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The interferon receptor-1 promoter polymorphisms affect the outcome of Caucasians with HBeAg-negative chronic HBV infection

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Keywords

hepatitis virus B – interferon receptor – polymorphisms – promoter

Abbreviations

CHB, Chronic Hepatitis B; HBeAg, Hepatitis B “e” Antigen; HBV, Hepatitis B Virus; HCC, Hepatocellular Carcinoma; IC, Inactive chronic HBV Carrier; IFN- α , Interferon- α ; IFNAR1/2, Interferon- α Receptor type 1/2; PBMC, peripheral blood mononuclear cell; SNP, single nucleotide polymorphism; VNTR, variable number tandem repeat.

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Abstract

Background & Aims: The outcome of HBeAg-negative chronic hepatitis B virus (HBV) patients who may remain in the inactive carrier state (IC) or progress to HBeAg-negative chronic hepatitis B may be affected by the host genetic profile. Genetic polymorphisms within not only the promoter but also the coding sequence of the interferon receptor 1 (IFNAR1) gene have been associated with susceptibility to chronic HBV infection, but their role on the outcomes of HBeAg-negative patients has not been evaluated. We examined the association of IFNAR1 promoter polymorphisms with the phase of chronic HBV infection in a demographically characterized Caucasian cohort of 183 consecutive HBeAg-negative chronic HBV patients. **Methods:** Using a combination of conventional and allele-specific polymerase chain reactions, bidirectional sequencing and DNA-fragment analysis, we performed typing of three Single Nucleotide Polymorphisms (SNPs -568G/C, -408C/T, -3C/T) and one Variable Number Tandem Repeat [VNTR -77(GT)n] within the IFNAR1 promoter sequence. **Results:** The genetic polymorphisms examined were found to be associated with the phase of HBeAg-negative chronic HBV patients. Using a multiple logistic regression model adjusting for age, gender and origin of the individuals, we found that patients with linked genotypes -408CT/-3CT were more likely to be ICs (OR = 2.42 vs. CC, $P = 0.036$). Also, given the partial linkage between SNP -568G/C and VNTR -77(GT)n, we found that linked genotypes -77(GT)n $\leq 8/\leq 8$ -568GC and -77(GT)n $\leq 8/\leq 8$ -568CC were detected more frequently among ICs (OR = 11.69, $P = 0.005$ and OR = 7.56, $P = 0.001$ vs. -77(GT)n $> 8/> 8$ -568GG respectively). **Conclusions:** These findings suggest that these genetic variations represent important factors associated with the clinical phase of HBeAg-negative chronic HBV infection.

The clinical course of hepatitis B virus (HBV) infection is highly variable, ranging from an asymptomatic carrier state to the development of cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC) (1, 2). In particular, Hepatitis B “e” Antigen (HBeAg)-negative patients, who represent the majority of chronic HBV cases worldwide, may be inactive chronic HBV carriers (IC) or present with the progressive HBeAg-negative chronic hepatitis B (CHB) (1–4).

The genetic profile of the host may play a pivotal role in the variability in the clinical course of CHB (5–7). In

particular, cytokines, like interferon (IFN) – α/β , represent one of the frontline defences against various viral infections, for both innate and adaptive immune responses (8, 9). Moreover, exogenous administration of IFN- α is one of the first line therapies for CHB in all guidelines (2, 10, 11). Type-I interferon receptor (IFNAR), composed by two subunits (IFNAR-1 and IFNAR-2), is involved in the effective binding and the downstream signalling of type-I IFNs (12–14). Both IFN-receptor subunits are required for IFN activity; however, IFNAR1 is essential for IFN induced signalling,

Key Points box

- INFAR1 promoter polymorphisms are associated with the phase of chronic HBV infection in a Caucasian cohort of HBeAg-negative chronic HBV patients, independently from their demographic and clinical characteristics.
- Polymorphisms -77(GT)_n and -568G/C are partially linked, while polymorphisms -408C/T and -3C/T are ultimately linked.
- Patients with linked genotypes -408CT_-3CT, 77(GT)_n ≤ 8/≤8_-568GC and -77(GT)_n ≤ 8/≤8_-568CC were detected more frequently among inactive carriers.
- Development of a sensitive and affordable method for the genotyping of the technically challenging locus of the microsatellite repeats of the VNTR -77(GT)_n.

and IFNAR2 is more involved in IFN binding (15). The promoter region, but also the coding sequence of the IFNAR-1 gene, seems to be the target of several mutations that can potentially affect the gene expression, the efficiency of the receptor and, therefore, the clinical outcome of HBV infection, as in previous studies (16–19). However, the majority of these studies have been carried out in non-Caucasian cohorts, focusing on the progression to chronicity during acute HBV infection.

In this study, we have estimated the genetic variations of four previously described (17, 20, 21) polymorphic sites within the INFAR-1 promoter sequence, in a Greek-Albanian cohort of patients with HBeAg-negative chronic HBV infection. There were three single nucleotide polymorphisms (SNPs) and one variable number tandem repeat (VNTR) located at positions -568, -408, -3 and -77, respectively, according to a published sequence (22). Moreover, we have evaluated the significance of these genetic variations as well as the main demographic patient characteristics as markers for the discrimination of HBV ICs from patients with HBeAg-negative CHB.

Patients and methods

Patients

A cohort of 183 consecutive HBeAg-negative chronic HBV patients seen at the liver clinics of Hippokration General Hospital of Athens was included in this study. Of them, 92 (50.3%) were HBV ICs and 91 (49.7%) were patients with HBeAg-negative CHB. Inactive chronic HBV carrier state was considered to be present in patients with persistently normal ALT values under strict follow-up (at least every 3 months) for at least 1 year and maximum HBV DNA levels <20 000 IU/ml. As it has been previously reported, none of these inactive carriers had inactive cirrhosis (23). HBeAg-negative

CHB was considered to be present in patients with elevated ALT on at least two occasions and HBV DNA ≥2000 IU/ml or in patients with HBV DNA ≥20 000 IU/ml regardless of ALT activity (2, 4). None of the patients included in this study had received any type of antiviral therapy before the diagnosis of the phase of chronic HBV infection had been established. The main demographic and clinical characteristics of these patients are included in Table 1.

Blood samples derived from all patients were centrifuged and peripheral blood mononuclear cells (PBMCs) obtained were stored in liquid nitrogen prior to use. This study has been approved by the Ethics Committee of Hippokration Hospital of Athens and the Bioethics Committee of Medical School University of Athens and all the subjects signed a written informed consent.

Polymorphisms' screening

Genomic-DNA purification from isolated PBMCs was performed using the QIA Symphony DNA MidiKit (Qiagen GmbH, Hilden, DL). Amplification of regions corresponding to nucleotides -656 to -324 and -185 to +108 of INFAR-1 promoter/gene sequence was achieved by PCR using two sets of primers (Invitrogen Corp., Carlsbad, CA). We examined for single nucleotide polymorphisms (SNPs) at three different positions -568G/C, -408C/T and -3C/T and for the number of binucleotide repeats at -77(GT)_n within the INFAR-1 promoter (17, 20, 21). Detection of genetic variation at previous positions was carried out by a combination of DNA sequencing and a new method using high-resolution gel electrophoresis.

Specifically for the SNP typing at positions -568G/C and the -408C/T we performed a PCR with primers (INFAR-1_For: 5'-CCCGTGGCTGTTCTCTCCAAGG-GAC-3', INFAR-1_Rev: 5'-GCACACACCTGCGCA GACCCAGC-3') amplifying a 333 bp sequence that contained the -568G/C SNP and the -408C/T SNP. Similarly, we used a second set of primers (INFAR-2_For: 5'-GCGCACAGGGGTGCTGCAATTAGGA-3', INFAR-2_Rev: 5'-AGGACGACCATCATCTGGGAGCCGC-3') amplifying a 293 bp sequence that contained the -77(GT)_n binucleotide repeat and the -3C/T SNP (Fig. 1A, B). Numbering of nucleotides is based on sequence X60459.1 (22), which includes 9 (GT) repeats at -77 position. PCR reactions were performed with the high-fidelity pfX-50 Taq polymerase (Invitrogen Corp., Carlsbad, CA). The thermal profile of the reactions involved optimization procedures, and determined as follows: initial denaturation at 94°C for 2 min; 35 cycles with denaturation at 94°C for 15 s, annealing at 65°C for 5 s and polymerization at 68°C for 15 s, followed by a final polymerization step at 68°C for 5 min. Purification of PCR products with PureLink™ Kit (Invitrogen Corp., Carlsbad, CA) was followed by bidirectional sequencing using BigDye Terminator Sequencing Kit (version 3.1) and the Avant 3100 Sequence Analyzer, while chromato-

Table 1. Demographic and clinical characteristics and type of HBV infection

		By type of HBV infection			Crude OR of being IC (95% CI)
	Total (N = 183)	IC* (N = 92)	CHBeAg(-)† (N = 91)	P value	
Demographic characteristics					
Gender, n (%)				0.021	
Male	122 (66.6)	54 (58.7)	68 (74.3)		1.00
Female	61 (33.3)	38 (41.3)	23 (25.3)		2.08 (1.11, 3.90)
Age					(per 10 year increase)
Mean, years (SD)	46.9 (15.9)	44.2 (14.4)	49.6 (16.9)	0.021	0.80 (0.67, 0.97)
Place of birth, n (%)‡				0.004	
Greece	111 (63.4)	45 (51.7)	66 (75.0)		1.00
Albania	53 (30.3)	36 (41.4)	17 (19.3)		3.11 (1.56, 6.19)
Other	11 (6.3)	6 (6.9)	5 (5.7)		1.76 (0.51, 6.12)
Clinical characteristics					
ALT before treatment				<0.001	
Abnormal	89 (48.6)	8 (8.7)	81 (89.0)		1.00
Normal	94 (51.4)	84 (91.3)	10 (11.0)		85.1 (32.0, 226.3)
ALT levels (IU/ml)					
Median (25th, 75th)	43.5 (22.4, 98.2)	23.2 (18.1, 33.0)	94.6 (62.0, 157.0)	<0.001	
HBV DNA serum levels (IU/ml)				<0.001	
>2000	104 (56.8)	17 (18.5)	87 (95.6)		1.00
≤2000	79 (43.2)	75 (81.5)	4 (4.4)		96.0 (30.9, 297.7)
HBV DNA (log10 IU/ml)					
Mean (SD)	4.00 (1.81)	2.73 (0.95)	5.50 (1.36)	<0.001	

Statistically significant associations in bold.

*Inactive carrier.

†Chronic Hepatitis B (HBeAg-).

‡Available for 175 of 183 subjects.

grams were evaluated using SeqScanner Software v1.0 (all by Applied Biosystems, Foster City, CA).

Linkage analysis of SNPs -408C/T and -3C/T

The linkage between SNPs -408C/T and -3C/T was examined with allele specific PCRs using previously described primers (For_408_C/T, Rev_3_C/T) (17) (Fig. 1A). These primers were SNP specific at their 3'-end and they were used in all combinations (For_408_T/Rev_3_C, For_408_T/Rev_3_T, For_408_C/Rev_3_T, For_408_C/Rev_3_C), only amplifying alleles with linked SNPs. PCR thermal conditions were optimized for Platinum Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA): initial denaturation at 94°C for 2 min; 30 cycles with denaturation at 94°C for 10 s, annealing at 69°C for 10 s and polymerization at 72°C for 29 s, followed by a final polymerization step at 68°C for 5 min.

Linkage analysis of SNP -568G/C and VNTR -77(GT)_n

The linkage between SNP -568G/C and the -77(GT)_n repeats were extensively examined with the development of one more allele specific PCR. The INFAR-1-For forward primer, which is located upstream of the 568G/C SNP and both Rev_3_C/T allele-specific reverse primers,

which are located downstream of the -77(GT)_n repeats locus, were used in both combinations (INFAR-1-For/Rev_3_C and INFAR-1-For/Rev_3_T) (Fig. 1A). Thermal conditions for AccuPrimeTaq DNA polymerase (Invitrogen Corp., Carlsbad, CA) were determined as follows: initial denaturation at 94°C for 2 min; 35 cycles with denaturation at 94°C for 15 s, annealing at 65°C for 5 s and polymerization at 68°C for 15 s, followed by a final polymerization step at 68°C for 5 min. Using this reaction in triple heterozygous samples (-568G/C, -77(GT)_{5/14}, -3C/T), the two alleles were separately amplified, giving products of different lengths corresponding to nucleotides -656 to +18 of the X60459.1 sequence. These allele specific PCR products had undergone bidirectional sequencing.

New method for typing number of -77(GT)_n binucleotide repeats

DNA sequencing of the amplicons spanning the -77(GT)_n repeats resulted to chromatographs with multiple peaks because of the high frequency of heterozygous genotypes with differential length of the two alleles. The high quality of sequencing reactions allowed us to estimate the number of binucleotide repeats of the different alleles. To confirm the sequencing results in -77(GT)_n - heterozygous individuals, a 109 bp region (nucleotides -

Fig. 1. (A) Schematic representation of the three SNPs, the VNTR, the primers used – pairs connected with dashed lines – and their relative position on the INFAR-1 promoter sequence. (B) (Upper): agarose-gel electrophoresis of PCR products from 12 random patients, encompassing -586 G/C and -408 C/T SNPs and (lower): PCR products from the same patients, encompassing -77VNTR and -3 C/T SNP. (Right): high-resolution agarose-gel electrophoresis of nested PCR products, from two homozygous (1,6) and four heterozygous (2–5) -77VNTR(GT)_n patients. The respective (GT)_n genotype per lane is: 1:5/5, 2:5/6, 3:5/9, 4:5/14, 5:9/14, 6:14/14. (M) stands for “marker”.

125 to -17, ref. X60459.1 (22)) was amplified in a nested PCR reaction, using the product of INFAR-2_FOR/REV primers described above as template and the primers: -77GT_FOR: 5'-AGGAGGAAAGGCGGTGCGTGG-3' and -77GT_REV: 5'-CGTAGAGGGGCGGTGAGAGC TAAGA-3' (Fig. 1A). These products were analysed by high-resolution agarose-gel electrophoresis using MetaPhor (Lonza, Rockland, ME) (4%, 80V, 1.5 h). To examine the resolution of the new method for detecting alleles of different lengths, we used homozygous samples as controls (-77(GT)_{5/5}, -77(GT)_{14/14}) (Fig. 1B).

Statistical analysis

The Chi-squared test, *t*-test and Wilcoxon rank-sum test were used to assess for the presence of association between the type of HBV infection and patients' demographic characteristics and genetic variants.

Multiple logistic regression analysis was used to identify variables independently associated with the type of HBV infection.

Results

Demographic and clinical characteristics and type of HBV infection

The demographic characteristics of the 183 chronic HBV patients were analysed to investigate potential

associations with the phase of chronic HBV infection (IC vs. HBeAg-negative CHB). ICs were significantly younger compared to patients with HBeAg-negative CHB (mean (SD) age: 44.2(14.4) vs. 49.6 (16.9) years, respectively, $P = 0.021$). In addition, females and patients of Albanian origin were more likely to be ICs compared with males (OR = 2.08, $P = 0.022$) and Greeks (OR = 3.11, $P = 0.001$) respectively. By definition, baseline serum levels of ALT were usually abnormal in HBeAg-negative CHB patients but not in ICs (89.0 vs. 8.7% respectively). Similarly, HBV DNA levels were higher in HBeAg-negative CHB patients as compared with ICs (mean (SD) log₁₀ levels: 5.50 (1.36) vs. 2.73 (0.95), $P < 0.001$). Of the ICs, 79 (85.9%) had HBV DNA <2000 IU/ml and 15 (16.3%) had HBV DNA between 2000 and 20 000 IU/ml. These data are summarized in Table 1.

Association of polymorphisms within INFAR-1 promoter and type of HBV infection

The alleles -408C/T and -3C/T were found to be ultimately linked (-408C to -3C and -408T to -3T), so they were analysed together. Univariable analysis revealed that individuals with heterozygous -408/-3 CT genotype were more likely to be ICs compared to those with -408/-3 CC genotype (OR = 2.47, $P = 0.005$) (Table 2). The same trend was not observed for the -408/-3 TT homozygous patients (OR = 1.64, $P = 0.272$), probably

Table 2. Genetic variations and type of HBV infection

		By type of HBV infection			
	Total (<i>N</i> = 183)	IC * (<i>N</i> = 92)	CHBeAg (-)† (<i>N</i> = 91)	<i>P</i> value	Crude OR of being IC (95% CI)
Genotypes/Alleles					
SNP -408/-3 C/T, <i>n</i> (%)					
CC	100 (54.6)	41 (44.6)	59 (64.8)	0.018	1.00
CT	68 (37.2)	43 (46.7)	25 (27.5)		2.47 (1.31, 4.67)
TT	15 (8.2)	8 (8.7)	7 (7.7)		1.64 (0.55, 4.89)
C	268 (73.2)	125 (67.9)	143 (78.6)	0.022	1.00
T	98 (26.8)	59 (32.1)	39 (21.4)		1.73 (1.08, 2.77)
SNP -568 G/C, <i>n</i> (%)					
GG	38 (20.8)	9 (9.8)	29 (31.9)	<0.001	1.00
CG	84 (45.9)	42 (45.6)	42 (46.2)		3.22 (1.36, 7.63)
CC	61 (33.3)	41 (44.6)	20 (22.0)		6.61 (2.63, 16.6)
G	160 (43.7)	60 (32.6)	100 (55.0)	<0.001	1.00
C	206 (56.3)	124 (67.4)	82 (45.0)		2.52 (1.65, 3.85)
VNTR -77GTn, <i>n</i> (%)					
>8/>8	32 (17.5)	7 (7.6)	25 (27.5)	<0.001	1.00
≤8/>8	79 (43.2)	37 (40.2)	42 (46.2)		3.14 (1.22, 8.11)
≤8/≤8	72 (39.3)	48 (52.2)	24 (26.4)		7.14 (2.70, 18.85)

Statistically significant associations in bold.

*Inactive carrier.

†Chronic Hepatitis B (HBeAg-).

because of the relatively small portion of this genotype in the population studied. Similarly, the -408/-3 T allele was detected more frequently among ICs (OR = 1.73, *P* = 0.022) vs. the -408/-3 C allele (Table 2).

Notably, both homozygous and heterozygous -568GC individuals were more likely to be ICs (OR = 6.61, *P* < 0.001 and OR = 3.22, *P* = 0.008 respectively) vs. those with -568GG genotype. The same trend was observed for the -568 C (vs. -568 G) allele (OR = 2.52, *P* < 0.001) (Table 2).

The number of GT repeats at the position -77 of INFAR-1 promoter sequence was also associated with the phase of chronic HBV infection. Homozygous carrying both alleles with ≤8 GT repeats, but also heterozygous ≤8/>8 GTn individuals, were more likely to be ICs, compared with those carrying both alleles with >8 GT-repeats (OR = 7.14, *P* < 0.001 and OR = 3.14, *P* < 0.001 respectively) (Table 2).

There was no significant difference in the polymorphisms prevalence between ICs with HBV DNA <2000 IU/ml and those with HBV DNA >2000 IU/ml (Table S1). The significant findings of this study in the prevalence of polymorphisms did not change when the 17 patients with normal ALT and HBV DNA of 2000–20 000 IU/ml were classified into the group of patients with HBeAg-negative CHB instead of ICs.

Multivariable analysis

Using a multiple logistic regression model, we examined the association between patients' genetic variations and the phase of chronic HBV infection adjusting for each

Table 3. Multiple logistic regression model: determinants of being inactive carrier

	OR of being IC (95% CI)	<i>P</i> value
Age (per 10 year increase)	0.98 (0.95, 1.01)	0.237
Gender		
Female vs. Male	3.56 (1.62, 7.80)	0.001
Place of birth		
Albania vs. Greece	3.27 (1.28, 8.35)	0.013
Other vs. Greece	2.55 (0.57, 11.44)	0.224
SNP -408/-3 C/T		
CT (vs. CC)	2.42 (1.06, 5.53)	0.036
TT (vs. CC)	0.36 (0.09, 1.49)	0.159
SNP -568 G/C		
CG (vs. GG)	3.21 (0.51, 20.13)	0.212
CC (vs. GG)	2.13 (0.25, 18.26)	0.488
VNTR -77GTn		
≤8/>8 (vs. >8/>8)	0.80 (0.10, 6.14)	0.831
≤8/≤8 (vs. >8/>8)	3.56 (0.37, 33.54)	0.267

Statistically significant associations in bold.

other as well as for patients' age, gender and place of birth. We found that heterozygous -408/-3 CT, but not homozygous -408/-3 TT patients were more likely to be ICs compared to those with -408/-3 CC genotype (-408/-3 CT vs. -408/-3 CC: OR = 2.42, *P* = 0.036 and -408/-3 TT vs. -408/-3 CC: OR = 0.36, **P* = 0.159) (Table 3).

Allele specific PCR and sequencing revealed that polymorphisms SNP -568G/C and VNTR -77(GT)n were partially linked (Table S2). These markers were not statistically significant associated with the IC state in the

multivariable analysis (Table 3). When we further evaluated them as linked polymorphisms, we found that -77 (GT) $n \leq 8/\leq 8$ -568GC and -77(GT) $n \leq 8/\leq 8$ -568CC linked genotypes were detected more frequently among ICs (OR = 11.69, $P = 0.005$ and OR = 7.56, $P = 0.001$ respectively) vs. the -77(GT) $n > 8/> 8$ -568GG genotype. This association was independent from the -408/-3 SNP, the age, the gender and the origin of the individuals (Table S3).

Linkage between polymorphisms and distribution of alleles

Our sequencing experiments suggested that alleles, at position -408 C/T were ultimately linked to those at position -3C/T. This association was confirmed with allele specific PCR in double heterozygous patients, followed by the sequencing of representative products – one allele at a time (Fig. 2A). Furthermore, we found that -568G/C SNP was also partially linked to the num-

ber of GT binucleotide repeats at position -77. The median (5th, 95th) number of GT repeats was 5 (5, 8) and 13 (9, 14) for alleles with -568C and -568G respectively ($P < 0.001$) (Fig. S1). Following a similar methodology, an allele specific PCR and a downstream sequencing analysis did confirm that -568C and -568G alleles were linked to -77(GT) $_5$ and -77(GT) $_{14}$ alleles, respectively, in double heterozygous (-568 CG, -77 (GT) $_{5/14}$) patients (Fig. 2B).

Evaluation of the new method for typing the -77 (GT) $_n$ VNTR

The discrimination of the alleles of different lengths within heterozygous -77(GT) $_n$ samples performed by a newly described method, based on high-resolution agarose-gel electrophoresis. The comparison between heterozygous and homozygous control samples suggested that our new method is capable of distinguishing heterozygous samples that differ for more than one GT

Fig. 2. Chromatographs and agarose-gel electrophoresis from allele specific sequencing and PCR respectively, indicating the allele linkage between: (A) SNP -408C/T and SNP -3C/T, and (B) SNP -568G/C and -77 VNTR (GT) $_n$. SNPs are included in boxes. Primers used are indicated on the top of each gel lane. (M) stands for "marker".

repeats in the -77 locus. The molecular weights of the bands revealed from these experiments, were in accordance with the computable weights (referring to the published sequence (22)) and with our sequencing results (Fig. 1B).

Discussion

The promoter sequence of the INFAR-1 gene presents extended diversity. In this study, a demographically well-characterized European-Mediterranean group of patients was thoroughly investigated. The genetic variations of three SNPs at positions -568, -408, -3 and of one VNTR at position -77 of the promoter sequence were found to be associated with the phase of chronic HBV infection. The -18G/A SNP described by Muldoon *et al.* (20) was not observed in the cohort studied. Alleles -568C, -408C, -3C and -77(GT)₅ were found to be dominant (Table 2, Table S4). The frequencies of the alleles, as far as their respective genotypes at -568 and -408 polymorphic sites, were comparable with those described by Zhou *et al.* (17). The frequencies of -77GT repeats were also comparable to the Caucasian group of patients used in the same study, as the five GT repeats were the most prevalent, the -77(GT)₉ allele was the second more prevalent followed by the -77(GT)₁₄ one. The 18-21 GT repeats were also absent in our study group.

The previously described (17) linkages between -403 SNP/-3 SNP and -568SNP/-77VNTR, were also revealed in our experiments. Furthermore, the development of an allele specific PCR followed by allele specific sequencing, allowed the confirmation of the relationship between the -568SNP and the -77VNTR (Fig. 2B). The partial linkage between these polymorphisms is reflected in the loss of statistical significance in the multivariable model that we have used in this study (Table 3) and is probably the reason why they have been linked in haplotypes in other studies (16, 17). In this study, apart from the -568 SNP and -77 VNTR alleles, a more significant role of the respective genotypes is arising, as far as the clinical course of HBV infection is concerned. Although they are partially linked, a more significant role of the -77VNTR locus was revealed during our analysis (Table S3).

Moreover, and, given the fact that regions in genome with binucleotide repeats are technically difficult to be examined via Sanger sequencing, we showed that a nested PCR followed by a high-resolution agarose-gel electrophoresis is a simple and affordable methodology to distribute and characterize alleles that differ in length in this locus (Fig. 1B).

The demographic characteristics of our patients were found to be associated with the phase of chronic HBV infection. It is known that chronic HBV infection progress more rapidly in males (24, 25). In accordance to this, we found that female patients were more likely to be ICs. Older age of the patients was also associated with higher probability of HBeAg-negative CHB, which is also compatible to previous findings (25). We further

found that the risk of CHB was higher for patients with a Greek than Albanian origin even after adjusting for differences in age, gender and genetic variants. Whether our findings can be affected by HBV genotypes is not known, HBV genotypes were not determined in our study, but the vast majority of our patients most probably had genotype D, which is responsible for >95% of HBeAg-negative Greek and Albanian patients living in Greece (26).

Our data suggest that there can be an intriguing association between the polymorphisms examined in our study and the phases of HBeAg-negative chronic HBV infection. Until now, other studies have come up with similar conclusions. Zhou *et al.* evaluated CHB patients and cases who spontaneously recovered after acute HBV infection and showed that alleles -568C, -408T, -3T and their related haplotype carrying <9 GT repeats at -77 locus were associated with higher probability of CHB, while -568G, -408C, -3C and >9 GT repeats were associated with spontaneous recovery after acute HBV infection (17). Here, for the first time, these polymorphisms appear to have an association with the phase of HBV infection in patients with established chronic HBV infection. Alleles -568C, -408/-3T (but also their respective genotypes) and ≤8 GT repeats at -77VNTR, were associated with the IC phase, whereas -568G, -408/-3C and >8 GT repeats were more frequent in HBeAg-negative CHB patients. It is interesting that the same genetic polymorphisms that were reported to be associated with progression to chronic HBV infection by Zhou *et al.* (17) were found to be associated with the milder phase of HBeAg-negative chronic HBV infection, the IC phase, in our study.

An effective expression of IFNAR-1 is essential for the downstream effects of IFN (27). The number of GT repeats has also been reported to affect the responsiveness of hepatitis C patients to IFN-based therapy where alleles with 5GT repeats (and genotypes GT_{5/5}/GT_{5/14}) were more frequent within the responders group (28). Our findings are in accordance with these results. The 5GT repeats were in dominance within the alleles carrying ≤8 GT repeats, which were significantly associated with the IC phase of chronic HBV infection. Moreover, the reported presence of >18 GT repeats only in Chinese population may be, at least partly, associated with the much higher prevalence of chronic HBV infection in Chinese than in Caucasian individuals who are infected with HBV.

In conclusion, the genetic polymorphisms of the promoter of the INFAR gene represent important factors associated with the clinical phase of HBeAg-negative chronic HBV infection. As the endogenous secretion, but also the exogenous administration of IFNα plays a key role in the defence against HBV, further investigations on the impact of these polymorphisms on the expression and the efficiency of the receptor need to be conducted, to certify the importance of the previous observations.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Polymorphisms in relation to HBV DNA levels in HBeAg-negative patients with persistently normal ALT and HBV DNA <20 000 IU/ml.

Table S2. Distribution of linked polymorphisms VNTR -77(GT)n and SNP -568G/C and type of HBV infection.

Table S3. Multiple logistic regression model: determinants of being inactive carrier, with linked polymorphisms at -568G/C and -77(GT)n.

Table S4. Distribution of alleles in VNTR -77 (GT)n.

Fig. S1. Distribution (percent -%) of GT repeats at -77 VNTR loci, in correlation with -568C (left) and -568G (right) alleles. Median, 5th, 95th percentile and range are included in table.