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Toll-Like Receptor 7 rs179008/Gln11Leu Gene Variants in Chronic Hepatitis C Virus Infection

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Hepatitis C virus (HCV) infection affects an estimated 3% of the world's population. The natural outcome of infection and the natural course of disease are highly variable. Sensing of viral single-stranded RNA (ssRNA) by Toll-like receptor 7 (TLR7) is likely involved in early pathogen detection and host response to viral infections. This study analyzed epidemiological and clinical data from 136 patients with HCV infection with regard to rs179008/Gln11Leu, a non-synonymous polymorphism within exon 3 of the X-linked TLR7 gene, the variant allele of which is suggested to code for a functionally impaired protein. Allele-specific transcript quantification (ASTQ) analyses in heterozygous females revealed individual skewed mosaicism in peripheral blood mononuclear cells (PBMCs). Thus, analyses were restricted to homo- and hemizygous individuals. Among the clinical and histological parameters studied, the variant allele T was found to be solely associated with the presence of portal lymphoid aggregates. Whereas hepatic viral load and expression of genes known to be induced in chronic HCV infection were not found to differ in patients with wild-type or variant TLR7 rs179008 genotype, significant lower gene expression of interleukin-29 (IL-29)/lambda$_1$ interferon (IFN-λ_1) and both of its receptor subunits was found for T homo- and hemizygous patients. Irrespective of the minor differences in disease phenotype including hepatic viral load, natural, and alpha interferon (IFN-α)-mediated outcome of infection, and disease activity and progression, the significant differences in hepatic IL-29/IFN-λ_1 and IFN-λ receptor gene expression between TLR7 rs179008 T and A allele patients might have implications for responsiveness to future IFN-λ-based approaches. ***J. Med. Virol. 82:1859–1868, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: toll-like receptor7 (TLR7); single-stranded RNA (ssRNA); hepatitis C virus (HCV); single nucleotide polymorphism (SNP); portal lymphoid aggregates

INTRODUCTION

Chronic infection caused by hepatitis C virus (HCV), an enveloped single-stranded RNA (ssRNA) virus [Choo et al., 1989], develops in 70–80% of patients [Schwabe et al., 2006]. Patients are at a high risk of developing severe disease as liver cirrhosis and hepatocellular carcinoma [Schwabe et al., 2006]. Toll-like receptors (TLRs) play a critical role in the innate immune sensing of the invasion of pathogenic microorganisms [Akira and Takeda, 2004]. Alpha interferon (IFN-α) is an important antiviral cytokine produced principally by plasmacytoid dentritic cells (pDCs), which circulate in the blood at low frequency and even lower in chronic hepatitis C [Kanto et al., 2004], through the stimulation of TLR7 and TLR9 [Hornung et al., 2005; Ito et al., 2005]. TLR7 senses unmethylated viral ssRNA [Diebold et al., 2004; Heil et al., 2004]. The expression of TLR7 in humans is mainly confined to the endosome–lysosome membrane of pDCs (including hepatic pDCs), hepatic natural killer cells [Seki and Brenner, 2008], and B lymphocytes [Hornung et al., 2002]. When the virus or virus-infected apoptotic cells are taken up by phagocytes, viral RNA is released in the highly acidified phagolysosome by degradation enzymes, leading to ssRNA release and recognition by TLR7. Upon TLR7 stimulation, a complex cascade is formed, starting with myeloid differentiation factor 88 (MyD88) and ending with the production of

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IFN-α/IFN-inducible genes and proinflammatory cytokines through the phosphorylation of interferon regulatory factor 7 and the liberation of nuclear factor-κB, respectively [reviewed in Akira et al., 2006; Schwabe et al., 2006; Seki and Brenner, 2008].

Macrophages overexpressing HCV non-structural proteins NS3, NS3/4A, NS4B, or NS5A show a strong suppression of TLR-MyD88-dependent signaling pathway. NS5A interacts with MyD88 to prevent cytokine production, such as interleukin-1 (IL-1), IL-6, and beta interferon (IFN-β) in response to TLR7 ligands [Abe et al., 2007]. In addition, pDCs from HCV patients have reduced deviation marker (HLA-DR) and IFN-α expression in response to TLR7 ligand, which is associated with an impaired activation of naive CD4 T cells [Yonkers et al., 2007]. TLR7 has been recently of particular medicinal chemistry interest because its small molecule ligands may serve as immune stimulants by enhancing endogenous IFN-α production and thus, they may complement IFN-α therapy of chronic HCV infection, especially in IFN-α-resistant patients. Horsmans and co-workers have applied a well-tolerated intravenous isatoribine treatment with only few mild to moderate adverse events for 1 week to chronic hepatitis C patients. It has resulted in viral load reduction regardless of the patients' HCV genotype, an induction of the antiviral immunity marker 2′,5′-oligoadenylate synthetase, and an increase in the levels of the gamma interferon (IFN-γ)-inducible protein 10 (IP-10) and neopterin, a marker of macrophage activation [Horsmans et al., 2005]. Moreover, a high-affinity ligand of TLR7, namely SM360320, has been found to inhibit HCV replication both through type I IFN production by leukocytes, and direct activation of antiviral mechanisms in infected hepatocytes [Lee et al., 2006].

TLR7 gene is located on the X-chromosome and contains three exons [Du et al., 2000]. Recently, the leucine (Leu) variant encoded by the T allele of the nonsynonymous single nucleotide polymorphism (SNP) rs179008, which is located within TLR7 exon 3 and leads to the replacement of the wild allele A-encoded glutamine (Gln) at codon 11 in the protein (Gln11Leu), has been correlated with higher susceptibility to HCV infection and less chances of response to an IFN-α-based therapy in chronic HCV-infected females [Schott et al., 2008]. Moreover, this variant has been associated with higher viral loads, accelerated progression to advanced immune suppression in human immune deficiency virus (HIV) infection, increased susceptibility to HIV-1 in women, and decreased IFN-α production after stimulation of healthy peripheral blood mononuclear cells (PBMCs) with the TLR7 ligand imiquimod [Oh et al., 2009].

Taking the X-linked location into account, the present study aimed to investigate the correlation between TLR7 rs179008 genotype and disease parameters in chronic hepatitis C, including the natural outcome of infection, that is, chronic versus self-limited course, histological features, and the initial virological response to an IFN-α-based treatment on the one hand, and hepatic expression of innate immunity genes on the other hand.

PATIENTS AND METHODS

Patients

From a total of 144 mainly Caucasian chronic hepatitis C patients who consulted the Liver Unit of the Department of Gastroenterology and Endocrinology at the University Medical Center Goettingen (UMG), Germany, between 1993 and 2006, 136 with complete data sets (mean age 45.0 ± 12.3, median 44 years, 60 females) were enrolled in epidemiological, biochemical, and histological analyses. Chronic infection was proven by detection of HCV-specific antibodies and HCV RNA in the patients' sera using a highly sensitive nested RT-PCR over a period of at least 6 months as described [Mihm et al., 1996a]. Before the start of therapy, liver biopsy procedures were performed and liver disease was confirmed in the course of a defined histological evaluation as described below. Biochemical liver disease parameters, that is, serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (γ-GT) were recorded in parallel. Patients with concomitant non-C viral infections and those with continued alcohol or other drug abuse were excluded.

A total of 55 patients (mean age 46.1 ± 12.3, median 45 years, 25 females) were treated with IFN-α_{2a} (Roferon A; Hoffman-La Roche, Basel, Switzerland) at an initial dose of 6×10^6 IU $3\times$ per week for at least 4 months (mean, 7.7 months; range 4–12 months). Depending on well-being and response parameters, both dose and duration were adapted individually. Initial virological response to therapy, which is defined as the elimination of HCV RNA below the limit of detectability during the first 4 months for a period of at least three consecutive months, was analyzed with regard to TLR7 rs179008 genotype.

Another group of 44 patients with self-limited HCV infection (mean age 37.0 ± 10.3, median 36 years, 14 females) was studied in addition. Spontaneous elimination was assured by the presence of anti-HCV antibodies in the absence of detectable amounts of HCV RNA (for detailed epidemiological and serological description of this cohort please refer to Wietzke-Braun et al. [2007]).

The study was approved by the local ethical committee and conformed to the ethical guidelines of the 2000 Declaration of Helsinki. Patients gave their informed consent.

Determination of HCV Genotype

HCV genotyping was performed using the Innolipa HCV II line probe assay (Innogenetics, Ghent, Belgium).

Histological Evaluation

Before the start of therapy, liver biopsies were taken from patients for histological evaluation. In

brief, sections (5–10 μm) from formalin-fixed and paraffin-embedded liver biopsies were stained with hematoxylin–eosin, trichrome, and Prussian blue. According to Desmet et al. [1994], necroinflammatory activity (grading, score 1–3) and architectural alterations (staging, score 0–4) were scored separately. Other lesions typical of hepatitis C such as the degree of steatosis (score 0–3), the presence or absence of portal lymphoid aggregates, and the presence or absence of bile duct damage were studied additionally as previously described [Mihm et al., 1997].

Preparation of PBMCs

PBMCs from ~30 ml of heparinized peripheral blood samples were prepared by Ficoll density centrifugation using guanidinium isothiocyanate as described [Boyum, 1984].

Isolation of Genomic DNA (gDNA) and Total Cellular RNA

gDNA was purified from PBMCs or from 2 ml samples of serum using the QIAamp DNA Mini or Midi Kits, respectively, following the blood and body fluid spin protocol (Qiagen, Hilden, Germany). The concentration and the purity of the DNA isolated from PBMCs were determined photometrically by reading the absorbance levels at 260 and 280 nm. The integrity of gDNA was ascertained through electrophoresis using a 0.6% agarose gel.

Total cellular RNA was prepared from available freshly isolated PBMCs and homogenized liver tissue samples by CsCl density gradient ultracentrifugation essentially as described [Mihm et al., 1996b].

Reverse Transcription

To get complementary DNA (cDNA), an amount of 1 μg of total cellular RNA was reverse transcribed by using random hexamers (6 μM) for priming as described previously [Mihm et al., 1996b].

Genotyping for the Variant Position rs179008/Gln11Leu

Allelic discrimination of the TLR7 exon 3-located SNP was performed by the commercially available TaqMan genotyping assay C_2259574_10 (Applied Biosystems, Foster City, CA). Reactions of 10 μl containing 4 ng of PBMCs-derived gDNA—or an aliquot corresponding to 6.7 μl serum—were performed in the sequence detection system StepOne-Plus (Applied Biosystems, Darmstadt, Germany) according to the supplier's instructions.

Allele-Specific Transcript Quantitation (ASTQ) of TLR7 rs179008 Variants

Discrimination and quantitation of TLR7 rs179008 transcript variants (A and T) was achieved by applying the commercially available TaqMan genotyping assay C_2259574_10 (Applied Biosystems, CA) on cDNA samples (3.2 ng). Heterozygote gDNA and homozygote gDNA and cDNA samples served as controls.

Quantification of Hepatic Gene Expression

Competitive RT-PCR was applied to quantify mRNA transcripts of HCV, the IFN-α/β inducible antiviral myxovirus resistance protein-1 gene (MxA), IFN-α and, as a reference gene, albumin, essentially as described [Mihm et al., 2004], and transcripts of IP-10, the gene encoding IFN-α/β-inducible p44 [Patzwahl et al., 2001], IFN-γ [Mihm et al., 1996b]. The relative number of interleukin-29 (IL-29)/lamda$_1$ interferon (IFN-λ_1), IFN-λ receptor subunits (IL-10Rβ and IL28Rα), and IFN-α/β receptor 2 (IFNAR2) mRNA transcripts was calculated by real-time RT-PCR using the sequence detection system ABI prism 7000 following the supplier's instructions (Applied Biosystems, Darmstadt) as described [Mihm et al., 2004; Doyle et al., 2006]. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) transcripts served as a housekeeping gene, using a commercially available TaqMan gene expression Assay on Demand (Hs 99999905 ml) (Applied Biosystems). Comparable results were found when relating the targets to β-actin transcripts (data not shown).

Statistical Analysis

Females and males were analyzed both separately (data shown in the text where necessary), and combined due to the ASTQ results. Quantitative parameters were described by mean and standard deviation if the data are normally distributed, or median and inter-quartile range (IQR) if the distribution is not normal. χ^2-test and independent samples t-test were applied where applicable. The level of significance was set to a screening level of 0.05. All tests were performed by using PC STATISTIK software package version 4.0 (Hoffmann-Software, Giessen, Germany).

RESULTS

Genotyping of HCV With Regard to TLR7 rs179008

A total of 136 patients with chronic hepatitis C (60 females/76 males) were genotyped for the bi-allelic SNP rs179008/Gln11Leu within exon 3 of the X-linked TLR7 gene (Table I). Genotype distribution in women followed Hardy–Weinberg equilibrium ($P = 0.318$). The frequency of the minor allele (MAF) was found to be close to that given for Caucasians in public databases and to be similar in females and males ($P = 0.175$).

ASTQ

Due to the X-linked location of TLR7, in females, the random inactivation of one X chromosome leads to cellular mosaicism with two populations of cells differing in the parental origin of the active X [Fish, 2008]. In heterozygous females, the presence of two kinds of cells might set up a competition between them [Migeon,

TABLE I. TLR7 rs179008 Genotype Distribution in Patients With Chronic or Self-Limited HCV Infection With Regard to Epidemiological and Biochemical Parameters

		TLR7 rs179008 genotype						
		Females			Males			
	n	AA	AT	TT	A	T	*P*	MAF
Patients with chronic HCV infection								
Number (%)	136	34 (56.7)	20 (33.3)	6 (10.0)	61 (80.3)	15 (19.7)	0.175[b]	0.267/0.197[g]
Age (mean ± SD)		45.8 ± 10.7	46.6 ± 11.5	50.0 ± 13.6	40.7 ± 11.2	46.9 ± 15.9	0.069[c]	
HCV types								
HCV type-1 n (%)	109	28 (53.9)	19 (36.5)	5 (9.6)	47 (82.5)	10 (17.5)	0.449[d]	0.279/0.175[g]
HCV non-1 n (%)	27	6 (75.0)	2 (25.0)	0 (0)	14 (73.7)	5 (26.3)		0.143/0.263[g]
Biochemical serum parameters								
AST (median, IQR)		23.0, 15–38	18.5, 13.5–31.5	45, 16–80	29, 18–67	36, 18–52		
No. patients with elevated/normal AST[a]	136	12/22	6/14	3/3	28/33	7/8	0.411[d]	
ALT (median, IQR)		32.5, 22–60	31.5, 20.5–44	58, 22.90	58, 36.5–129	78, 47–92		
No. patients with elevated/normal ALT[a]	136	13/21	7/13	3/3	36/25	12/3	0.078[d]	
γ-GT (median, IQR)		19.5, 10–45	19.5, 13.5–41	17, 5–34	39, 23.5–62.5	24, 14–72		
No. patients with elevated/normal γ-GT[a]	136	10/24	5/15	2/4	17/44	5/10	0.419[d]	
Patients with self-limited HCV infection								
Number (%)	44	7 (50.0)	6 (42.9)	1 (7.1)	26 (86.7)	4 (13.3)	0.766[e]	0.286/0.133[g]
Age (mean ± SD)		39.3 ± 10.4	38.0 ± 13.2	30	36.0 ± 9.9	40.5 ± 9.7	0.722[f]	

[a]Markedly elevated serum activities of transaminases (>2-fold of the upper normal limit) were considered. Upper normal limits for females/males, respectively, are: 15 U/L/19 U/L for AST; 19 U/L/23 U/L for ALT; and 18 U/L/28 U/L for γ-GT.
[b]χ^2-test was applied to compare MAF between females and males.
[c]Independent samples *t*-test was applied, the trend was valid only in males ($P = 0.083$).
[d]χ^2-test was applied to compare A with T homo- and hemizygous patients (combined females males).
[e]χ^2-test was applied to compare TLR7 rs179008 genotype distribution in patients with chronic- and patients with self-limited HCV infection.
[f]Independent samples *t*-test was applied.
[g]MAF is given for females/males, respectively.

2006]. Non-random inactivation (skewing) has been implicated for discrete cell populations, for example, dendritic cells [Fish, 2008; Migeon, 2006]. In order to find out whether heterozygous females should be assigned either to the wild-type, the variant genotype or to be considered as a separate, that is, true heterozygous group, ASTQ was performed to quantify the relative proportion of A and T allele transcript variants in RNA preparation from freshly isolated PBMCs (Fig. 1). gDNA, a natural source of equal amounts of A and T sequences served as a control. RNA preparations from three heterozygous women were found to contain nearly equal numbers of both alleles' transcripts, whereas material from four heterozygous females was found to contain an excess of either A or T, two women each, respectively (Fig. 1A).

Because of the limited amount of available samples, 5 heterozygous females were further identified among a total of 42 healthy blood donors. ASTQ with these five samples yielded comparable results (equal expression in two samples, an excess of A in three samples) (data not shown).

On the basis of these findings we decided to restrict analyses to the comparison between A and T homo- and hemizygous individuals.

Epidemiological and Biochemical Characteristics

Demographic analysis revealed a slight trend of the variant allele carriers to be older than those who carry the wild-type allele (47.8 ± 15.0 vs. 42.5 ± 11.2, respectively, $P = 0.069$). This difference, however, was clearer in males ($P = 0.083$) (Table I).

As expected for a European population, most patients (80.1%) were infected with HCV type-1 (including subtypes 1b, 1a, and 1a + 1b), while 19.9% were infected with HCV non-1 (including mainly subtypes 3a, and a minority of 2a and 2b). No significant difference among the distribution of HCV types (or subtypes, data not shown) infections according to patients' TLR7 rs179008 genotype was found (Table I).

AST, ALT, and γ-GT serum activities were recorded as indicators of liver injury in chronic hepatitis C. Although T homo- and hemizygotes seemed to have higher AST and ALT but lower γ-GT serum levels than the A counterparts, the proportion of A or T homo- and hemizygote patients among those with markedly elevated transaminase activities, that is, greater than twofold of the upper normal limit, was not found to be significantly different (Table I).

Genotyping of Individuals With Self-Limited HCV Infection

The T allele is suggested to confer enhanced susceptibility to chronic HCV infection as MAF has been found to be significantly lower in healthy individuals [Schott et al., 2008]. To address the question whether this enhanced susceptibility is due to a higher incidence of infection or to an impaired capacity to self-eliminate

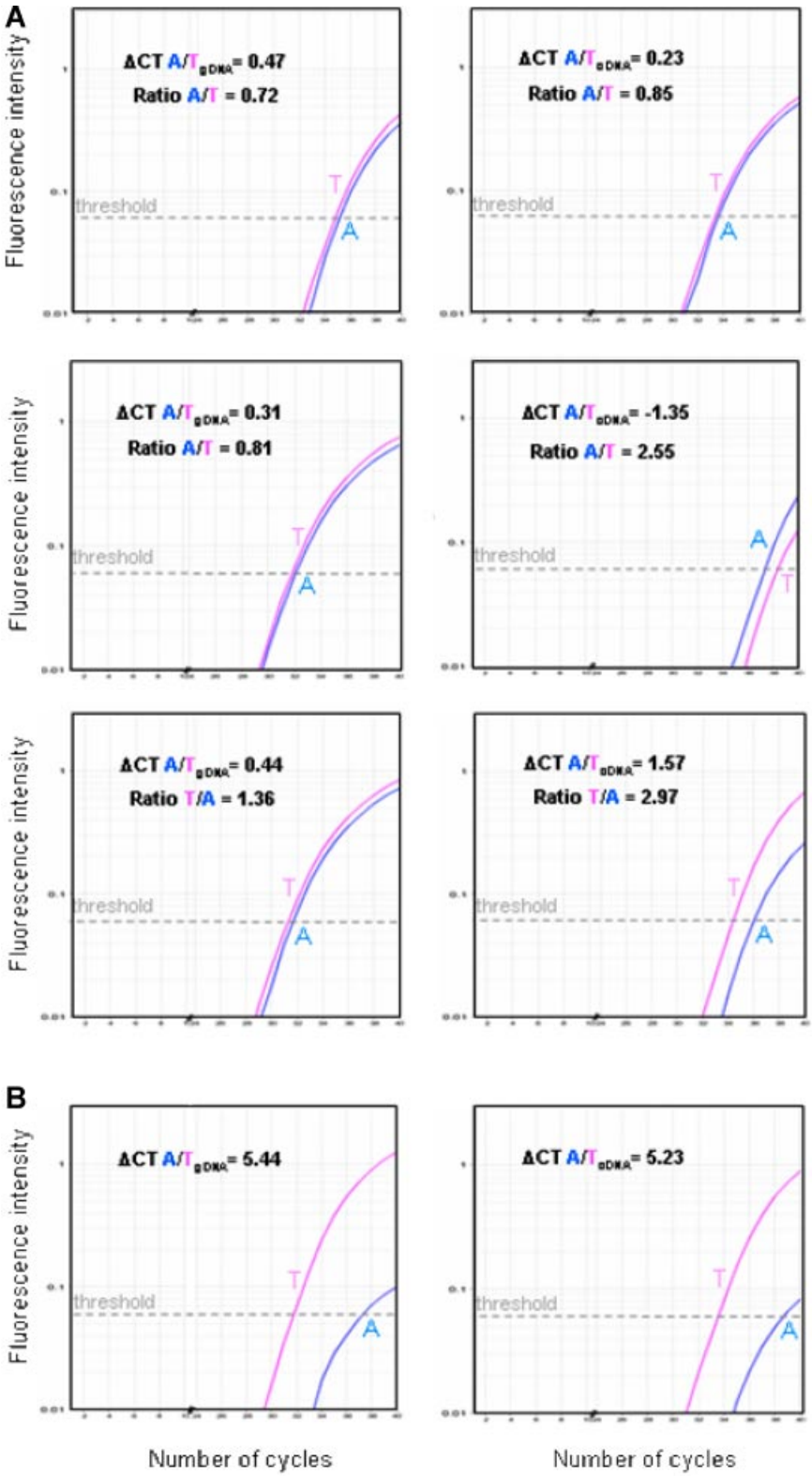

Fig. 1. TLR7 ASTQ in heterozygous female hepatitis C patients. **A**: ASTQ carried out on corresponding gDNA and cDNA samples from three representative TLR7 rs179008 heterozygous female patients revealed either nearly equal amounts of both alleles (**top**), a 2.7-fold excess of the A allele variant (**middle**), or a 2.6-fold excess of the T allele variant (**bottom**). Analyses were made in duplicate, therefore the mean ΔC_T is given but one representative amplification plot is shown. **B**: ASTQ carried out on one representative pair of corresponding gDNA and cDNA samples from a TLR7 rs179008 homozygote T patient yielded only non-specific signal for the allele A as defined by a 10-fold less fluorescence intensity in the plateau phase at the end of the reaction.

the virus, TLR7 rs179008 genotype distribution of the chronic hepatitis C patients was compared to a group of 44 patients with self-limited HCV infection [Wietzke-Braun et al., 2007]. The proportion of T homo- and hemizygous individuals or even of heterozygous female patients was not found to be significantly higher in chronic HCV patients (15.4% and 14.7%, respectively) in comparison to patients with self-limited HCV infection (11.4% and 13.6%, respectively) (Table I).

Hepatitis C Histological Manifestations

To investigate whether a functionally impaired TLR7 protein might be related to histological features of chronic hepatitis C, liver biopsy specimens were taken and evaluated histologically with regard to hepatitis activity, fibrosis progression, steatosis stage, portal lymphoid aggregates, and bile duct damage (Table II). TLR7 rs179008 genotype distribution showed no association with the first three characteristics. Nevertheless, a higher frequency of the minor allele T among patients with portal lymphoid aggregates was observed ($P = 0.013$), this difference, however, was only valid in males ($P = 0.032$) (Table II). Whereas only 30.5% of A carriers were found to have bile duct damage, 52.4% of T carriers did have the lesion ($P = 0.051$) (Table II).

Noteworthy, it was reported recently that patients homozygous for the variant allele T of the endotoxin receptor CD14 SNP rs2569190/C-159T have more frequent portal lymphoid aggregates than C carriers [Askar et al., 2009]. Interestingly, analyzing CD14 C-159T genotype separately in the two genders revealed that the significant association was only confined to males ($P = 0.004$) (unpublished observation).

Response to an IFN-α_{2a} Monotherapy

A total of 55 patients were treated with IFN-α_{2a} as described in the Patients and Methods Section. The initial virological response to therapy is defined as the elimination of HCV-RNA below the limit of detectability during the first 4 months for a period of at least three consecutive months. Some patients kept undetectable viral RNA levels till completing therapy, that is, end-of-treatment response, or even for a period of at least continuous 6 months after the last dose of IFN-α_{2a}, that is, sustained virological response. While 22 (63%) of the A carriers responded, at least initially, to the therapy, only 3 (30%) of the T counterparts (and 30% of the heterozygous females) were responders ($P = 0.069$) (Table III). Similar results were found considering a larger cohort of 145 patients treated with an IFN-α-based therapy (data not shown).

Hepatic Gene Expression

To examine whether a functionally impaired TLR7 protein might be related to hepatic gene expression in chronic HCV infection, innate immunity gene transcripts were quantified in freshly derived liver tissue samples. Data were related both to GAPDH and to albumin as reference genes. When A homo- and hemizygous samples were compared to T homo- and hemizygous samples, no significant difference was found with

TABLE II. Histological Manifestations of Chronic Hepatitis C Patients With Regard to TLR7 rs179008 Genotype

Histological manifestations	TLR7 rs179008 genotype					
	Females n (%)			Males n (%)		
	AA	AT	TT	A	T	P^a
Hepatitis activity						
Mild	20 (57.1)	12 (34.3)	3 (8.6)	35 (85.4)	6 (14.6)	0.156
Moderate	13 (59.1)	6 (27.3)	3 (13.6)	22 (75.9)	7 (24.1)	
Severe	1 (33.3)	2 (66.7)	0 (0)	4 (66.7)	2 (33.3)	
Fibrosis						
Absent	5 (55.7)	3 (33.3)	1 (11.1)	9 (100.0)	0 (0)	0.222
Mild	18 (56.3)	11 (34.4)	3 (9.4)	29 (80.6)	7 (19.4)	
Modorate	6 (54.6)	4 (36.4)	1 (9.1)	11 (37.3)	4 (26.7)	
Marked	4 (66.7)	1 (16.7)	1 (16.7)	5 (62.5)	3 (37.5)	
Cirrhosis	1 (50.0)	1 (50.0)	0 (0)	7 (87.5)	1 (12.5)	
Steatosis						
Absent	15 (60.0)	7 (28.0)	3 (12.0)	27 (77.1)	8 (22.9)	0.509
Mild	12 (50.0)	10 (41.7)	2 (8.3)	20 (83.3)	4 (16.7)	
Moderate	3 (42.9)	3 (42.9)	1 (14.3)	9 (75.0)	3 (25.0)	
Marked	4 (100.0)	0 (0)	0 (0)	5 (100.0)	0 (0)	
Portal lymphoid aggregates						
Absent	25 (64.1)	11 (28.2)	3 (7.7)	39 (88.6)	5 (11.4)	**0.013**[b]
Present	9 (42.9)	9 (42.9)	3 (14.3)	22 (68.8)	10 (31.3)	
Bile duct damage						
Absent	26 (63.4)	12 (29.3)	3 (7.3)	40 (85.1)	7 (14.9)	0.051
Present	8 (42.1)	8 (42.1)	3 (15.8)	21 (72.4)	8 (27.6)	

[a] χ^2-test was applied on A and T homo- and hemizygous patients, to compare mild versus moderate and severe hepatitis activity, absent, mild versus moderate and marked fibrosis and cirrhosis, and absent, mild versus moderate and marked steatosis.
[b] The difference is valid only in males ($P = 0.032$).
Number in bold refers to an analysis irrespective of gender.

TABLE III. Initial Virological Response to an IFN-α_{2a} Monotherapy in Chronic Hepatitis C Patients With Regard to TLR7 rs179008 Genotype

	TLR7 rs179008 genotype					
	Females			Males		
	AA	AT	TT	A	T	*P*
No. non-responsive patients (%)	6 (35.3)	7 (41.2)	4 (23.5)	7 (70.0)	3 (30.0)	0.069[a]
No. responsive patients (%)	4 (50.0)	3 (37.5)	1 (12.5)	18 (90.0)	2 (10.0)	

[a]χ^2-test was applied to compare A with T homo- and hemizygous patients.

regard to the amount of hepatic viral RNA (Fig. 2A), or the genes that have been shown to be enhanced in chronic HCV infection when compared to healthy liver tissue as IP-10, p44, MxA, or IFN-γ [Mihm et al., 2004] (Fig. 2B). In contrast, T homo- and hemizygotes were found to express significant lower amounts of IL-29/IFN-λ_1 ($P = 0.015$), IL-10 receptor beta (IL-10Rβ) ($P = 0.001$), and IL-28 receptor alpha (IL-28Rα) ($P = 0.003$), which constitute the two components of IFN-λ heterodimeric receptor, as well as lower amounts of IFN-α and $IFNAR_2$ (Fig. 2B).

DISCUSSION

The TLR7 rs179008/Gln11Leu is located in the signal sequence of TLR7, adjacent to the typical basic residues in the N-terminal part of this sequence. Signal peptide degeneracy modulates posttranslational modification, localization, quantity, and thus the functionality of the affected protein [Hegde and Bernstein, 2006]. In the studied cohort of 136 chronic hepatitis C patients, no significant association was found between TLR7 rs179008 and any of the epidemiological or biochemical characteristics, inflammation activity (grading), or fibrosis progression (staging). These results are in concordance with previous findings [Schott et al., 2007]. Considering the presence of portal lymphoid aggregates, a significant higher frequency of the T hemizygosity was found among male patients. Portal lymphoid aggregates are defined as densely packed collection of small lymphocytes within the portal tract with or without the formation of a germinal center [Luo et al., 1999]. Their presence, which is suggested to play an immunological albeit indeterminate role in chronic HCV liver injury similar to the mechanism of autoimmune hepatitis [Hino et al., 1992; Mosnier et al., 1993], has been found to be significantly correlated with hepatic inflammatory activity and bile duct damage [Freni et al., 1995; Wong et al., 1996; Luo et al., 1999; Askar et al., 2009]. Interestingly, the common allele A trended to be low-frequented among patients with bile duct damage ($P = 0.051$) (Table II).

Portal lymphoid aggregates have been recently found to be more frequent among patients homozygote for the T allele of CD14 rs2569190/C-159T [Askar et al., 2009], this association, however, was valid only in males as it is for TLR7 rs179008 in the present study. Noteworthy, sex itself is neither found to be associated with portal lymphoid aggregates [Mihm et al., 1997; Luo et al., 1999; Askar et al., 2009], nor with CD14 rs2569190 genotype distribution [Askar et al., 2009]. Freni et al. [1995] described the cellular composition of this manifestation as a core of B cells—which do express TLR7—mixed with many T helper/inducer lymphocytes, and an outer ring prominently formed by T suppressor/cytotoxic lymphocytes, and a rarely identifiable germinal center. Taken together, being the first observation of its kind, although its real biological mechanism is still to be found out, replication in an independent larger cohort, and correction of multiple testing are required, TLR7 rs179008 might have a role in the formation of portal lymphoid aggregates.

TLR7 rs179008 T allele has been found recently to be over-represented and predictive of unfavorable outcome of IFN-α therapy in female patients with chronic HCV infection [Schott et al., 2008]. In the present study, we investigated the distribution of this SNP among patients who spontaneously resolved HCV infection. Comparing the two cohorts with regard to TLR7 rs179008 genotype did not reveal any significant difference (Table I). Unfortunately, this analysis lacks statistical power due to the small cohort of self-limited individuals, yet it did not give any preliminary indication for an altered capacity of resolving the infection spontaneously. Moreover, the observed slight (but still non-significant) trend of the TT/T patients to be non-responders to a mono- or a combined IFN-α-based therapy and to have lower hepatic expression of both IFN-α and $IFNAR_2$ might confirm—in general—the previous findings of Schott et al. [2007] with cautious limitations due to the novel ASTQ analyses in this study that let us omit female heterozygotes from our analyses.

The impaired receptor appeared not to affect HCV hepatic viral load, accordingly, no further effect was observed on p44, MxA, IFN-γ, or IP-10, genes known to be upregulated in chronic HCV infection in the absence of hepatic type I IFN induction [Mihm et al., 2004]. The minor allele T, however, was found to be significantly associated with lower hepatic mRNA expression of IL-29/IFN-λ_1 and both IL-10Rβ and IL-28Rα (Fig. 2B). This suggests that, rather than being useful in forecasting the current IFN-α-based therapy outcomes, genotyping for TLR7 rs179008 might be predictive for response to IL-29/IFN-λ_1-based therapy approaches [Sheppard et al., 2003] currently being in phase 2 of clinical development.

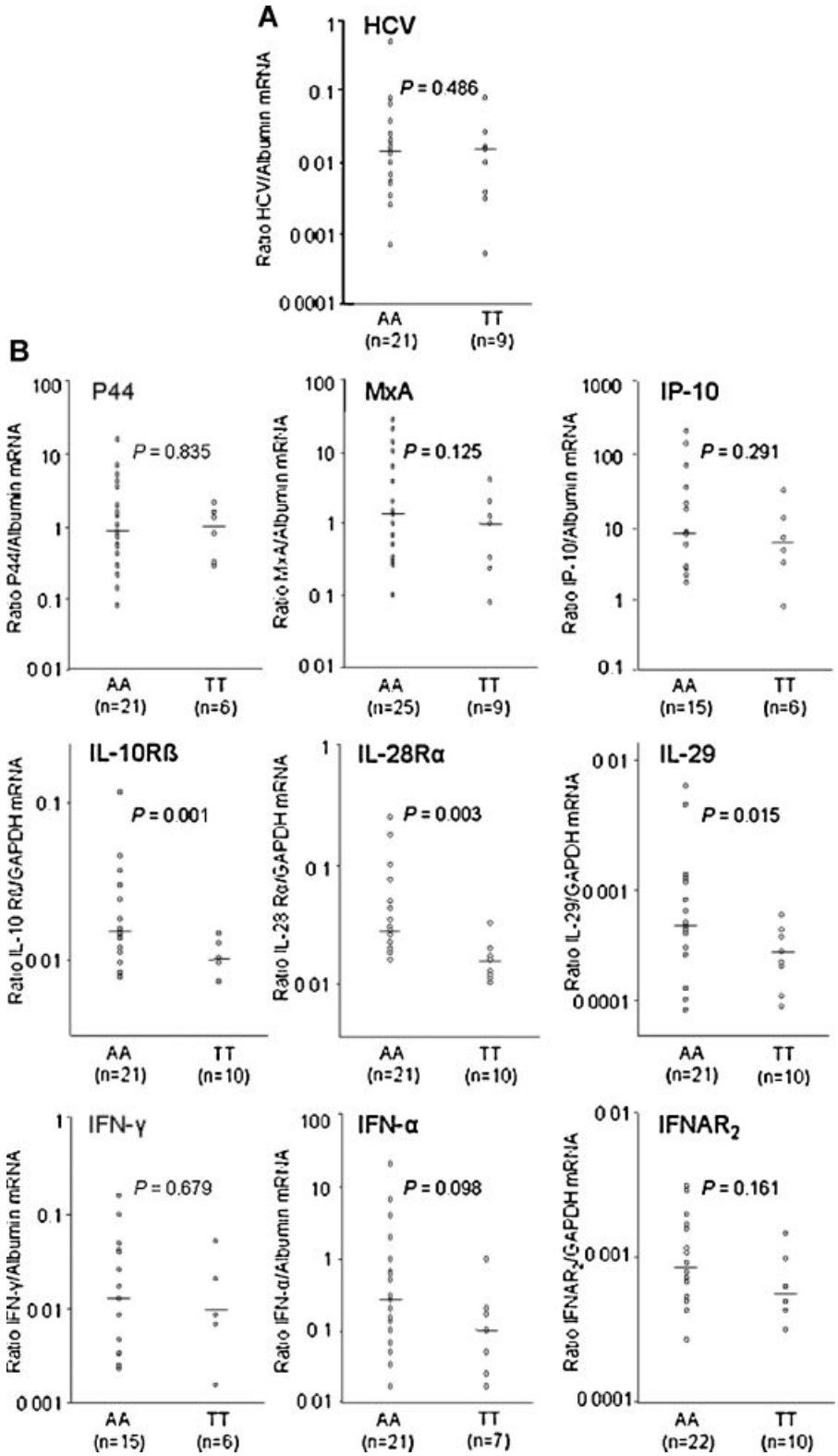

Fig. 2. Hepatic gene expression in chronic hepatitis C patients with regard to TLR7 rs179008 genotype. Total cellular RNA from liver biopsy specimens taken from homo- and hemizygous patients with chronic hepatitis C was quantified with respect to (**A**) HCV RNA, (**B**) p44, MxA, IP-10, IFN-γ, and IFN-α in relation to albumin mRNA transcripts by using competitive quantitative RT-PCR, and IL-10Rβ, IL-28Rα, IL-29, and $IFNAR_2$ in relation to GAPDH mRNA by using quantitative real-time RT-PCR assays. Data are given as ratios of the target to reference gene $\times 10^{-3}$. Medians are indicated by horizontal bars. Levels of significance are given. Similar results were obtained when data were related to β-actin (data not shown).

Three independent genome-wide association studies (GWASs) have reported recently on several SNPs in the intergenic region between the genes coding for IL-28A/IFN-λ_2 and IL-28B/IFN-λ_3 on chromosome 19 to be associated with response outcomes to an IFN-α-based therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and with spontaneous clearance of HCV [Thomas et al., 2009]. The minor non-responder allele of rs8099917 in IL-28B/IFN-λ_3 gene has been found, moreover, to be associated with lower IL-28 mRNA expression in PBMCs [Suppiah et al., 2009; Tanaka et al., 2009]. These GWASs have identified as well many SNPs in several genes to be of minor predictability for IFN-α-based therapy outcomes, TLR7, however, not to be among them.

Taken together, despite of significant decreased hepatic gene expression in TLR7 rs179008 T compared to A allele patients, that might be due to improper virus sensing and that might affect responsiveness to IL-29/IFN-λ_1 rather than IFN-α, differences in phenotype of disease including hepatic viral load, natural outcome of infection, and disease activity and progression appear to be minor with the exception of the presence of portal lymphoid aggregates in T hemizygous males. Further investigations will elucidate the impact of this polymorphism on responsiveness to endogenous and probably exogenous IFN-λ.

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