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ORIGINAL ARTICLE

Polymorphisms of Toll-like receptors 2 and 4 in chronically infected hepatitis C patients from north-east Croatia

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Abstract Chronic infection with hepatitis C virus (HCV) is caused by an inadequate immune response. Experimental data suggest that the impaired activation of Toll-like receptors (TLRs) 2 and 4 contributes to chronic infection. We assessed the distribution of three single-nucleotide polymorphisms (SNPs) in the TLR2 (Arg753Gln) and TLR4 (Asp299Gly/Thr399Ile) genes in individuals from north-east Croatia and their effect on the outcome of antiviral therapy. The study consisted of 60 chronically infected patients and 40 healthy subjects. TLR polymorphisms were determined by the PCR-based melting curve analysis. HCV genotyping was performed using the Linear Array Hepatitis C Virus Genotyping Test. Thirty-three patients were treated with standard interferon and ribavirin therapy, and their viral load was evaluated at weeks 28 and 53 after the beginning of therapy. The majority of chronic infections were caused by genotype 1 (77%), followed by genotypes 3 (15%) and 4 (7%). Patients with genotype 1 had higher viral loads than patients infected with other genotypes ($P = 0.0428$). Healthy individuals and patients with chronic infection had similar frequencies of TLR2-Arg753Gln and TLR4-Asp299Gly/Thr399Ile SNPs. Heterozygous and homozygous TLR4-Asp299Gly/Thr399Ile polymorphisms correlated with higher viral loads and delayed responses to antiviral therapy. We have provided the first evidence that TLR4 polymorphisms influence the success of antiviral therapy in our region. This suggests that therapeutic strategies should be adjusted not only according to HCV genotype but also to individual TLR polymorphism(s).

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Introduction

Hepatitis C infection (HCV) is a global public-health problem. According to the World Health Organization (WHO), the worldwide spread and late onset of visible signs of HCV disease makes it a silent pandemic. HCV infection is often symptomless or accompanied by non-specific symptoms such as fatigue, weakness, appetite loss, and muscle and joint pain [1, 2]. The majority of infections remain undetected and lead to chronic disease (CHCV), liver cirrhosis and hepatocellular carcinoma (HCC). Out of 170 million chronically infected people worldwide, 5 million live in western Europe [3]. CHCV is one of the main reasons for liver transplantation in Europe, the USA, and Australia [4, 5]. CHCV is also more refractory to antiviral treatment and increases healthcare costs.

Although the prevalence of HCV infection in the general population of Croatia is low (1.4%), two hundred cases of acute infection and a similar number of chronic infections with HCV have been registered in Croatia every year [6]. The standard-of-care therapy with pegylated interferon and ribavirin (pegIFN-RBV) has been used and approved in Croatia since the Consensus Conference on Viral Hepatitis in 2005. HCV-infected patients are treated if their medical records confirm the presence of HCV RNA in the serum or plasma; persistently elevated level of alanine

aminotransferase (ALT); F2 liver fibrosis stage assessed using the Ishak scoring system; co-infection with hepatitis B virus (HBV), human immunodeficiency virus (HIV) or HCV genotypes 2 or 3; liver transplantation, and/or severe extra-hepatic symptoms.

Hepatitis C virus has six genotypes and 90 subtypes. Genotypes 1, 2 and 3 are distributed worldwide, while others are more restricted. In Croatia, genotypes 1 and 3 are the most prevalent genotypes, while the prevalence of genotypes 2 and 4 is very low [7]. HCV classification is important not only for epidemiological studies but also for guiding antiviral therapy. When compared to infections with other genotypes, genotype-1-infected patients have higher viral loads, suffer from more-severe liver disease and respond less to therapy [8]. Interferon-ribavirin therapy heals 40-50% of genotype-1-infected patients and 70-80% of genotype-2- and 3-infected patients [9].

An inadequate immune response is one of the major causes of chronic HCV disease. The Toll-like receptors (TLRs) are evolutionarily conserved proteins that play a crucial role in innate and acquired immunity. They recognize a wide range of pathogen-associated molecular patterns (PAMPs) including lipopolysaccharides, peptidoglycans, lipoteichoic acid, and viral DNA and RNA. This stimulates the secretion of inflammatory cytokines and interferon (IFN) and maturation of naïve Th lymphocytes (Th0) into inflammatory (Th1) and helper (Th2) lymphocytes [10, 11].

A growing amount of evidence suggests that single-nucleotide polymorphisms (SNPs) in the TLR genes impair the ability to respond properly to infection [12]. For example, a heterozygous Arg753Gln SNP in the TLR2 gene is more common in children suffering from repeated bacterial infections [13]. The same SNP is associated with tuberculosis, staphylococcal septic shock, heart restenosis, Lyme disease and cytomegalovirus (CMV) disease after liver transplantation [14–17]. Similarly, a homozygous Arg753Gln SNP in the TLR2 gene correlates with transplant rejection and higher mortality of CHCV patients [18]. Moreover, two SNPs in the TLR4 gene, Asp299Gly and Thr399Ile, are associated with sepsis induced by Gram-negative bacteria, respiratory syncytial virus (RSV)-related bronchiolitis in newborns, and repeated lung inflammation in young children [19–21]. Their correlation with rheumatoid arthritis has also been reported [22] but disputed by others [23–26]. Finally, some TLR 2 and 4 polymorphisms correlate with higher HCV loads and more-severe infection. This may be due to the impaired recognition of two HCV proteins, HCV core and NS3 [11, 27, 28].

Based upon these findings, we investigated whether TLR2-Arg753Gln, TLR4-Asp299Gly and TLR4-Thr399Ile polymorphisms correlate with the susceptibility of HCV-infected patients from north-east Croatia to chronic disease.

Materials and methods

Study population

This study comprised 100 individuals from north-east Croatia: 40 healthy (control group) and 60 diagnosed with chronic infection (CHCV group). All CHC patients, HCV antibody and HCV RNA positive, were referred to our department by general practitioners for measurement of HCV RNA viral load in order to assess disease prognosis and response to treatment. The control group consisted of 24 men and 16 women, and their mean age was 41 ± 12 years (age range, 22-68). The CHCV group consisted of 35 men and 25 women, and their mean age was 44 ± 12 years (age range, 23-67). Their blood and plasma samples were collected at the outpatient clinic of the Institute of Public Health of Osijek-Baranja County.

Participants were older than 18 and informed about the study goals and protocol. All of them signed informed consent before joining the study. The study was approved by the Ethics Committee of the Institute of Public Health of Osijek-Baranja County and performed according to ethical principles of the 1975-1983 Helsinki declarations.

HCV detection and genotyping

RNA from study participants was analyzed for HCV using a High Pure System Viral Nucleic Acid Kit (Roche Diagnostics). RNA from HCV-positive samples (CHCV group) was isolated using an Amplicor HCV Specimen Preparation Kit, v 2.0 (Roche Diagnostics) and genotyped using the Linear Array Hepatitis C Virus Genotyping Test (Roche Diagnostics). All procedures were performed according to the manufacturer's instructions.

Detection of TLR polymorphisms

One hundred CHCV patients and healthy individuals were genotyped for TLR2-Arg753Gln (rs5743708), TLR4-Asp299Gly (rs4986790) and TLR4-Thr399Ile (rs4986791) polymorphisms. DNA was extracted from 200 µl of whole blood samples using a High Pure PCR Template Preparation Kit (Roche Diagnostics). Polymorphism analysis was performed by real-time polymerase chain reaction (PCR) using a Light Cycler instrument.

1. TLR2 polymorphism assay. *PCR was performed using 5 µl of DNA, 0.5 µM TLR2 primers (sense 5'-AGTGAGCGGGATGCCTACT-3', antisense 5'-GACTTTATCGCAGCTCTCAGATTTAC-3'), 0.225 µM TLR2*

sensor probe (5'-CAAGCTGCAGAAGATAATGAAC ACCAAG-3'-FL), 0.225 μM TLR2 anchor probe (640-5'-CCTACCTGGAGTGGCCCATGGACG-3'), 4 mM $MgCl_2$, and Light Cycler FastStart DNA master mix in a total volume of 20 μl. PCR amplification consisted of an initial denaturation step (95°C for 10 min) followed by 40 cycles of denaturation (95°C/0 s, 20°C/s), annealing (55°C for 10 s) and extension (72°C for 18 s). The melting curve analysis involved 1 cycle at 95°C for 0 s and 53°C for 30 s, followed by an increase of temperature to 80°C at a slope of 0.1°C/s. Melting temperature values were graphically represented as the negative first derivative of the change in fluorescence with temperature (-dF/dT). The wild-type TLR2 rs5743708 had a melting peak of 70.0°C, whereas the SNP that resulted in the Arg753Gln substitution had a melting peak of 62.9°C.

2. *TLR4 polymorphism assay*. PCR for the detection of the Asp299Gly mutation was performed using 5 μl of DNA, 0.5 μM TLR4 primers (sense 5'-ATTTAAA GAAATTAGGCTTCATAAGCT-3' and TLR4 antisense (5'-CCAAGAAGTTTGAACTCATGGTAA-3'), 0.225 μM TLR4 sensor probe (5'-ACTACCTCGAT GATATTATTGACTTATT-3'-FL), 0.225 μM TLR4 anchor probe (640-5'-AATTGTTTGACAAATGTT TCTTCATTTTCC-3'), 4 mM $MgCl_2$, and Light Cycler FastStart DNA master mix in a total volume of 20 μl. PCR for the detection of the Thr399Ile mutation was performed as above using the following primers: TLR4 primers-2 (sense 5'-AAGAAAT-TAGGCTTCATAAGCT-3' and TLR4 antisense-2 (5'-CCAAGAAGTTTGAACTCATGGTAA -3') at 0.5 μM, 0.225 μM TLR4 sensor-2 probe (5'-CTTG AGTTTCAAAGGTTGCTGTTCTCAAAGT-3'-FL), and 0.225 μM TLR4 anchor-2 probe (705-5'-ATTTTGGGACAACCAGCCTAAAGTAT-3'). The PCR amplification consisted of an initial denaturation step (95°C for 10 min) followed by 45 cycles of denaturation (95°C/5 s, 20°C/s), annealing (54°C/10 s, 20°C/s) and extension (72°C/25 s, 20°C/s). The melting curve analysis involved 1 cycle at 95°C for 20 s and 40°C for 20 s, followed by an increase of temperature to 85°C at a slope of 0.2°C/s. Melting temperature values were graphically represented as the negative first derivative of the change in fluorescence with temperature (-dF/dT). The wild-type TLR4 rs4986790 had a melting peak of 60.3°C, whereas the SNP that resulted in the Asp299Gly substitution had a melting peak of 52.8°C. The wild-type TLR4 rs4986791 had a melting peak of 65.2°C, whereas the SNP that resulted in the Thr399Ile substitution had a melting peak of 59.5°C. TLR4 SNPs were identified using multiplex PCR.

Table 1 Age- and sex-dependent distribution of HCV genotypes in CHCV patients

Age group (years)	Gender	Genotype			
		1	2	3	4
21-30	M (3)	1	0	2	0
	W (6)	5	0	1	0
31-40	M (11)	7	0	2	2
	W (6)	2	0	2	2
41-50	M (9)	8	0	1	0
	W (3)	2	0	1	0
51-60	M (10)	10	0	0	0
	W (7)	7	0	0	0
>61	M (2)	2	0	0	0
	W (3)	2	1	0	0
Total	60	46	1	9	4
Mean age		47 ± 12	63	34 ± 8	34 ± 2
Age range		23-67		23-48	33-37
Median age		50		36	33

M, men; W, women

Antiviral treatment and determination of viral load

Quantitative determination of HCV RNA (COBAS® TaqMan® HCV Test, Roche Diagnostics) was performed in 60 CHCV patients. Thirty-three of them were treated according to the standard-of-care therapy for patients with chronic HCV infection. The therapy consisted of the combined administration of peginterferon (pegIFN) and ribavirin (RBV) for 24 (genotypes 2 and 3) or 48 weeks (genotypes 1 and 4). Viral loads were re-measured at weeks 28 and 53 after the beginning of treatment. The efficiency of antiviral treatment was estimated from the time course of viremia.

Statistical analysis

A χ^2 test was used for the analysis of age- and sex-specific distribution of HCV infection. The chi-square (χ^2) and Fisher-Freeman-Halton tests were used to determine the significance of the TLR allele distribution between control and CHCV groups. The nonparametric Mann-Whitney U test was used to compare the distribution of two variables. The magnitude of association between TLR polymorphisms and chronic disease was expressed as an odds ratio (OR) with a 95% confidence interval (95% CI). The Hardy-Weinberg test of the equilibrium of genotype frequencies was performed using the exact test (HWsim program, 50000 Monte Carlo simulation steps). The linkage disequilibrium Lewontin coefficients D' and r^2 were calculated to reflect allelic linkage (Haploview program http://www.broadinstitute.org). All differences were considered

Table 2 Allele frequencies of TLR2 and TLR4 polymorphisms

	TLR2 Arg753Gln (rs5743708)		TLR4 Asp299Gly (rs4986790)		TLR4 Thr399Ile (rs4986791)	
Nucleotide	G	A	A	G	C	T
Allele frequency	0.98*	0.02*	0.88#	0.12#	0.88#	0.12#

A, adenine; T, thymine; C, cytosine; G, guanine

* Hardy-Weinberg equillibrium rs5743708 [P(CHCV) = 1; P(control) = 1]

Chi^2 test [P(CHCV) = 0.011; P(control) = 0.0006]

to be significant at $P < 0.05$. Multivariate analysis of age and HCV genotype in relation to HCV infection was performed for both sexes using Statistica 12.0 software (StatSoft). The HCV genotype distribution was analyzed using Microsoft Office Excel 2003 (Microsoft).

Results

Distribution of chronic HCV infection in relation to age, sex and viral genotype

Table 1 shows the age- and sex-dependent distribution of CHCV in north-east Croatia. No significant difference was observed between men and women ($P = 0.9669$). Table 1 shows that the genotype 1 was the most common genotype in CHCV patients (77%). This was followed by genotypes 3 (15%), 4 (7%) and 2 (one patient). Chronic infections with genotype 1 or genotype 2 were more common in older patients (median age: 50 and 63 years, respectively) than chronic infections with genotype 3 or genotype 4 (median age: 36 and 33 years, respectively).

Linkage disequilibrium and Hardy-Weinberg analysis of TLR2 and TLR4 SNPs

Both in CHCV patients and healthy individuals, the G allele of TLR4-Asp299Gly had a high level of linkage disequilibrium with the C allele of TLR4-Thr399Ile SNP (Table 2). The linkage disequilibrium Lewontin (D') and correlation (r^2) coefficients were 1, similar to previous reports [19, 31]. The TLR2-Arg753Gln SNP matched the Hardy-Weinberg equilibrium in both study groups ($P = 1$). On the other hand, the TLR4-Asp299Gly and TLR4-Thr399Ile SNPs did not obey Hardy-Weinberg principle (CHCV group, $P = 0.011$; control group, $P = 0.0006$). The primers used to genotype both SNPs were successfully used in other genotyping, suggesting that the absence of equilibrium was due to the small sample size or chance and not a technical issue.

Distribution of TLR2 and TLR4 SNPs in CHCV patients and healthy individuals

Table 3 compares the distribution of TLR2 and TLR4 polymorphisms between 60 CHCV and 40 healthy individuals from north-east Croatia. In both study groups, the frequency of the wild-type (WT) allele of the TLR2-Arg753Gln SNP was significantly higher than the frequency of its heterozygous (HE) and homozygous mutant (MT) alleles ($P < 0.0001$ each). A significantly higher prevalence of the WT than mutated alleles was also observed for two TLR4 polymorphisms ($P < 0.0001$, CHCV and control group). None of the tested individuals showed homozigosity for the TLR2-Arg753Gln SNP.

In comparison to healthy subjects, CHCV patients did not exhibit higher odds for any of the analyzed TLR2 and TLR4 polymorphisms (Table 3). To the contrary, the odds ratio (OR) for having an HE allele in the TLR2 gene and chronic disease was < 1 (OR 0.32; CI 0.03-3.68). Similarly, the odds ratio of having an MT allele in the TLR4 gene and chronic HCV disease was < 1 (OR 0.40; CI 0.11-1.54). These differences could be misleading, however, as they were not statistically significant.

Table 3 also shows the gender-specific distribution of TLR polymorphisms in CHCV patients and control groups. In women, the heterozygous TLR2-Arg753Gln allele was found only in healthy women (5%, 2/16), whereas the heterozygous TLR4-Asp299Gly/Thr399Ile alleles were found only in women with CHCV (3%, 2/25). In CHCV patients, the homozygous TLR4-Asp299Gly/Thr399Ile alleles were found only in men (7%, 4/35). However, as none of these differences were statistically significant, another study with a larger number of participants is necessary to determine whether some TLR polymorphisms exhibit a gender-specific distribution.

Viral loads in CHCV patients in relation to viral genotypes and TLR polymorphisms

Fig. 1 shows that genotype-1-infected patients had significantly higher initial viral loads than those infected with genotype 3 or genotype 4 (Mann-Whitney U test; $P = 0.0428$; $Z = 2.0257$). Moreover, the Mann-Whitney U test showed that the HE and MT carriers of TLR4-Asp299Gly/Thr399Ile polymorphisms had higher viral loads than the WT carriers (Fig. 2). None of the tested individuals were homozygous for the TLR2-Arg753Gln SNP, and only one of the treated CHCV patients was heterozygous. His viral load was > 2 log at the beginning of treatment and undetectable during two follow-up examinations (not shown).

Table 3 Sex-specific distribution and comparison of frequencies of TLR2 and TLR4 polymorphisms between CHCV and control subjects

		Gender		Fisher-Freeman-Halton test				Statistics	
		M (%)	W (%)	*P*		OR	95% CI	χ^2	*P*
TLR2 Arg753Gln (rs5743708)									
CHCV	WT	56.6	41.7		WT vs. HE	59.00	7.92-439.75	41.71	<0.0001
n = 60	HE	1.7	0	1.0000	WT vs. MT	119.00	7.19-1969.08	44.37	<0.0001
	MT	0	0		HE vs. MT	3.00	0.12-75.12	0.99	0.3193
Control	WT	60	35		WT vs. HE	19.00	4.29-84.13	23.74	<0.0001
n = 40	HE	0	5	0.1539	WT vs. MT	77.00	4.57-1296.47	28.74	<0.0001
	MT	0	0		HE vs. MT	5.00	0.23-107.44	1.95	0.1623
CHCV vs. control					HE vs. WT	0.32	0.03-3.68	0.92	0.3384
					MT vs. WT	-	-	-	-
TLR4 Asp299Gly/Thr399Ile (rs4986790/rs4986791)									
CHCV	WT	50	38		WT vs. HE	17.66	5.23-59.66	33.11	<0.0001
n = 60	HE	2	3	0.1810	WT vs. MT	13.25	4.51-38.92	30.93	<0.0001
	MT	7*	0		HE vs. MT	0.75	0.16-3.50	0.13	0.7133
Control	WT	42.5	37.5		WT vs. HE	16.00	3.59-71.30	19.96	<0.0001
n = 40	HE	5	0	0.3159	WT vs. MT	5.33	2.01-14.15	12.68	0.0004
	MT	12.5	2.5		HE vs. MT	0.33	0.06-1.75	1.82	0.1771
CHCV vs. control					HE vs. WT	0.91	0.14-5.72	0.01	0.9160
					MT vs. WT	0.40	0.11-1.54	1.86	0.1723
					HE vs. MT	2.25	0.25-20.13	0.54	0.4642

WT, wild type; HE, heterozygous mutation; MT, homozygous mutation; CHCV, chronic hepatitis C virus

* MT allele of the TLR4 rs4986790 and rs4986791 was detected only in male CHCV patients

Efficiency of antiviral therapy in CHCV patients

The overall efficiency of antiviral therapy in 33 CHCV patients is presented in Fig. 3. Before the beginning of antiviral treatment, all patients except one had > 2 logarithms of viral copies/ml. Follow-up examinations at weeks 28 and 53 showed undetectable HCV viral loads in 33.3% and 57.5% of patients, respectively. A < 2-log decline was detected in 27.3% and 12.1% of patients at weeks 28 and 53, respectively. On the other hand, unchanged viral loads were observed in 27.3% and 18.2% of patients at weeks 28 and 53, respectively. Finally, a > 2-log increase in viral loads was detected in 12.1% of patients at both follow-ups. Comparison of the efficiency of antiviral therapy at weeks 28 and 53 showed that the prolonged application of therapy leads to a statistically higher decrease HCV viral loads (chi-square; $P = 0.0481$, $\chi^2 = 3.91$).

Antiviral treatment appeared to be less efficient in genotype 1-infected patients than in those infected with other genotypes (Fig. 4). For example, the first follow-up examination at week 28 detected no HCV RNA in 30.8% (8/26) of genotype-1-, 40% (2/5) of genotype 3- and 50% (1/2) of genotype 4-infected patients. These numbers were higher during the second follow-up examination at 53 weeks: no RNA was detected in 57.7% (15/26) of genotype-1-, 60% (3/5) of genotype-3- and 50% (1/2) of genotype-4-infected patients. However, 38.5% (10/26) of genotype-1- and 20% (1/5) of genotype-3- but none of genotype-4-infected patients showed a > 2 log increase in HCV RNA at this time point. Additional statistical analysis was not carried out because the genotype 3 and genotype 4 subgroups had an insufficient number of patients.

HE and MT carriers of TLR4-Asp299Gly/Thr399Ile polymorphisms seemed to have a delayed response to antiviral therapy (Fig. 5). While 37.9% (11/29) of WT carriers showed undetectable HCV RNA at week 28, all HE and MT carriers (2/2 each) still contained > 2 log of HCV RNA. At week 53, HCV RNA was undetectable in 55.1% (16/29) of WT, 50% (1/2) of HE and 100% (2/2) of MT carriers. Finally, no therapy response was observed in 34.5% (10/29) of WT and 50% (1/2) of HE carriers. Due to an insufficient number of patients in subgroups HE and MT, additional statistical analysis was not done.

Discussion

To our knowledge, this is the first report on TLR2 and TLR4 polymorphisms and their correlation with viral load in CHCV patients from Croatia. Our results suggest that

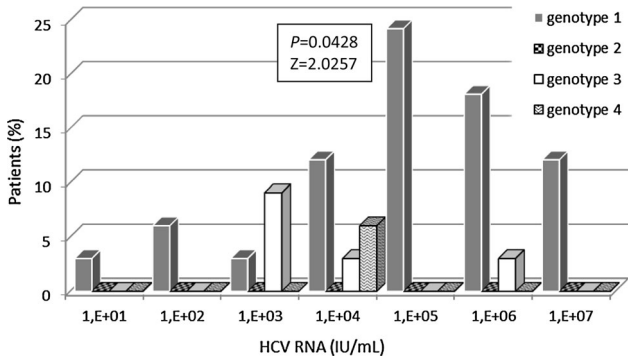

Fig. 1 Viral load in relation to HCV genotype

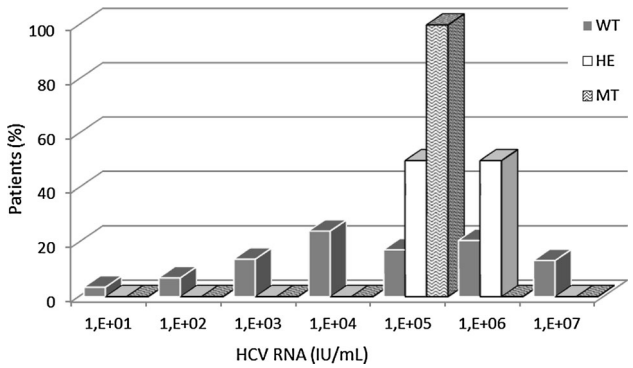

Fig. 2 Viral load in relation to TLR4-Asp299Gly/Thr399Ile polymorphisms (WT, wild-type; HE, heterozygous; MT, homozygous)

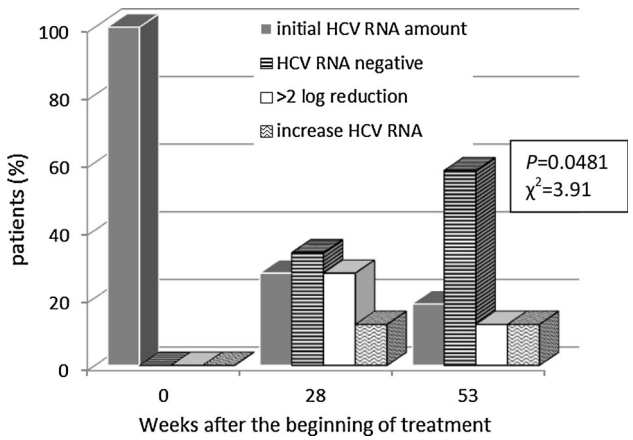

Fig. 3 Virological response to antiviral therapy in CHCV patients

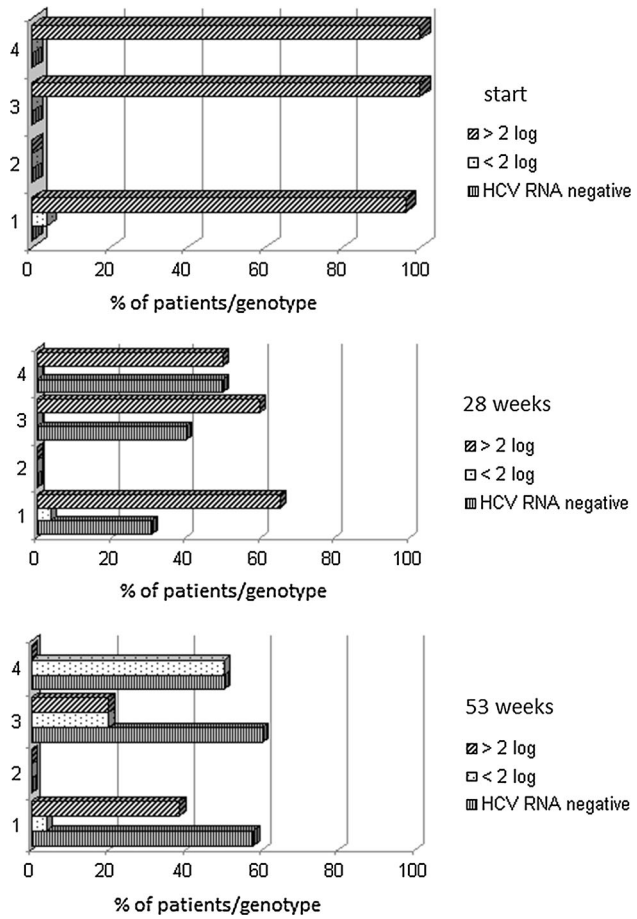

Fig. 4 Virological response to therapy in relation to HCV genotype

the antiviral therapy of chronically infected patients should be response-guided and consider individual TLR4 polymorphisms.

The CHCV patients analyzed in this study were mainly infected with genotype 1 (77%), followed by genotype 3 (15%), which was similar to the distribution of HCV genotypes in other regions of Croatia and Europe [7]. When compared to a study from southern Croatia however, the median age of our genotype-1-infected patients was higher and the prevalence of genotype 3 infection was lower [7]. This is possibly due to the higher proportion of young and/or intravenous drug users in the latter report. In our study, the highest HCV prevalence was in 31- to 40- and 51- to 60-year-old individuals. This is similar to reports from Italy, central Africa, Japan and China, in which the highest rate of HCV infection was found in patients older than 50 [4, 29]. Contrary to our study, the highest HCV prevalence in western Croatia was found in 21- to 30-year-old symptomatic individuals (44%, n = 76). This could also be explained by the higher number of drug users in that region [30]. Finally, we did not observe any significant difference in the HCV prevalence between men and women.

The distribution of TLR2-Arg753Gln, TLR4-Asp299Gly and TLR4-Thr399Ile polymorphisms in 40 healthy and 60 CHCV patients from north-east Croatia was comparable to that in the European population (National

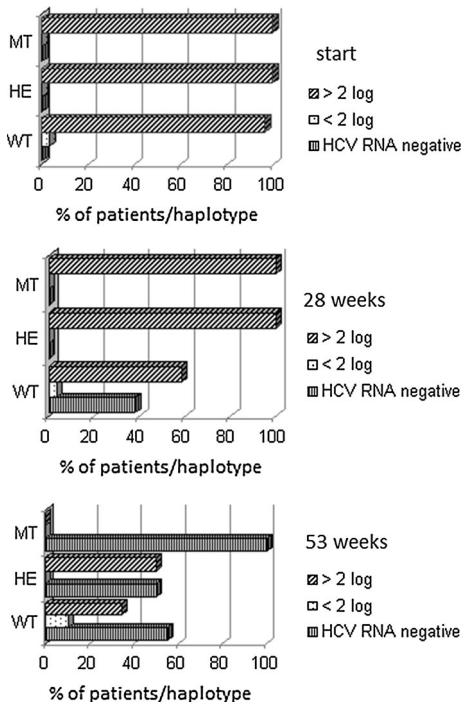

Fig. 5 Virological response to therapy in relation to TLR4-Asp299Gly/Thr399Ile polymorphisms (WT, wild-type; HE, heterozygous; MT, homozygous)

Center for Biotechnology Information, NCBI). As expected from previous studies, the two SNPs in the TLR4 gene were in linkage disequilibrium [19, 31, 32]. Gender-specific different frequencies of TLR SNPs between healthy individuals and CHCV patients were not significant.

Although the highest viral loads were observed in genotype-1-infected patients, their virological response to therapy at week 53 after the beginning of treatment was nevertheless comparable to the responses of genotype-3- and 4-infected patients. At this time point, 57.7% of genotype 1, 60% of genotype 3, and 50% of genotype 4 patients showed no HCV RNA. The efficiency of antiviral therapy in our genotype-3- and 4-infected patients was thus lower than in other studies [33, 34]. Whether this could be due to the more advanced chronic disease in our patients is not known.

The HE and MT carriers of TLR4-Asp299Gly/Thr399Ile SNPs in our study had higher viral loads in the plasma than WT carriers. A similar observation was reported by Pine at al. for the HIV patients co-infected with HCV [31]. It remains elusive how these polymorphisms would affect the viral copy number.

Another interesting finding of our study was that the HE and MT carriers of TLR4-Asp299Gly/Thr399Ile polymorphisms showed a delayed response to antiviral treatment with pegIFN-RBV. In contrast to WT carriers, the viral loads of HE and MT carriers showed no decrease when measured during the first follow-up examination at week 28. The second follow-up at week 53 showed that a half of HE and all MT carriers of TLR4-Asp299Thr/Thr399Ile SNPs responded to antiviral therapy. This suggests that the antiviral treatment of patients with this type of polymorphism should be more closely monitored and continued longer than is recommended as the standard-of-care therapy for CHCV patients.

The mechanism by which TLR4-Asp299Gly/Thr399Ile may influence HCV viral load is currently unknown. TLR4 is located on antigen-presenting cells and recognizes HCV nonstructural protease NS3/4, which induces the release of cytokines, chemokines and their receptors, which are particularly important in response to the virus. This makes TLR4 an essential element of the immune response, especially in the early treatment response. Therefore, TLR4-Asp299Gly/Thr399Ile SNPs have been implicated in delayed responses to antiviral therapy and higher viral loads [10, 26, 34].

In conclusion, our results provide the first evidence of a direct association between the TLR4-Asp299Gly/Thr399Ile polymorphisms and increased viral copy number in CHCV patients from north-east Croatia. As suggested previously, this could explain their delayed response to antiviral therapy [8]. Patients with high viral loads could thus benefit from a modified antiviral therapy, such as its prolongation to 72 weeks. However, as this approach often leads to adverse effects [35], the healthcare system in our country should also consider some alternative approaches. In France, for example, the protease inhibitor telaprevir is given to patients who are unresponsive to pegIFN-RBV therapy. Such alternative treatment has been shown to increase their therapy response to 70% [34, 35].

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