Association of two variants of the interferon-alpha receptor-1 gene with the presentation of hepatitis B virus infection

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ABSTRACT. Interferon-α (IFNα) is a critical mediator of immunity to hepatitis B virus (HBV) infection. Although IFN has been used in the treatment of viral hepatitis for more than a decade, the role of IFN-α-receptor in HBV infection has not been intensively studied. We have evaluated the impact of two variants of the *IFNAR1* gene on the outcome of HBV infection. Four hundred and fifty eight HBV-infected Vietnamese patients, with well-characterised clinical profiles including all forms of hepatic disease, and 160 non-infected, healthy Vietnamese individuals were enrolled in the study. Of these patients, 54 had acute hepatitis B, 88 had chronic hepatitis B, 118 had liver cirrhosis, 146 had a hepatocellular carcinoma and 52 were asymptomatic carriers of HBV. We analysed two SNPs for unequal distribution between these groups. The first SNP, rs1012335 is situated in intron 3 of the interferon alpha receptor 1 (IFNAR1). A C at position 17470 in the IFNAR1 on both chromosomes was detected more frequently in HBV-infected patients compared to healthy controls (OR: 2.6; 95% CI: 1.46-4.72, p < 0.001). The same homozygosity is also associated with higher concentrations of AST and ALT (aspartate and alanine amino-transferase) in the plasma of the patients. The second SNP (rs2257167) is situated in exon 4, causing a change of amino acids from Val (GTT) to Leu (CTT). Subjects having GTT on both chromosomes were more frequent in the healthy control group (OR: 0.54, 95% CI: 0.35-0.84, p = 0.004) and had lower plasma ALT concentrations. The findings indicate that two variants of the IFNAR1 gene are associated with the clinical presentation of HBV infection.

Keywords: hepatitis B virus (HBV), interferon-alpha receptor-1 (IFNAR-1), hepatocellular carcinoma, polymorphism

Hepatitis B virus (HBV) infection can cause a broad spectrum of liver diseases, including asymptomatic carriage or cryptic hepatitis, self-limiting acute hepatitis, chronic infection, liver cirrhosis, primary hepatocellular carcinoma and fulminant hepatitis leading to liver failure. The reasons for these variations in the course of HBV infection is unknown, but they are probably related to a number of factors including the viral genotype [1], and the level of viremia [2]. Epidemiological investigations in humans have suggested a strong genetic component that affects the individual's susceptibility to infectious pathogens. A number of host factors are discussed as potential causes of this variability: histocompatibility leukocyte antigens (HLA) class I and II, polymorphisms in cytokine genes, the mannan-binding lectin (MBL), as well as the vitamin D receptor [3-5]. A pivotal molecule in HBV infection may be the interferon-α/β receptor (IFNR). The mRNA content of this receptor in a cell is thought to play an important role in hepatitis C virus infection itself and as a predictor of the outcome of interferon therapy [6, 7].

IFNRs are present on most cells and consist of a multiprotein complex of three subunits from 95, 115, and 135 kD [8]. Among these the IFNαR1 chain, a135 kD glycoprotein, plays a central role in signal transduction generated by IFNα/β binding to cells. The respective IFNαR1 gene is situated on chromosome 21q22.11, a locus which has been shown to be linked with the persistence of hepatitis B [9]. This locus also contains genes coding for the cytokine receptor-II family: interferon-alpha receptor-2 (IFNAR2), interleukin-10 receptor-B (IL10RB) and interferon-gamma receptor-2 (IFNGR2) [10]. Polymorphisms in these receptors have been associated with viral infections or autoimmune disease. In IFNαR1, a dinucleotide polymorphism was found to be associated with responsiveness to interferon treatment [11], and a single nucleotide polymorphism (SNP) was found to be correlated to higher levels of serum alanine transferase. [12]. Recently, two novel variants of the IFNAR1 gene (G17470C and Leu168Val) have been studied in the context of *Plasmodium falciparum* malaria in the Gambia [13]. One of the SNP (rs1012335) is situated in intron 3 in the IFNAR1 gene and is a C to G transversion at position 17470; the second SNP (rs2257167) is located in

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exon 4 and affects the first base of the Val codon (GTT), causing a change of amino acid to Leu (CTT). A protective effect could be shown for these polymorphisms in cerebral malaria. Here we investigated these IFNαR1 gene variants and their association with clinical presentation of HBV infection. We analysed these two IFNαR1 variants in a case-controlled study and showed that these variants are associated with clinical presentation of HBV infection.

PATIENTS AND METHODS

Study subjects

Four hundred and fifty eight HBV-infected individuals were included in this study. Of these, 54 had acute hepatitis B (AHB), 88 had chronic hepatitis B (CHB), 118 had liver cirrhosis (LC), 146 had a hepatocellular carcinoma (HCC) and 52 were asymptomatic carriers of HBV (Asym) who had had no previous liver disease and who were healthy at the time of examination. The classification into these five groups was done according to diagnostic criteria described elsewhere [14]. All HBV-infected patients were enrolled at Tran Hung Dao Hospital, Bach Mai Hospital, 103 Military Hospital, Hanoi, and the Centre of Haemodialysis, Hanoi, Vietnam in 2000 and 2002. All subjects were confirmed positive for HBsAg but negative for antibodies against hepatitis C virus (anti-HCV) and human immunodeficiency virus (anti-HIV). None of the study participants had a history of alcohol or drug abuse and none had received any antiviral or immunosuppressive therapy before or during the course of this study. Biopsies were taken from all patients with suspected chronic HBV. They were then classified on the basis of detailed histological examination, into those with or without evidence of either cirrhosis or carcinoma. In the latter case, the degree of differentiation was noted. All individuals included in the HCC group had late stage carcinoma. Individuals with neither cirrhosis nor carcinoma were attributed a histological activity index according to a scheme described elsewhere [15]. As a control group, 160 healthy, Vietnamese blood donors were enrolled, who were examined for hepatitis infections and found to be negative. The study was approved by the Institutional Review Board of the Tran Hung Dao Hospital, Hanoi. Informed consent was given by all participants.

Liver biochemistry tests, hepatitis B virus markers, cancer markers and HBV-DNA quantification has been described in detail elsewhere [14].

Investigation of the INFα receptor1 (IFNAR1) gene

Genomic DNA was isolated from whole blood using a commercial purification kit (Qiagen, Hilden, Germany). To determine the G17470C and Leu168Val variants of the IFNAR1, the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were performed.

To investigate the single nucleotide polymorphisms (SNPs) at position 17470 (G→C) of the INFAR1 gene, the following primers were used: 5'-CTT TCC CTG TAG TAG TGG TTC T-3' (IFNAR-F1) and 5'-CTG TAG TGA GCC GTG ATT GT-3' (IFNAR-R1), (Qiagen, Germany) attaining a 420-base pair (bp) fragment of the IFNAR-1 gene. The reaction mix (50 μL) contained Taq DNA polymerase (0.5 units); 0.2 μM of each primer; 2.5 mM $MgCl_2$; 2 μM 0.4 μL dNTP mix and distilled water. Thirty five cycles were performed after denaturation at 95°C for 5 minutes as follows: 95°C for 30s, 60°C for 30s and 72°C for 30s (Thermocycler Biometra, Germany). A final extension time of 10 minutes at 72°C was added. PCR products were analysed by agarose gel electrophoresis.

Restriction fragment length polymorphism analysis was chosen to distinguish the various alleles. For restriction analysis of amplified genomic fragments, a 10 μL aliquot of each PCR reaction was digested with endonuclease *Bsm*AI at 55°C for 1 hour following the manufacturer's instruction (New England Biolabs). *Bsm*AI cleaves the 420 bp PCR product of the G17470C variant into two fragments of 359 bp and 61 bp. After digestion, all fragments were run on a 3% agarose gel, stained with ethidium bromide and visualised under UV light.

To analyze the Leu168Val variant of the IFNAR1, the following primers (Qiagen, Germany) were used: 5'-AGC TTT CTA TCC TAT CTG TAT G- 3' (IFNAR-F2) and 5'-TTC GCC TAA TTT TTC TCT-3' (IFNAR-R2). The PCR conditions were the same as described above, except for the annealing temperature, which was 54°C for 30 s. The amplification product length was 345-bp. Digestion with *Dde*I was performed following the manufacturer's instruction (New England Biolabs). *Dde*I generates three fragments of 60bp, 78bp and 207bp, the latter is cut into two fragments of 86bp and 121bp, if the PCR product contains the variant.

Statistical analysis

The non-parametric Mann-Whitney U (2-group comparisons) or Kruskal-Wallis (> 2-group comparison) test, respectively, were used to determine the significance of differences in continuous variables. Normally distributed values were compared using ANOVA (Bonferroni/Dunn corrected). Pearson's chi^2 test or Fisher's exact tests were used for groups associations. The level of significance in all cases was set at $p < 0.05$. As software for the all test mentioned above and the calculations of odds ratios, we used StatView, STATA and Jmp.

Haplotype estimations were calculated using Arlequin (http://lgb.unige.ch/arlequin).

RESULTS

Clinical symptoms and liver parameters of HBV-infected patients

In total, 458 HBV-infected individuals with well-characterised clinical profiles including all forms of hepatic disease (asymptomatic HBV carriers, acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma) were enrolled in this study. The criteria for diagnosis are described elsewhere [14]. The clinical details and the results of the biochemical analyses from the dif-

Table 1
Characteristics of HBV infection segregated according to clinical status

Characteristics	AHB (n = 54)	CHB (n = 88)	LC (n = 118)	HCC (n = 146)	ASY (n = 52)
HBcAb IgM(+)	54/54	0/88	ND	ND	ND
HBcAb IgG(+)	0/54	88/88	ND	ND	ND
ALT(IU/L)	1183*	291	71	73	< 30
	[20-4593]	[11.8-2244]	[10-591]	[8-355]	
AST(IU/L)	1117*	289	125	90	< 30
	[27-4425]	[17.2-1782]	[12-1104]	[14-513]	
Total bilirubin (mg/dL)	181*	90	65	46	< 17
	[11-558]	[2-795]	[1-752]	[2-488]	
direct bilirubin (mg/dL)	130*	56	38	18	ND
	[5-512]	[2.1-472]	[0.9-405]	[1-322]	
prothrombin (% of standard)	84	74	51*	73	>90
	[25-100]	[26-100]	[20-100]	[31-100]	
Age (years)	36*	42	50	53	35
Gender (M/F)	46/8	70/18	96/22	122/24	40/12

AHB: acute hepatitis B; CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma; ASY: asymptomatic; HBcAb: hepatitis B surface antigen antibodies; AST and ALT: aspartate and alanine amino-transferase; ND: not done, IU: international units. Values given are medians. * $p < 0.001$ for comparison with all other groups.

ferent patients groups at the time of the study are shown in *table 1*.

In accordance with other studies, patients with acute HBV infections were younger than those in all other groups ($p < 0.001$, ANOVA test). As expected, serum levels of ALT, AST and bilirubin in the AHB group were significantly higher than in all other groups ($p < 0.001$; Mann-Whitney U test; *table 1*), and prothrombin time in patients with liver cirrhosis was significantly longer when compared to all other groups ($p < 0.001$). The AST/ALT ratio increased significantly from AHB > CHB > HCC > LC ($p < 0.001$, Kruskal-Wallis test [5]), indicating a positive correlation with liver function impairment and the progressively severe, pathological processes [16].

Interferon-alpha receptor-1 gene variants: frequencies in HBV-infected and healthy persons

In total, 164/618 (27%) individuals carried the wild-type IFNAR1 gene and 124/618 (20%) were homozygous C/C for G17470C; 330/618 individuals (53%) were found to be heterozygous at position 17470 *(table 2A)*. The C/C genotype was associated with an increased risk of HBV infection, being significantly more frequent in HBV-infected individuals regardless of their clinical presentation compared to healthy controls. Twenty three percent of the virus-carrying individuals (107/428 individuals) were homozygous C/C for G17470C and HBV positive; 77% (351/428 individuals) had a G on at least one chromosome; in the healthy group 11% (17/160) were homozygous C/C for G17470C *versus* 89% (n = 143) with at least one G on one chromosome ($p < 0.001$, Fisher's exact test). The odds ratio of being infected and carrying the C/C genotype was 2.55 with a 95% CI = 1.46-4.73, $p < 0.001$ (Fisher's exact test). When the patient groups presenting a different clinical status were compared separately against the healthy group, the genotype C/C for G17470C was shown to be a susceptibility marker for all groups except in the comparison of the asymptomatic HBV carriers with the healthy controls *(table 3)*.

For the Val168Leu polymorphism, we found 450/618 (73%) were carrying a mutated codon 168 on one or both chromosomes: 316/618 (51%) were heterozygous and 134/618 (22%) were the homozygous C/C for the Val618Leu polymorphism *(table 2A)*. The presence of the altered codon on both chromosomes had a protective effect, with an odds ratio of 0.54 with a CI = 0.35-0.83; (Fisher's exact $p = 0.004$; *table 3*). When comparing the non-infected individuals with the other groups, Leu168-Val are associated with protection only in the comparison with the chronic forms CHB and HCC *(table 3)*.

All four possible haplotypes were found in the Vietnamese population *(table 2B)*. The distribution of the haplotypes between the groups was significantly different ($p < 0.001$ Pearson's chi^2; *table 2*). The "most protective" haplotype had G at both positions. This haplotype, compared to all other haplotypes, was significantly more frequently found in the healthy controls than in the patient groups (40 individuals out of 160 (25%) *versus* 58 individuals out of 406 (13%); $p < 0.001$, Fisher's exact test; OR: 0.43; 95% CI: 0.31-0.60, $p < 0.001$ Fisher's exact test).

Association of IFNAR1 gene variants and clinical parameters

The genetic association of G17470C with susceptibility to HBV is also noticeable in the analysis of the genotypes and the various clinical parameters. The plasma levels of AST and ALT, as a measurement of liver damage, are increased in those individuals homozygous G at G17470C, compared to all of the others (140 ± 95 IU/mL *versus* 102 ± 63 IU/mL; $p = 0.023$; respectively 112 ± 76 IU/mL *versus* 69 ± 42 IU/mL; $p = 0.011$ by Mann-Whitney U test; *figure 1*).

In patients with chronic HBV infections (defined as CHB, HCC and LC individuals), the serum levels of both ALT and AST in patients carrying the C/C genotype at codon G17470C of IFNAR-1 were higher than in all

Table 2
Frequencies and distribution of the genotypes (**A**) and haplotypes (**B**) within the groups
A) shows the number (N) of patients (with the percentage in brackets) having the genotype, with the allele indicated above the column. G17470C represents the SNP rs1012335; L168V represents the SNP rs2257167; C/C or G/G indicates homozygosity at these positions, G/C indicate heterozygosity. **B**) indicates the number of chromosomes analysed carrying one of the haplotypes G-C; G-G; C-C; C-G, and their frequency in brackets.

A)

G17470C					**L168V**				
		N (%)					**N (%)**		
	N (%)	**C/C**	**C/G**	**G/G**		**N (%)**	**G/G**	**C/G**	**C/C**
Healthy					Healthy				
HBV neg	160 (100)	17 (11)	93 (58)	50 (31)	HBV neg	160 (100)	48 (30)	65 (41)	47 (29)
HBV pos	458 (100)	107 (23)	237 (52)	114 (25)	HBV pos	458 (100)	86 (19)	251 (55)	121 (26)
Asym	52 (100)	11 (21)	26 (50)	15 (29)	Asym	52 (100)	14 (27)	22 (42)	16 (31)
AHB	54 (100)	14 (26)	31 (57)	9 (17)	AHB	54 (100)	10 (19)	33 (61)	11 (20)
CHB	88 (100)	28 (32)	35 (40)	25 (28)	CHB	88 (100)	14 (16)	50 (57)	24 (27)
LC	118 (100)	25 (21)	74 (63)	19 (16)	LC	118 (100)	23 (19)	68 (58)	27 (23)
HCC	146 (100)	29 (20)	71 (49)	46 (32)	HCC	146 (100)	25 (17)	78 (53)	43 (29)
Total	618 (100)	124 (20)	330 (53)	164 (27)	Total	618 (100)	134 (22)	316 (51)	168 (27)
	p = 0.001 over all groups (*) p = 0.001 HBV pos *versus* HBV neg (**)					p = 0.047 over all groups (*) p = 0.003 HBV pos *versus* HBV neg (**)			

B)

Haplotype	**All**	**Healthy/HBV neg**	**HBV pos**	**Asymp**	**AHB**	**CHB**	**LC**	**HCC**
G – C	463 (0.374)	113 (0.355)	303 (0.373)	46 (0.446)	43 (0.399)	59 (0.336)	82 (0.349)	119 (0.408)
G – G	195 (0.158)	80 (0.249)	106 (0.131)	10 (0.092)	6 (0.054)	26 (0.147)	30 (0.126)	44 (0.150)
C – C	189 (0.153)	46 (0.142)	136 (0.161)	8 (0.073)	12 (0.110)	39 (0.221)	40 (0.168)	45 (0.154)
C – G	389 (0.315)	81 (0.255)	267 (0.329)	40 (0.388)	47 (0.436)	52 (0.296)	84 (0.357)	84 (0.288)
Total	1236 (1)	320 (1)	812 (1)	104 (1)	108 (1)	176 (1)	236 (1)	292 (1)
	p < 0.001 over all groups* p < 0.0001 HBV pos *versus* HBV neg**							

P values are calculated by Pearson's chi^2 (*) or 2-sided Fisher's exact tests (**).

others (91 ± 56 IU/mL *versus* 61 ± 30 IU/mL, p = 0.013; 127 ± 78 IU/mL *versus* 78 ± 42; p = 0.005; Mann-Whitney U test, *figure 1*). The exon mutation Val168Leu, in contrast, was not significantly different between the same groups as regards the AST measurements. The ALT values however, supported the protective role of this variant. Here we found significantly higher levels of ALT in the plasma of patients having C on one or both chromosomes, G/G was associated with lower ALT levels in the chronic cases (49 ± 29 IU/mL *versus* 66 ± 34 IU/mL; p = 0.031, Mann-Whitney U test).

DISCUSSION

Several human genetic markers have been tested for associations with hepatitis B virus infections (reviewed in [3, 4]). SNPs in the promoter regions of the genes coding for TNF or TGF1ß play a beneficial role in the outcome of a hepatitis B virus infection, because they cause higher levels of mRNA or higher plasma levels of the respective cytokine. On the other hand, polymorphisms leading to deficiency of MBL [17] or IFNα [5] are associated with aggravation of the disease. Because of its importance in antiviral therapy, the interferon-signaling pathway has been investigated for associated genetic factors [18]. Recently, a genome-wide family study was performed revealing a region of chromosome 21q22 as a locus for hepatitis B persistence [9]. In that study involving a cohort from the Gambia, variants of the genes for IFNAR2 and IL10RB were characterized as being responsible for persistence.

Here we analysed two polymorphisms in the IFNAR1 gene, which is also part of this gene complex. One mutation is situated in an intron, the second causes an exchange from Leu to Val. So far it is not known whether these alterations have a functional effect. Although the physical properties of these two hydrophobic amino acids are very similar, there are examples of substitutions from Leu to Val with considerable consequences. In the enzyme ribulose-biphosphate carboxylase, a Leu to Val exchange causes reduced inhibition without affecting the active site [19]. In humans, the oestradiol hydoxylase activity is affected by the same exchange [20]. The

Table 3
Risk calculation according to the SNP genotypes and the various clinical presentations of hepatitis B

G17470C	N (%)					L168V	N (%)				
	C/C	C/G + G/G	OR	95% conf int.	P		G/G	C/G + C/C	OR	95% Conf int.	P
Healthy						**Healthy**					
HBV neg	17 (11)	143 (89)	na	na	na	HBV neg	48 (30)	112 (70)	na	na	na
HBV pos	107	351	2.6	1.46-4.72	< 0.0001	HBV pos	86	372	0.54	0.35-0.84	0.004
Asym	11 (21)	41 (79)	2.26	0.88-5.57	ns	Asym	14 (27)	38 (73)	0.86	0.39-1.81	ns
AHB	14 (26)	40 (74)	2.94	1.22-6.94	0.012	AHB	10 (19)	44 (81)	0.53	0.22-1.18	ns
CHB	28 (32)	60 (68)	3.93	1.90-8.21	< 0.001	CHB	14 (16)	74 (84)	0.44	0.21-0.89	0.015
LC	25 (21)	93 (79)	2.26	1.10-4.71	0.020	LC	23 (19)	95 (81)	0.57	0.31-1.13	ns
HCC	29 (20)	117 (80)	2.09	1.05-4.25	0.026	HCC	25 (17)	121 (83)	0.48	0.27-0.86	0.011
	124 (20)	494 (80)					134 (22)	484 (78)			

The homozygous individuals for C17470 or Val168 were grouped and compared to the healthy controls. Odds ratios (OR) were calculated by comparing the healthy HBV-negative control groups to the patients presenting different outcomes: 95% conf int. indicates the 95% confidence interval. Only significant P values are given. ns: not significant; na: not applicable. For other abbreviations see *table 1*.

reverse exchange was found in a structurally abnormal insulin isolated from a diabetic patient [21] and in factor XII causing an acceleration of thrombin activation and influencing the clot architecture [22].

Also, SNPs in introns can have a substantial impact on expression by affecting bases directly at exon/intron boundaries: base changes can introduce novel splice sites or abolish correct splicing totally [23]. The resulting mRNA may be impaired in function. Mutations can alter intronic splice enhancer sites also leading to reduced mRNA levels in the cell [24]. In some cases, the mechanism by which SNPs affect gene function is not known but the respective phenomena can have a dramatic impact on protein function and may lead to severe conditions

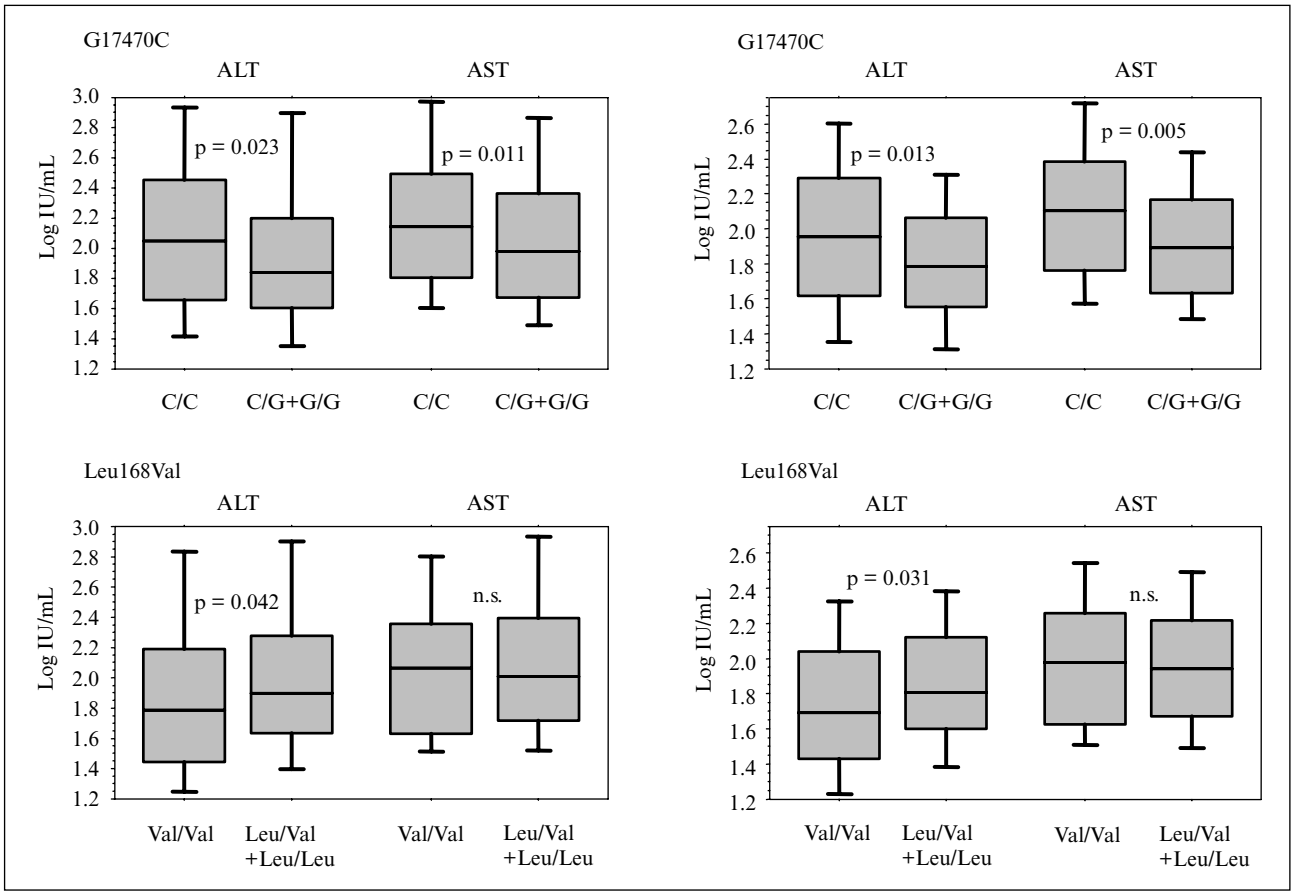

Figure 1
ALT and AST measurements according to the genotypes for the positions G17470C (upper panel) and Leu168Val (lower panel). Plots on the left-hand side summarize all patients enrolled in the study. Plots on the right-hand side include only patients with chronic hepatitis B (*i.e.* CHB, HCC, LC). Individuals homozygous for 17470C (C/C) or 168Val (Val/Val) were compared with all others (C/G+G/G; respectively Leu/Val+Leu/Leu). P values for significance are given above the respective plots.

such as myocardial infarction, rheumatoid arthritis and dementia [25-27].

The IFNAR1 receptor mutations G17470C and Leu168-Val were tested in a malaria patient cohort [13]. In the study presented here, the genotypes showed a consistent resistance/susceptibility profile in favour of the same alleles. However, haplotypes were not associated with the severity of malaria but with HBV presentation. This difference could be due to the fundamentally different compartments in which the different pathogens exert "their" pathology. All patients studied here had liver complications, whereas the pathology of malaria affects blood cells resulting in anemia, or epithelial cells involved in cyto-adherence leading eventually to cerebral malaria.

IFNAR1 expressed in the liver correlates with treatment of viral hepatitis C, reflecting a close relationship between the affected cell and invading pathogen [28-30]. Whether the polymorphisms analysed here influence IFNAR1 expression and treatment response in HBV remains to be elucidated.

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