hepatogastroenterology* 2012;59:190–197.
- 16 Kimkong I, Tangkijvanich P, Hirankarn N: Association of interferon-alpha gene polymorphisms with chronic hepatitis B virus infection. *Int J Immunogenet* 2013;40:476–481.
- 17 Zhou J, Smith DK, Lu L, Poon VKM, Ng F, Chen D-Q, et al: A non-synonymous single nucleotide polymorphism in IFNAR1 affects susceptibility to chronic hepatitis B virus infection. *J Viral Hepat* 2009;16:45–52.
- 18 Song LH, Toan NL, Xuan NT, Uhlemann A-C, Boldt ABW, Duy DN, et al: A promoter polymorphism in the interferon alpha-2 gene is associated with the clinical presentation of hepatitis B. *Mutat Res* 2006;601:137–143.
- 19 Diop G, Hirtzig T, Do H, Coulonges C, Vasilescu A, Labib T, et al: Exhaustive genotyping of the interferon alpha receptor 1 (IFNAR1) gene and association of an IFNAR1 protein variant with AIDS progression or susceptibility to HIV-1 infection in a French AIDS cohort. *Biomed Pharmacother* 2006;60:569–577.
- 20 Kreil S, Waghorn K, Ernst T, Chase A, White H, Hehlmann R, et al: A polymorphism associated with STAT3 expression and response of chronic myeloid leukemia to interferon α . *Haematologica* 2010;95:148–152.
- 21 Sambrook J, Russell DW: Molecular cloning: a laboratory manual; in: Protocol 1 – Isolation of High-Molecular-Weight DNA from Mammalian Cells Using Proteinase K and Phenol, ed 3. New York, Cold Spring Harbor Laboratory Press, 2001, pp 6.4–6.11.
- 22 Jiang E, Li F, Jing C, Li P, Cui H, Wang B, et al: High-resolution melting analysis as a developed method for genotyping the PD susceptibility loci in LRRK2 gene. *J Clin Lab Anal* 2015;29:299–304.
- 23 Vali Z, Raz A, Bokharai H, Nabavi M: Development of a high-resolution melting analysis method based on SYBR Green-I for rs7216389 locus genotyping in asthmatic child patients. *Avicenna J Med Biotechnol* 2014;6:72–80.
- 24 European Association for the Study of the Liver: EASL Clinical Practice Guidelines: management of chronic hepatitis B virus infection. *J Hepatol* 2012;57:167–185.
- 25 Leong D, Gupta A, Bai H, Wan G, Yoong L, Too H, et al: Absolute quantification of gene expression in biomaterials research using real-time PCR. *Biomaterials* 2007;28:203–210.
- 26 Manly KF: Reliability of statistical associations between genes and disease. *Immunogenetics* 2005;57:549–558.
- 27 Ait-Goughoulte M, Lucifora J, Zoulim F, Durantal D: Innate antiviral immune responses to hepatitis B virus. *Viruses* 2010;2:1394–1410.
- 28 Pan CQ, Zhang JX: Natural history and clinical consequences of hepatitis B virus infection. *Int J Med Sci* 2005;2:36–40.
- 29 Kondo Y, Kobayashi K, Ueno Y, Shiina M, Niitsuma H, Kanno N, et al: Mechanism of T cell hyporesponsiveness to HBcAg is associated with regulatory T cells in chronic hepatitis B. *World J Gastroenterol* 2006;12:4310–4317.
- 30 Uno K, Suginoishi Y, Kakimi K, Moriyasu F, Hirotsaki M, Shirakawa T, et al: Impairment of IFN-alpha production capacity in patients with hepatitis C virus and the risk of the development of hepatocellular carcinoma. *World J Gastroenterol* 2005;11:7330–7334.
- 31 Wu D, Liu F, Li J, Liu C, Liu L, Tang H, et al: Intrahepatic IFN-alpha expression in liver specimens from HBV-infected patients with different outcomes. *Eur Rev Med Pharmacol Sci* 2013;17:2474–2480.
- 32 Li Y, Wang J-J, Gao S, Liu Q, Bai J, Zhao X-Q, et al: Decreased peripheral natural killer cells activity in the immune activated stage of chronic hepatitis B. *PLoS One* 2014;9:1–8.
- 33 Bream J, Ping A, Zhang X, Winkler C, Young H: A single nucleotide polymorphism in the proximal IFN-gamma promoter alters control of gene transcription. *Genes Immun* 2002;3:165–169.
- 34 Toyonaga T, Hino O, Sugai S, Wakasugi S, Abe K, Schichiri M, et al: Chronic active hepatitis in transgenic mice expressing interferon- γ in the liver. *Proc Natl Acad Sci USA* 1994;91:614–618.
- 35 Young H, Klinman D, Reynolds D, Grzegorzewski K, Nii A, Ward J, et al: Bone marrow and thymus expression of interferon-gamma results in severe B-cell lineage reduction, T-cell lineage alterations, and hematopoietic progenitor deficiencies. *Blood* 1997;89:583–595.
- 36 Ribeiro CSS, Visentainer JEL, Moliterno RA: Association of cytokine genetic polymorphism with hepatitis B infection evolution in adult patients. *Mem Inst Oswaldo Cruz* 2007;102:435–440.
- 37 Corrêa Bde M, Lopes EP, Albuquerque Mde F, Dourado L: Association between HLA-DRB1* polymorphisms and hepatitis B infection in a Brazilian population. *Rev Assoc Med Bras* 2012;58:537–542.