Major Article

Revista da Sociedade Brasileira de Medicina Tropical 48(2):136-142, Mar-Apr, 2015
http://dx.doi.org/10.1590/0037-8682-0008-2015

Toll-like receptor 3 gene polymorphisms are not associated with the risk of hepatitis B and hepatitis C virus infection

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ABSTRACT

Introduction: The present study investigated the prevalence of two single-nucleotide polymorphisms (SNPs) in the *Toll-like receptor 3* (*TLR3*) gene in patients infected with hepatitis B virus (HBV) and hepatitis C virus (HCV). **Methods:** Samples collected from HCV (n = 74) and HBV (n = 35) carriers were subjected to quantitative real-time PCR (qPCR) to detect the presence of the SNPs rs5743305 and rs3775291 in *TLR3* and to measure the following biomarkers: alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), and prothrombin time (PT). A healthy control group was investigated and consisted of 299 HCV- and HBV-seronegative individuals. **Results:** No significant differences in allele, genotype and haplotype frequencies were observed between the investigated groups, and no association was observed between the polymorphisms and histopathological results. Nevertheless, genotypes TA/AA (rs5743305) and GG (rs3775291) appear to be associated with higher levels of ALT ($p<0.01$), AST ($p<0.05$) and PT ($p<0.05$). In addition, genotypes TT (rs5743305; $p<0.05$) and GG (rs3775291; $p<0.05$) were associated with higher GGT levels. **Conclusions:** This genetic analysis revealed the absence of an association between the polymorphisms investigated and susceptibility to HBV and HCV infection; however, these polymorphisms might be associated with a greater degree of biliary damage during the course of HCV infection.

Keywords: TLR3. rs5743305. rs3775291. HCV. HBV.

INTRODUCTION

Toll-like receptor 3 (TLR3) is a major intracellular receptor that recognizes viral double-stranded RNA (dsRNA)[1] and initiates inflammatory responses against deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses, such as hepatitis B virus (HBV) and hepatitis C virus (HCV). Therefore, TLR3 plays an important role in the immune response to infections caused by such viruses and might influence the chronicity of such viruses, eventually leading to liver cirrhosis and hepatocellular carcinoma[2,3,4,5]. Furthermore, polymorphisms in the *Toll-like receptor 3* gene have been associated with susceptibility to or the clinical progression of infection[6,7,8,9].

Single-nucleotide polymorphism (SNP) rs5743305 (-1077 T>A) is located in the promoter region of the *TLR3* gene and might influence its transcription. SNP rs3775291 (1234 C>T) is a nonsynonymous mutation (Leu412Phe) located in the fourth exon and occurs very frequently in HCV carriers who require liver transplantation[6]. In addition, rs3775291 is a risk factor for chronic HBV infection[10] and has been associated with resistance to human immunodeficiency virus (HIV) infection[11,12].

Given these reports concerning the influence of the *TLR3* gene on infections caused by HCV and HBV, the present study investigated the prevalence of *TLR3* SNPs rs5743305 and rs3775291 in hepatitis patients who visited a northern Brazil hospital that is a regional reference for the treatment of hepatic diseases.

METHODS

Study population

In this study, samples were collected from 74 HCV carriers and 35 HBV carriers at the liver disease outpatient clinic of Holy House of Mercy of Para Foundation Hospital [*Hospital da Fundação Santa Casa de Misericórdia do Pará* (FSCMPA)].

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Received 10 January 2015
Accepted 18 March 2015

The selected participants were consecutive patients, of both genders, with chronic HCV or HBV infection; their age ranged from 22 to 80 years. The control group consisted of 299 HCV- and HBV-seronegative individuals of both genders and aged 19 to 68 years. All of the recruited individuals signed an informed consent form.

The inclusion criteria were as follows: individuals aged 18 years or older of either gender, hepatitis B surface antigen (HBsAg)-positive for more than six months, HCV RNA-positive, and with or without a persistent elevated alanine aminotransferase (ALT) level. Individuals without a history of hepatobiliary disease or autoimmune hepatitis who did not meet the inclusion criteria, individuals co-infected with hepatitis D virus (HDV) and/or HIV, and patients who were previously or currently treated for HBV or HCV were excluded from the study.

This study was approved by the Research Ethics Committee of the Federal University of Para, protocol nº 684.432/2014, in accordance with resolution nº 466/2012 from the National Health Council, which addresses regulatory standards and guidelines for research involving humans.

Obtained samples and histopathological analysis

Blood samples were collected in vacuum tubes containing diaminoethanetetraacetic acid (EDTA) as an anticoagulant, and the plasma was separated by centrifugation and stored at -20°C until use. Biopsies were performed using a Tru-Cut needle and were guided by ultrasound. Each sample was divided into two parts. One part was subjected to histopathological examination after hematoxylin-eosin (HE), chromotrope aniline blue (CAB), Gomori's reticulin and Shikata's orcein staining in the Department of Anatomic Pathology at Federal University of Para (UFPA). The diagnosis followed the French classification METAVIR (METAVIR Cooperative Study Group), which scores the activity of portal and periportal inflammatory infiltrates from 0 to 3 and any structural changes from 0 to 4. The remaining portion of each biopsy specimen was sent for genetic analysis at the Laboratory of Virology of the Institute of Biological Sciences/Federal University of Para (ICB/UFPA) and was stored at -70°C until the time of use.

Genetic analysis

Genomic DNA was extracted from total peripheral blood leukocytes following the protocol of a nucleic acid isolation kit (Gentra Systems, Inc., USA). The procedure included the following steps: cell lysis, protein precipitation, DNA precipitation, and DNA hydration. Data related to ALT, aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), and prothrombin time (PT) quantification were collected from the Virus Laboratory's database and the participants' clinical records.

The extracted DNA was subjected to real-time polimerase chain reaction (qPCR) using a Step One Plus Sequence Detector (Life Technologies, Foster City, CA, USA). For each assessed polymorphism, the assays included one pair of primers and one pair of probes VIC® (VIC fluorescent dye) and FAM® (fluorescein amidite) labeling were used for each allele of the corresponding polymorphisms. Each reaction included 3.5µL H_2O, 5.0µL TaqMan® Universal PCR Master Mix [2X], 0.5µL TaqMan® Assay Mix [20X], and 1µL DNA in a 10-µL final volume. The following temperature cycles were used for the amplification and detection of alleles: 60°C for 30 seconds, 95°C for 10 minutes, and 50 cycles of 92°C for 30 seconds and 60°C for 90 seconds. Assays C_393058_10 and C_1731425_10 (Life Technologies, Foster City, CA, USA) were used for polymorphisms rs5743305 and rs3775291, respectively.

Statistics

Genotype and allele frequencies were estimated by direct counting. Genotype frequencies and expected Hardy-Weinberg equilibrium were estimated with the chi-square test (χ^2) using the BioEstat 5.0 software[13]. A simple logistic regression analysis was performed to investigate the association of genetic models (i.e., codominant, dominant, recessive and overdominant) with the risk of HCV and HBV infection. The risk of disease progression was calculated as an odds ratio (OR) with a 95% confidence interval (CI).

The allele, genotype and haplotype frequencies were compared between groups using the chi-square test (χ^2). To determine whether the variables AST, ALT, GGT and PT followed a normal distribution, D'Agostino's test was used, and the mean values of the laboratory markers were compared using analysis of variance (ANOVA). A p-value <0.05 was considered statistically significant using the BioEstat 5.0 software[13].

RESULTS

All studied groups were found to be in Hardy-Weinberg equilibrium, and no significant differences in the allele, genotype and haplotype frequencies of the investigated polymorphisms were observed (**Table 1**). Nine haplotypes were observed in the patient and control groups, with haplotype TT/GA as the most frequent in the control and HCV-positive groups and haplotype TT/GG as the most frequent in the HBV-positive group (**Table 1**).

Comparisons of the polymorphisms, inflammatory activity, degree of fibrosis and presence of cirrhosis in the chronically infected HCV and HBV patients (**Table 2**) revealed no significant associations. Additionally, none of the suggested genetic models (i.e., codominant, dominant, recessive or overdominant) was associated with the risk of infection with HBV and HCV (**Table 3**).

The mean values of ALT, AST, GGT and PT followed a normal distribution. The presence of allele A in genotypes TA/AA (rs5743305) was associated with higher AST ($p<0.05$) levels in HCV carriers compared to HBV-infected individuals (**Figure 1**). The same results were found with respect to the GGT level in genotypes TT (rs5743305; $p<0.05$) and GG (rs3775291; $p<0.05$).

Alanine aminotransferase levels were higher ($p<0.01$) in HCV-infected subjects carrying the GG genotype (rs3775291) than in HBV-infected patients. However, GGT levels were higher in HCV patients than in HBV patients, independent of the polymorphism analyzed.

Finally, among the HCV-infected subjects, PT was higher in individuals exhibiting genotypes TA/AA (rs5743305; $p<0.05$) and GG (rs3775291; $p<0.05$) than in the HBV-infected group.

TABLE 1 - Allele, genotype and haplotype frequencies of *Toll-like receptor 3* gene polymorphisms in the control group, chronic HCV carriers and chronic HBV carriers.

	Control		HCV-positive		HBV-positive		p1	p2
Genetic profile	n	%	n	%	n	%	p value	p value
Polymorphism								
rs5743305								
TT	147	49.2	40	54.1	20	57.1		
TA	129	43.1	29	39.2	12	34.3		
AA	23	7.7	5	6.7	3	8.6	0.7516	0.6028
T	423	70.7	109	73.7	52	74.3		
A	175	29.3	39	26.3	18	25.7	0.5485	0.6308
rs3775291								
GG	134	44.8	24	41.4	26	55.3		
GA	132	44.2	30	51.7	18	38.3		
AA	33	11.0	4	6.9	3	6.4	0.4612	0.3439
G	400	66.9	78	67.2	70	74.5		
A	198	33.1	38	32.8	24	25.5	0.9728	0.1788
Haplotypes								
rs5743305/rs3775291								
TT/GG	53	17.7	10	17.3	10	31.2	0.9275	0.5010
TT/GA	70	23.4	18	31.0	8	25.0		
TT/AA	24	8.0	2	3.5	1	3.1		
TA/GG	67	22.4	12	20.7	4	12.5		
TA/GA	54	18.1	11	19.0	6	18.8		
TA/AA	8	2.7	2	3.4	-	-		
AA/GG	14	4.7	2	3.4	3	9.4		
AA/GA	8	2.7	1	1.7	-	-		
AA/AA	1	0.3	-	-	-	-		

Notes: Conventional symbol used: (-) numerical data equal zero. p1 = comparison between control group and HCV-positive group. p2 = comparison between control group and HBV-positive group. HBV: hepatitis B virus; HCV: hepatitis C virus.

DISCUSSION

The results of this study suggest that the investigated polymorphisms are not associated with susceptibility to HBV and HCV infection or with the inflammatory activity, the degree of fibrosis or cirrhosis. Nevertheless, the results did suggest that polymorphisms might play a role in disease progression because genotypes TA/AA (rs5743305) and GG (rs3775291) appear to be associated with a greater degree of biliary and liver damage in HCV-infected individuals. These findings are inconsistent with the results reported by Askar et al.[8], who found no relationship between polymorphism rs5743305 and HCV chronicity.

TLR3 plays a significant role in HCV and HBV infection because it is responsible for the production of interferon (IFN) and for the activation of interferon-stimulated genes (ISGs) and IFN regulatory factor 3 (IRF-3), which helps to maintains a low rate of viral replication[14] [15] [16]. Therefore, we suggest that the more severe liver damage that was observed in the HCV patients carrying genotypes TA/AA (rs5743305) and GG (rs3775291) occurs due to the increased production of proinflammatory cytokines, particularly TNF (tumor necrosis factors), IL-1 (interleukin 1), IL-6 and IFN-α, β and γ, by hepatic cells during liver injury; these cytokines are somehow related to the pathogenesis of the investigated viruses.

TLR3 activation efficiently suppresses HBV replication through the stimulation of IFN-β in murine liver cells[17].

TABLE 2 - Correlation between liver histopathology (METAVIR classification) and polymorphisms in the *TLR3* gene in chronically infected HCV and HBV patients.

Genetic and infection profile	Inflammatory activity					Fibrosis degree					Cirrhosis				
	0 to 1		2 to 3		*p value*	0 to 2		3 to 4		*p value*	yes		no		*p value*
HCV															
rs5743305	n	%	n	%		n	%	n	%		n	%	n	%	
TT	20	64.5	11	35.5		22	71.0	9	29.0		11	27.5	29	72.5	
TA/AA	16	54.1	12	42.9	0.7546	19	67.9	9	32.1	0.9809	7	20.6	27	20.4	0.6754
rs3775291															
GG	9	47.4	10	52.6		14	73.7	5	26.3		4	16.7	20	88.3	
GA/AA	19	73.1	7	26.9	0.1483	18	69.2	8	30.8	0.9941	11	32.3	23	67.7	0.2987
HBV															
rs5743305															
TT	9	81.8	2	18.2		10	90.9	1	9.1		2	10.0	18	90.0	
TA/AA	4	66.7	2	33.3	0.9162	5	83.3	1	16.7	0.7397	4	26.7	11	73.3	0.4024
rs3775291															
GG	12	85.7	2	14.3		12	85.7	2	14.3		5	19.2	21	80.8	
GA/AA	7	77.8	2	22.2	0.9413	8	88.9	1	11.1	0.6822	4	19.1	17	80.9	0.7217

METAVIR: METAVIR scoring system; TLR3: Toll-like receptor 3; HBV: hepatitis B virus; HCV: hepatitis C virus. *Chi-square test with Yates' correction.

However, HBV polymerase inhibits IFN-β promoter region activity, thus blocking both IFN-β expression and TLR3-mediated antiviral activity[18]. In the case of HCV infection, an increase in HCV envelope protein E1E2 is associated with reduced TLR3 expression[16]. In addition, the reduced amounts of intracellular Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) that occur during HCV infection also impair TLR3 signaling[14]. Thus, we believe that the action of the polymorphisms investigated in this study is more relevant in HCV infection than in HBV infection, as HCV directly affects gene expression as an escape mechanism, which might be hindered by the location of polymorphism rs5743305 in the promoter region. As HBV reduces INF-β expression, the polymorphisms investigated in this study might lack this activity during infection.

Certain studies have demonstrated that allele A (rs3775291) reduces the ability of TLR3 to recognize double-stranded RNA, leading to decreased activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)[19] [20]. Therefore, this allele might represent a protective factor against liver failure, as the inflammatory response would not be exacerbated during the course of an HCV infection; in contrast, there is no relationship between this allele and HBV infection.

In chronic viral hepatitis, TLR3 expression is increased in intrahepatic biliary epithelial cells[21] and likely plays a role in the onset of biliary damage. Our results suggest that polymorphisms in the *TLR3* gene (i.e., rs5743305 and rs3775291) might be associated with increased GGT levels in patients infected with HCV but not in patients infected with HBV.

The results of this study suggest that the influence of the investigated polymorphisms might be greater in HCV infection, contributing to increased liver damage in comparison to HBV infection. Finally, further studies with larger cohorts are needed to confirm or refute the results and conclusions reported here.

ACKNOWLEDGMENTS

We thank all of the subjects who participated in this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

This work was partially supported by grants from the Brazilian National Council for Scientific and Technological Development [CNPq (480128/2013-8)], the Office of the Dean of Research and Graduate Studies [*Pró-Reitoria de Pesquisa e Pós-Graduação* (PROPESP/UFPA)], and the Research Support and Development Foundation [*Fundação de Apoio e Desenvolvimento da Pesquisa* (FADESP)].

TABLE 3 - Genetic models and the risk of HCV and HBV infection.

		Frequencies								Frequencies						
	SNP	control		HCV					SNP	control		HCV				
Genetic model	genotype	n	%	n	%	OR	95% CI	p value *	genotype	n	%	n	%	OR	95% CI	p value *
	rs3775291								rs3775291							
Codominant	GG	134	44.8	24	41.4	1			GG	134	44.8	26	55.3	1		
	GA	132	44.2	30	51.7	0.9838	0.64 – 1.51	0.9405	GA	132	44.2	18	38.3	0.6931	0.42 – 1.14	0.1466
	AA	33	11.0	4	6.9				AA	33	11.0	3	6.4			
Dominant	GG	134	44.8	24	41.4	1			GG	134	44.8	26	55.3	1		
	GA+AA	165	55.2	34	58.6	1.1505	0.65 – 2.03	0.6298	GA+AA	165	55.2	21	44.7	1.5245	0.82 – 2.83	0.1815
Recessive	GG+GA	266	89.0	54	93.1	1			GG+GA	266	89.0	44	93.6	1		
	AA	33	11.0	4	6.9	0.5971	0.20 - 1.75	0.3485	AA	33	11.0	3	6.4	1.8195	0.53 – 6.19	0.3379
Overdominant	GG+AA	132	44.1	28	48.3	1			GG+AA	132	44.2	29	61.7	1		
	GA	167	55.9	30	51.7	1.3555	0.77 - 2.38	0.2899	GA	167	55.8	18	38.3	1.2735	0.68 – 2.39	0.4526
	rs5743305								rs5743305							
Codominant	TT	147	49.2	40	54.0	1			TT	147	49.2	20	57.1	1		
	TA	129	43.1	29	39.2	0.8603	0.4755	0.4723	TA	129	43.1	12	34.3	0.8326	0.47 – 1.48	0.5301
	AA	23	7.7	5	6.8				AA	23	7.7	3	8.6			
Dominant	TT	147	49.2	40	54.0	1			TT	147	49.2	20	57.1	1		
	TA+AA	152	50.8	34	46.0	0.8220	0.49 –1.37	0.4517	TA+AA	152	50.8	15	42.9	1.3787	0.68 – 2.80	0.3710
Recessive	TT+TA	276	92.3	69	93.2	1			TT+TA	276	92.3	32	91.4	1		
	AA	23	7.7	5	6.8	0.8696	0.32 – 2.37	0.7847	AA	23	7.7	3	8.6	1.1250	0.32 – 3.96	0.8544
Overdominant	TT+AA	170	56.9	45	60.8	1			TT+AA	170	56.9	23	65.7	1		
	TA	129	43.1	29	39.2	0.8493	0.50 – 1.43	0.5397	TA	129	43.1	12	34.3	0.6874	0.33 – 1.43	0.3175

HBV: hepatitis B virus; HCV: hepatitis C virus; SNP: single-nucleotide polymorphism; OR: odds ratio; CI: confidence interval. *Simple logistic regression.

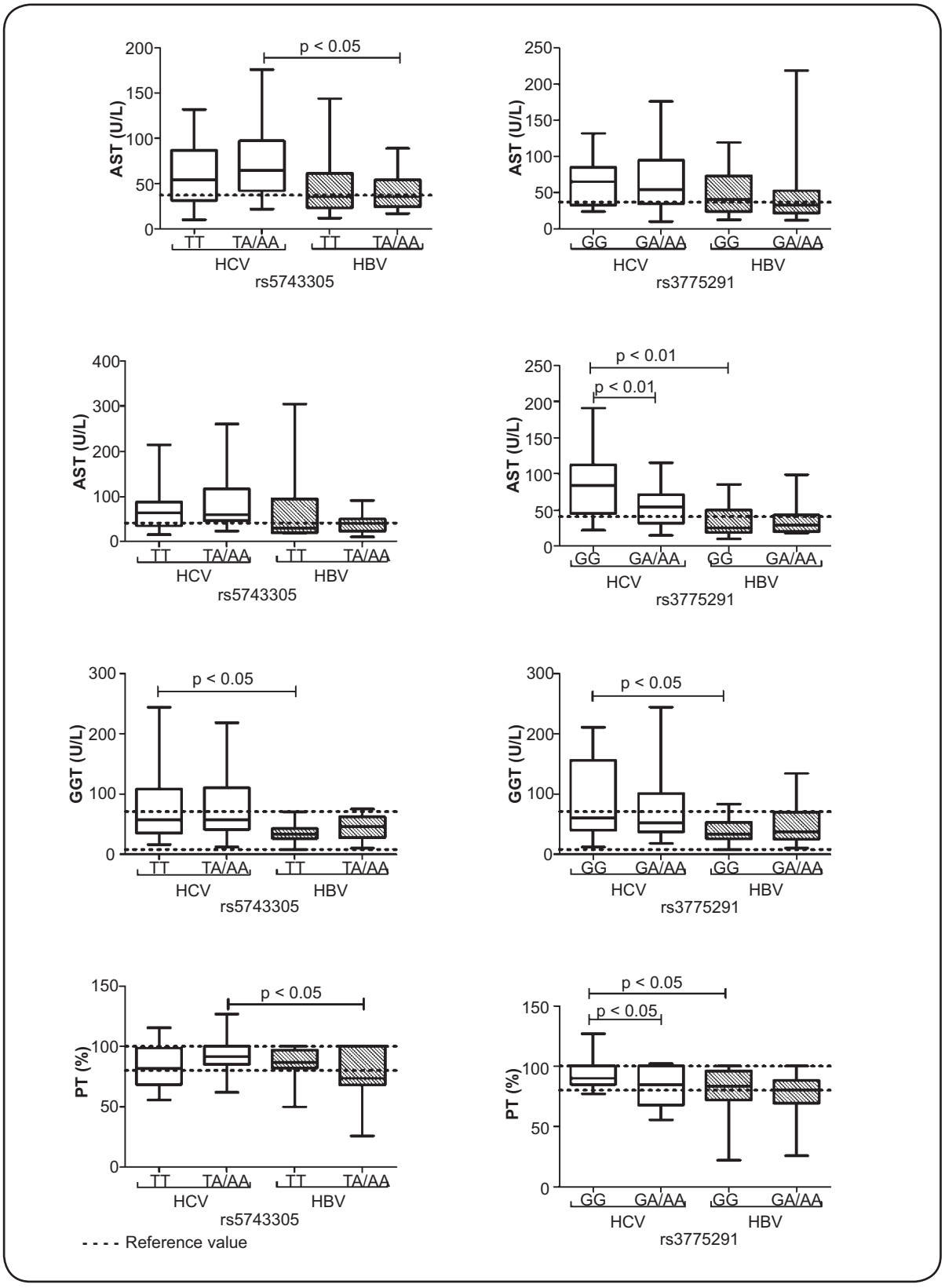

FIGURE 1 - ALT, AST, GGT, and PT levels according to the genotype of *Toll-like receptor 3* gene polymorphisms rs5743305 and rs3775291 in HBV and HCV carrier groups. ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transpeptidase; PT: prothrombin time; HBV: hepatitis B virus; HCV: hepatitis C virus.

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