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ORIGINAL ARTICLE

Basic Study

Prevalence of *IFNL3* rs4803217 single nucleotide polymorphism and clinical course of chronic hepatitis C

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Abstract

AIM

To evaluate the association of *IFNL3* (*IL28B*) SNP rs4803217 with severity of disease and treatment outcome in chronic hepatitis C (CHC).

METHODS

The study enrolled 196 CHC Polish patients (82 women and 114 men in age 20-64) infected with hepatitis C virus (HCV) genotype 1. They were treatment naïve and qualified to pegylated interferon alpha (PEG-IFN-α) and ribavirin (RBV) therapy. The analyzed baseline parameters included: degree of inflammation, stage of fibrosis, viral load as well as alanine aminotransferase (ALT), asparagine aminotransferase (AST) and total bilirubin (TBIL). The analysis of response to therapy included: sustained virological response (SVR), defined as undetectable serum HCV RNA level six month after completion of 48-wk therapy, and relapse, defined as achieving undetectable viral load at the end of treatment but not SVR. HCV genotyping and HCV RNA quantification were performed using commercially available tests. DNA was isolated from peripheral blood mononuclear cells or from buccal cell swabs. In addition to rs4803217, also single nucleotide polymorphisms (SNPs) (rs12979860, rs8099917 and rs12980275) of known significance in predicting of HCV clearance were analyzed. SNPs were determined by high resolution melt analysis and confirmed by sequencing of amplicons.

RESULTS

Frequency of rs4803217 genotypes in studied group was as follows: 27.55%; 54.59% and 17.86% for CC, CA and AA, respectively. The rs4803217 SNP, similar to other analyzed SNPs, was not associated with severity of CHC (grade of inflammation, stage of fibrosis, baseline viral load as well as biochemical parameters: ALT, AST, TBIL). It was demonstrated that the rs4803217C allele is associated with SVR (C *vs* A: $P < 0.0001$; dose of C allele: $P = 0.0002$) and non-relapse (C *vs* A: $P = 0.001$; dose of C allele: $P = 0.002$). Moreover, it was found that patients with CC genotype have significantly higher response rates as compared with CA/AA patients ($P < 0.0001$), whereas patients carrying A allele are significantly predisposed to relapse after treatment ($P = 0.0007$). Moreover, the association of rs4803217 with SVR was comparable to that of rs12979860 and stronger as observed for rs12980275 and rs8099917. Association of rs4803217 with relapse, was the strongest as compared with the other SNPs. The analysis of combined rs4803217 and rs8099917 genotypes demonstrated that additional genotyping of rs8099917 had no significant impact on the prediction of SVR. Multivariate analysis revealed that among analyzed SNPs only rs4803217 is an independent predictor of SVR ($P = 0.016$) and relapse ($P = 0.024$).

CONCLUSION

The rs4803217 SNP is a strong, independent and superior predictor of SVR and relapse in HCV genotype 1 infected CHC patients treated with PEG-IFN-α and RBV.

Key words: Hepatitis C virus; Chronic hepatitis C; Interferon lambda 3; Interleukin 28B; rs4803217

Core tip: The rs4803217 single nucleotide polymorphism of *IFNL3* (*IL28B*) gene, which encodes, interferon lambda 3 (interleukin 28B), has been proposed as a causal variant that may influence hepatitis C virus (HCV) clearance. In the present study it was found that rs4803217 is a strong and independent predictor of sustained virological response and relapse in HCV genotype 1 infected chronic hepatitis C patients treated with pegylated interferon alpha and ribavirin. Moreover, it was indicated that rs4803217 seems to be much better predictor of therapy outcome than well-establish *IFNL3* SNPs (rs12979860, rs8099917 and rs12980275).

Świątek-Kościelna B, Kałużna E, Strauss E, Nowak J, Bereszyńska I, Gowin E, Wysocki J, Rembowska J, Barcińska D, Mozer-Lisewska I, Januszkiewicz-Lewandowska D. Prevalence of *IFNL3* rs4803217 single nucleotide polymorphism and clinical course of chronic hepatitis C. *World J Gastroenterol* 2017; 23(21): 3815-3824 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i21/3815.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i21.3815

INTRODUCTION

Hepatitis C virus (HCV) infection is considered as a global health problem. World Health Organization (WHO) reported that 130-170 million people are chronically infected with HCV worldwide and that 3-4 million people are newly infected per year[1,2]. In Poland, the estimated number of people infected is approximately 730000[3]. Spontaneous viral clearance occurs in about 20% of HCV-infected individuals, whereas others (75%-85%) develop chronic infection (chronic hepatitis C, CHC), which is considered as a major cause of liver fibrosis. 10%-15% of HCV-infected subjects progress to liver cirrhosis and 1%-4% of them develop hepatocellular carcinoma (HCC)[4]. It is estimated that about 350000 people die each year from HCV-related liver disease[2]. The treatment of CHC with pegylated interferon alpha (PEG-IFN-α) and ribavirin (RBV) in patients infected with HCV genotype 1, predominant genotype in Poland, results in sustained virological response (SVR), defined as undetectable serum HCV RNA six month after completion of therapy, only in about 50% of cases[5]. Moreover, it is estimated that about one-third of CHC patients with undetectable serum HCV RNA at the

end of treatment (end of treatment response, ETR) experience virological relapse[6]. Response to anti-HCV treatment as well as course of HCV infection may be influenced by age, gender, ethnicity, HCV genotype, viral load[7] as well as genetic factors[8].

IFNL3 (IFN-λ3, IL28B) along with IFNL1 (IFN-λ1, IL29) and IFNL2 (IFN-λ2, IL28A) belong to the interferon-λ (IFNL) cytokine family, all of which are encoded by genes clustered on chromosome 19. IFNLs, similar to IFN-α and β, act through the Janus kinase and signal transducer and activator (JAK-STAT) pathway and upregulate transcription of interferon stimulated genes (ISGs), which are required to control viral infection. Moreover, it was found that IFNL3 affects the adaptive immune response as well as has the ability to inhibit HCV replication *in vitro*[9,10]. A series of genome-wide association studies (GWAS) have independently shown that single nucleotide polymorphisms (SNPs) near the *IFNL3* (*IL28B*) gene are strongly associated with response to IFN-based therapy in CHC patients[11-14] and with natural clearance of HCV RNA[14,15]. Among them rs12979860[11] (actually located within intron 1 of *IFNL4*), rs8099917[12,13] and rs12980275[13] have a repeatedly proven role in predicting SVR in HCV genotype 1 patients. The exact mechanism responsible for this strong relationships remain, however, unclear.

In 2014, McFarland *et al*[16] reported the identification of a functional SNP rs4803217, located in the 3′ untranslated region (UTR) of *IFNL3* (*IL28B*) which is in strong linkage disequilibrium (LD) with rs12979860 in Caucasian and Asian population. They performed *in vitro* analyses and found that occurrence of this SNP results in altered stability of the transcript. The authors indicated that favorable rs4803217 allele is associated with decreased HCV-induced degradation of IFNL3 mRNA and in this way with enhanced HCV elimination. Based on these results, the rs4803217 SNP has been identified as a causal variant that may affect HCV clearance[16]. Number of studies in which rs4803217 was analyzed in CHC patients regarding disease severity and therapy outcome as well as in relation with *IFNL3* (*IL28B*) SNPs of known significance in HCV infection (rs12979860, rs8099917 and rs12980275) is, however, limited.

The study aims at analyzing the association between *IFNL3* SNP rs4803217, compared with well-established SNPs (rs12979860, rs8099917 and rs12980275), and severity of liver disease as well as treatment outcome in Polish CHC patients infected with HCV genotype 1. The goal was realized through the analysis of relationship of above-mentioned SNPs with degree of inflammation, stage of fibrosis, baseline level of HCV RNA and biochemical parameters (alanine aminotransferase ALT, asparagine aminotransferase AST, total bilirubin TBIL) as well as SVR and relapse after PEG-IFN-α and RBV combined therapy.

MATERIALS AND METHODS

Patients

The study population included 196 patients (82 women and 114 men in age 20-64) diagnosed with CHC. All patients were of Polish Caucasian origin (Wielkopolska region). They were infected with HCV genotype 1 (1a: *n* = 16, 1b: *n* = 173, 1a + 1b: *n* = 7) and were treatment-naïve. The exclusion criteria included: coexistence of hepatitis B or human immunodeficiency virus infection as well as other chronic liver diseases.

All patients were qualified for 48-wk therapy with standard doses of PEG-IFN-α-2a (PEGASYS®, Roche; 180 or 135 μg per week; *n* = 78) or PEG-INF-α-2b (PEGINTRON®, Schering-Plough; 1.5 μg/kg of body mass per week; *n* = 118) combined with weight-based dose of RBV (COPEGUS®, Roche or REBETOL®, Schering-Plough; 1000 mg per day if body weight was < 75 kg or 1200 mg per day if body weight ⩾ 75 kg). The liver biopsy was performed in 114 patients before therapy. It provides information on the grade (degree of inflammation that reflects ongoing liver disease injury) and the stage (amount of currently established fibrosis). The histologic status of liver biopsy specimens was scored using the Scheuer scoring system. Biochemical parameters analyzed at the start of therapy included baseline level of ALT (available: *n* = 196; abnormal: *n* = 129, 65.82%), AST (available: *n* = 194; abnormal: *n* = 88, 45.36%) and TBIL (available: *n* = 189; abnormal: *n* = 10, 10.58%). The median value for baseline viral load was 7.945×10^4 (range: 0.0063×10^4 to 2030×10^4).

Blood samples were obtained before (on the day of treatment initiation), at week 4, 12, 24 and 48 of treatment as well as 24 wk after the end of treatment. In 62 cases buccal swabs samples were obtained before therapy initiation.

The study was conducted in compliance with the relevant laws and guidelines in accordance with the ethical standards of the Declaration of Helsinki and was approved by the local ethical committee of the Poznan University of Medical Sciences (no. 650/12).

DNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated from 5 ml of venous ethylenediaminetetraacetic acid (EDTA)-blood by Histopaque®-1077 (Sigma-Aldrich, United States) gradient centrifugation (1.077 g/mL). Genomic DNA was extracted from 1×10^6 PBMCs using QIAamp® DNA Mini and Blood Mini Kit (Qiagen, Germany) or from buccal swabs using Invisorb® Spin Tissue Mini Kit (Stratec molecular, Germany), according to the manufacturer's instructions.

IFNL3 SNPs genotyping

The genotyping of *IFNL3* gene SNPs were determined

by high resolution melt (HRM) analysis. The PCR amplification reactions were performed in a final volume of 12.5 μL. Each reaction contained: 10 ng of genomic DNA, 0.7 μmol/L of each primer and 1x HRM PCR Master Mix (Qiagen, Hilden, Germany; containing HotStarTaq® Plus DNA Polymerase, Type-it HRM PCR Buffer with EvaGreen® dye, Q-Solution® and dNTP mix). The following PCR conditions were used: 95 ℃ for 5 min, followed by 45 cycles of pre-incubation at 95 ℃ for 10 s, annealing at 55 ℃ (rs12979860, rs8099917), 56 ℃ (rs4803217) or 58 ℃ (rs12980275) for 30 s (rs12979860) or 35 s (rs8099917, rs12980275, rs4803217) and extension at 72 ℃ for 10 s. The HRM step was performed from 80 to 95 ℃ (rs12979860), 65 to 85 ℃ (rs8099917), 70 to 85 ℃ (rs12980275) or 70 to 90 ℃ (rs4803217), raising the temperature by 0.1 ℃/s degree at each step. All reactions were performed in triplicate on a Rotor-Gene® Q apparatus (Qiagen, Hilden, Germany). Three controls (homozygous wild type, heterozygous, and homozygous mutant), earlier confirmed by sequencing, were included in each run. The obtained plots were analyzed if confidence percentage threshold was ⩾ 90% for at least two of the three replicates.

To confirm the genotyping results, PCR products from at least 15% of samples for each SNPs were further purified with thermosensitive Exonuclease I and FastAP Alkaline Phosphatase (Fermentas, Thermo Fisher Scientific, United States) and sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit on an ABI Prism 3130XL Analyzer (Applied Biosystems, Foster City, CA, United States) according to manufacturers' protocols.

Primer sequences and PCR product sizes are shown in Supplementary Table 1.

HCV genotyping and HCV RNA quantification

Viral RNA was isolated from serum using Invisorb® Spin Virus RNA Mini Kit (Stratec Molecular, Berlin, Germany). HCV genotyping was performed using VERSANT® HCV Genotype 2.0 Assay (LiPA; Siemens Medical Solution Diagnostics, Tarrytown, NY, United States). Serum HCV RNA qualitative and quantitative detections were performed by RT-PCR technique using GeneProof HCV PCR Kit (GeneProof, Brno, Czech Republic) with the limit of detection of 36.173 IU/mL. In order to determine response to treatment, serum HCV RNA levels were prospectively evaluated before initiation of treatment (week 0), at week 4, 12, 24 of treatment, at the end of treatment (week 48) and 24 wk after the end of treatment.

Statistical analysis

Genotype frequencies were tested for Hardy-Weinberg equilibrium by the χ^2 test (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Haploview v4.2 software was used for linkage disequilibrium (LD) analysis.

Qualitative variables were expressed as number and percentage and compared between groups using Pearson's χ^2 test of Fisher exact test as appropriate and OR and 95%CI were calculated. Quantitative variable were expressed as median and range and compared using Mann-Whitney *U* test.

In a univariate analysis of factors associated with treatment outcome (SVR, relapse) in addition to SNPs, other parameters assessed at the start of therapy (such as: age and gender, HCV genotype, baseline viral load, type of IFN, baseline biochemical /ALT, AST, TBIL/ and histopathological/grade and stage of CHC/parameters) were taken into account. In univariate analysis of the factors related with severity of CHC (grading, staging) in addition SNPs, only patients' gender and HCV genotype were considered because other possible cofounders were not assessed at the moment of liver biopsy. Variables that were significantly associated with treatment outcome/CHC severity were included in a multivariate logistic regression analysis.

All calculations were performed using GraphPad Prism software v6.04 and Statistica software v8.0. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Groups of patients

Patients were divided into the following groups: *G0-G1* - patients with mild hepatic tissue inflammation (n = 48), *G2-G4* - patients with obvious hepatic tissue inflammation (n = 66), *S0-S1* - patients with mild liver fibrosis (n = 69), *S2-S4* - patients with severe liver fibrosis (n = 45), "response group" - patients who achieved SVR after completion of 48-wk therapy (n = 54), "non-response group" - patients without SVR (n = 83) and patients who terminated therapy at week 12 or 24 due to its ineffectiveness (n = 43), "relapse group" - patients who achieved ETR (at week 48) but not SVR (n = 39), "non-relapse group" - patients who achieved ETR and SVR (n = 38).

IFNL3 SNPs allele and genotype frequencies

Allele and genotype frequencies of *IFNL3* (*IL28B*) SNPs in studied group (n = 196) are shown in Table 1. The genotype distribution of all four SNPs showed no deviation from the Hardy-Weinberg equilibrium.

It was found that the rs4803217 SNP was in strong LD with rs12979860 (r^2 = 0.75) and rs12980275 (r^2 = 0.68). Therefore, the frequencies of allele of these SNPs are almost identical. In consequence, it can be assumed that there is no point to include these variants in the combination analysis. Lower association was found between rs4803217 and rs8099917 (r^2 = 0.36). The combination of rs4803217 and rs8099917 resulted in the following combined genotypes: CA/TG (53.06%), CC/TT (20.41%), AA/GG (8.16%), AA/TG (7.65%), CC/TG (7.14%), CA/GG (1.53%).

Table 1 Frequencies of alleles and genotypes of analyzed *IFNL3* (*IL28B*) single nucleotide polymorphism in the group of patients enrolled (n = 196) n (%)

SNP	Allele frequency	Genotype frequency
rs4803217 (C/A)	C: 215 (54.85) A: 177 (45.15)[1]	CC: 54 (27.55) CA: 107 (54.59) AA: 35 (17.86)
rs12979860 (C/T)	C: 212 (54.08) T: 180 (45.92)[1]	CC: 53 (27.04) CT: 106 (54.08) TT: 37 (18.88)
rs8099917 (T/G)	T: 252 (64.29) G: 140 (35.71)[1]	TT: 75 (38.27) TG: 102 (52.04) GG: 19 (9.69)
rs12980275 (A/G)	A: 227 (57.91) G: 165 (42.09)[1]	AA: 64 (32.65) AG: 99 (50.51) GG: 33 (16.84)

[1]MAF (minor allele frequency). SNP: Single nucleotide polymorphism.

Association between IFNL3 gene SNPs and severity of disease

It was found that there is no association between the rs4803217 SNP and degree of inflammation as well as stage of fibrosis. Moreover, no relation was indicated between rs4803217 variants and baseline viral load and level of biochemical parameters (ALT, AST, TBIL). Similarly, no statistically significant associations were found concerning other SNPs (Supplementary Table 2). Moreover, as we indicated previously[17], patients' gender and HCV genotype has no influence on histological grading and staging of CHC in studied group (Supplementary Table 2[17]).

Association between IFNL3 gene SNPs and CHC therapy outcome

Sustained virological response: It was found that the C allele of the rs4803217 SNP is associated with SVR (OR_{crude} = 4.848, 95%CI: 2.871-8.186, P < 0.0001; dose of C allele: OR_{crude} = 7.588, 95%CI: 3.858-14.922, P < 0.0001). Moreover, significantly higher response rates were observed in patients with CC genotype (70.83%) as compared with CA/AA (15.15%) (OR_{crude} = 13.6, 95%CI: 6.219-29.77, P < 0.0001). Furthermore, it was found that AA genotype is associated with non-response: SVR was observed in 8.82% of AA patients and in 34.93% of CC/CA patients (OR_{crude} = 5.547, 95%CI: 1.617-19.035, P = 0.003) (Table 2).

Statistically significant associations with SVR was observed also for other analyzed SNPs. The favorable alleles of rs12979860, rs8099917 and rs12980275 were C, T and A, respectively. Moreover, it was found that rs12979860CC, rs8099917TT and rs12980275AA homozygous genotypes were significantly associated with SVR, whereas rs12979860TT and rs12980275GG genotypes with non-response (Table 2).

In the analysis of other factors which may influence anti-HCV treatment outcome, we previously indicated that stage of fibrosis (S) have significant effect[17]. It was demonstrated that mild liver fibrosis (S0-S1) is associated with "responder" (SVR) status (OR = 0.347, 95%CI: 0.133-0.903, P = 0.027) (Supplementary Table 3[17]). After adjustment for staging, relationship with SVR, observed in dominant model and in allelic dosage analysis, remained significant for all SNPs besides rs8099917 (for rs4803217: CC/CA + AA: $OR_{adjusted}$ = 8.357, 95%CI: 3.013-23.181, P < 0.0001; dose of C allele: $OR_{adjusted}$ = 4.310, 95%CI: 1.956-9.5, P = 0.0002) (Table 2).

When the association with SVR was compared between analyzed SNPs, it was found that for rs4803217 it is slightly lower but the most similar to observed for rs12979860 ($OR_{adjusted}$ = 8.357 *vs* 9.765 in recessive model and 4.310 *vs* 5.574 in allele dose model for rs4803217 *vs* rs12979860, respectively). Lower $OR_{adjusted}$ values were observed for rs12980275 (5.225 and 3.596 in recessive and dose allele model, respectively) whereas the lowest for rs8099917 (2.097 and 1.896 in recessive and dose allele model, respectively). When OR_{crude} values were compared, it was found that the association with SVR observed for rs4803217 is even the strongest in each model (recessive model: 13.6 *vs* 11.636 *vs* 2.588 *vs* 8.249; dominant model: 5.547 *vs* 4.255 *vs* 2.473 *vs* 3.737; dose allele model: 7.588 *vs* 6.072 *vs* 2.190 *vs* 4.705 for rs4803217 *vs* rs12979860 *vs* rs8099917 *vs* rs12980275, respectively) (Table 2).

In a multivariate logistic regression analysis all SNPs in allele dose model together with stage of fibrosis were included. It was found that only rs4803217 and stage of fibrosis are independent predictors of SVR (OR = 4.979, 95%CI: 1.344-18.444; P = 0.016 and OR = 3.27, 95%CI: 1.108-9.698, P = 0.031 for rs4803217 and stage of fibrosis, respectively) (Table 3).

In the analysis of combined rs4803217 and rs8099917 genotypes, it was found that additional genotyping of rs8099917 had no significant impact on the prediction of SVR (Table 4).

Relapse: It was observed that rs4803217C allele is associated with non-relapse (OR = 0.339, 95%CI: 0.173-0.667, P = 0.001; dose of C allele: OR = 0.296, 95%CI: 0.134-0.653, P = 0.002). By stratifying patients on the basis of genotype (CC *vs* CA/AA), CC patients showed lower relapse rate (25%) compared with CA/AA patients (65.31%), which means that patients carrying A allele (CA/AA) are significantly predisposed to relapse after treatment (OR = 0.177, 95%CI: 0.063-0.5, P = 0.0007). No significant association was found when CC and CA genotypes were compared with AA genotype (Table 5).

Statistically significant associations with relapse were observed also for rs12979860 and rs12980275. It was found that rs12979860C allele and rs12980275A allele are associated with non-relapse and that patients carrying rs12979860T (CT/TT) or rs12980275G (AG/GG) allele have significantly higher chance to relapse. No significant results were obtained when CC + CT *vs*

Table 2 Association between *IFNL3* (*IL28B*) single nucleotide polymorphism and sustained virological response after pegylated interferon alpha and ribavirin treatment in chronic hepatitis C patients

SNP	Allele/ genotype	Response (n = 54), n (%)	Non-response (n = 126), n (%)	Response *vs* non-response	OR_{crude} (95%CI)/$OR_{adjusted}$ (95%CI)[1]	P_{crude}/$P_{adjusted}$[1]
rs4803217 (C/A)	C/A	85 (78.70)/23 (21.30)	109 (43.25)/143 (56.75)	C/A	4.848 (2.871-8.186)	< 0.0001[a]
	CC	34 (62.96)	14 (11.11)	CC/CA + AA	13.6 (6.219-29.770)/8.357 (3.013-23.181)	< 0.0001[a]/< 0.0001[a]
	CA	17 (31.48)	81 (64.29)	CC + CA/AA	5.547 (1.617-19.035)/3.240 (0.849-12.367)	0.003[a]/0.082
	AA	3 (55.56)	31 (24.60)	Dose of C allele	7.588 (3.858-14.922)/4.310 (1.956-9.500)	< 0.0001[a]/0.0002[a]
rs12979860 (C/T)	C/T	82 (75.93)/26 (24.07)	108 (42.86)/144 (57.14)	C/T	4.205 (2.533-6.980)	< 0.0001[a]
	CC	32 (59.26)	14 (11.11)	CC/CT + TT	11.636 (5.351-25.306)/9.765 (3.407-27.990)	< 0.0001[a]/< 0.0001[a]
	CT	18 (33.33)	80 (63.49)	CC + CT/TT	4.255 (1.424-12.715)/4.413 (0.927-21.011)	0.006[a]/0.059
	TT	4 (7.41)	32 (25.40)	Dose of C allele	6.072 (3.195-11.538)/5.574 (2.345-13.246)	< 0.0001[a]/< 0.0001[a]
rs8099917 (T/G)	T/G	80 (74.07)/28 (25.93)	149 (59.13)/103 (40.87)	T/G	1.975 (1.2-3.251)	0.007[a]
	TT	29 (53.70)	39 (30.95)	TT/TG + GG	2.588 (1.344-4.981)/2.097 (0.844-5.210)	0.004[a]/0.107
	TG	22 (40.74)	71 (56.35)	TT + TG/GG	2.473 (0.690-8.867)/2.397 (0.482-11.926)	0.153/0.280
	GG	3 (5.56)	16 (12.70)	Dose of T allele	2.190 (1.266-3.788)/1.869 (0.911-3.837)	0.0048[a]/0.0845
rs12980275 (A/G)	A/G	85 (78.70)/23 (21.30)	120 (47.62)/132 (52.38)	A/G	4.065 (2.410-6.857)	< 0.0001[a]
	AA	35 (64.81)	23 (18.25)	AA/AG + GG	8.249 (4.021-16.923)/5.225 (1.999-13.658)	< 0.0001[a]/0.001[a]
	AG	15 (27.78)	74 (58.73)	AA + AG/GG	3.737 (1.244-11.223)/4.075 (0.848-19.589)	0.013[a]/0.076
	GG	4 (7.41)	29 (23.02)	Dose of A allele	4.705 (2.591-8.542)/3.596 (1.659-7.791)	< 0.0001[a]/0.001[a]

[1]Adjusted for staging; [a]$P < 0.05$. SNP: Single nucleotide polymorphism.

Table 3 Multivariate analysis of factors associated with outcome of chronic hepatitis C treatment with pegylated interferon alpha and ribavirin

Variable	OR (95%CI)	P value
Response (SVR)[1]		
rs4803217 (C/A)	4.979 (1.344-18.444)	0.016[a]
rs12979860 (C/T)	1.499 (0.446-5.042)	0.510
rs8099917 (T/G)	0.594 (0.247-1.429)	0.242
rs12980275 (A/G)	1.658 (0.598-4.594)	0.328
Stage of fibrosis	3.278 (1.108-9.698)	0.031[a]
Relapse[2]		
rs4803217 (C/A)	0.134 (0.023-0.789)	0.024[a]
rs12979860 (C/T)	2.381 (0.444-12.773)	0.303
rs12980275 (A/G)	1.046 (0.369-2.961)	0.932

[1]Model summary: χ^2 (5) = 56.028; $P < 0.0001$, constant: $P < 0.0001$. rs4803217, rs12979860, rs8099917, rs12980275 (dose allele model) an stage of fibrosis were included in the analysis; [2]Model summary: χ^2 (3) = 12.337, $P < 0.00632$, constant: $P = 0.019$. rs4803217, rs12979860, and rs12980275 (dose allele model) were included in the analysis. [a]$P < 0.05$ (independent factors). SVR: Sustained virological response.

TT (rs12979860) and AA + AG *vs* GG (rs12980275) genotypes were compared. Moreover, no association with relapse was reported for the rs8099917 SNP (Table 5). Therefore, analysis of combined rs4803217 and rs8099917 genotypes was not performed.

As demonstrated previously[17], no significant association was found between relapse after treatment and other potentially influenced baseline factors (Supplementary Table 3[17]). Therefore results obtained for SNPs were not further adjusted.

When OR values were compared between SNPs in each model, it was observed that among studied SNPs, rs4803217 displays the strongest association with relapse (OR = 0.177 and 0.296 in recessive and dose allele model), whereas rs12979860 and rs12980275 are associated similarly to each other (OR = 0.287 and 0.323 in recessive model, 0.442 and 0.495 in dose allele model for rs12979860 and rs12980275, respectively) yet slightly weaker when compared with rs4803217 (Table 5).

In a multivariate logistic regression analysis the rs4803217, rs12979860 and rs12980275 SNPs were included. It was found that rs4803217 was the only one factor independently associated with relapse (OR = 0.134, 95%CI: 0.023-0.789, P = 0.024) (Table 3).

DISCUSSION

McFarland *et al*[16] proposed the rs4803217 SNP as a causal variant that has influence on innate immune IFN response in the liver. They indicated that rs4803217G variant reduces the binding of AU-rich element (ARE)-binding proteins, impairing the degradation of the *IFNL3* mRNA. Moreover, they showed that in the case of protective rs4803217G

Table 4 Association of *IFNL3* (*IL28B*) rs4803217 and rs8099917 combined genotypes with response to pegylated interferon alpha and ribavirin treatment

Genotypes/combined genotypes	SVR (n = 54), n	Non-response (n = 126), n	OR (95%CI)	P value
rs4803217CC/rs8099917TT	22	13	0.697 (0.276-1.759)	0.442
rs4803217CC/rs8099917TG	12	1	4.941 (0.586-41.699)	0.156
rs4803217CC[1]	34	14	Ref.	-
rs4803217CA/rs8099917TT	5	9	2.813 (0.832-9.504)	0.136
rs4803217CA/rs8099917TG	9	71	0.641 (0.267-1.542)	0.388
rs4803217CA/rs8099917GG	2	1	10.125 (0.865-118.471)	0.083
rs4803217CA	16	81	Ref.	-
rs4803217AA/rs8099917TT	1	2	5.167 (0.355-75.138)	0.298
rs4803217AA/rs8099917TG	1	14	0.738 (0.070-7.736)	1.000
rs4803217AA/rs8099917GG	1	15	0.689 (0.066-7.192)	1.000
rs4803217AA	3	31	Ref.	-

[1]Combination of rs4803217CC and rs8099917GG genotypes was not observed. SNP: Single nucleotide polymorphism; SVR: Sustained virological response.

Table 5 Association between *IFNL3* (*IL28B*) gene single nucleotide polymorphism and relapse after pegylated interferon alpha and ribavirin treatment in chronic hepatitis C patients

SNP	Allele/genotype	Relapse (n = 39), n (%)	Non-relapse (n = 38), n (%)	Relapse *vs* non-relapse	OR (95%CI)	P value
rs4803217 (C/A)	C/A	38 (48.72)/40 (51.28)	56 (73.68)/20 (26.32)	C *vs* A	0.339 (0.173-0.667)	0.001[a]
	CC	7 (17.95)	21 (55.26)	CC *vs* CA + AA	0.177 (0.063-0.500)	0.0007[a]
	CA	24 (61.54)	14 (36.84)	CC + CA *vs* AA	0.332 (0.081-1.364)	0.113
	TT	8 (20.51)	3 (7.89)	Dose of C allele	0.296 (0.134-0.653)	0.002[a]
rs12979860 (C/T)	C/T	40 (51.28)/38 (48.72)	52 (68.42)/24 (31.58)	C *vs* T	0.486 (0.252-0.937)	0.030[a]
	CC	8 (20.51)	18 (47.37)	CC *vs* CT + TT	0.287 (0.105-0.783)	0.013[a]
	CT	24 (61.54)	16 (42.11)	CC + CT *vs* TT	0.538 (0.143-2.013)	0.351
	TT	7 (17.95)	4 (10.53)	Dose of C allele	0.442 (0.211-0.925)	0.028[a]
rs8099917 (T/G)	T/G	56 (71.79)/22 (28.21)	52 (68.42)/24 (31.58)	T *vs* G	1.175 (0.589-2.344)	0.647
	TT	21 (53.85)	17 (44.74)	TT *vs* TG + GG	1.304 (0.526-3.233)	0.566
	TG	14 (35.90)	18 (47.37)	TT + TG *vs* GG	0.750 (0.156-3.560)	1.000
	GG	4 (10.26)	3 (7.89)	Dose of T allele	1.174 (0.582-2.365)	0.649
rs12980275 (A/G)	A/G	45 (57.69)/33 (42.31)	56 (73.68)/20 (26.32)	A *vs* G	0.487 (0.247-0.961)	0.037[a]
	AA	12 (30.77)	22 (57.89)	AA *vs* AG + GG	0.323 (0.127-0.825)	0.017[a]
	AG	21 (53.85)	12 (31.58)	AA + AG *vs* GG	0.647 (0.167-2.503)	0.737
	GG	6 (15.38)	4 (10.53)	Dose of A allele	0.495 (0.245-0.998)	0.046[a]

[a]$P < 0.05$. SVR: Sustained virological response.

variant, two HCV-induced miRNAs (miR-208b and miR-499a-5p) cannot bind to the *IFNL3* mRNA and therefore cannot inhibit its expression. The association observed in the study is consistent with the fact that the probability of HCV clearance in the African population in which the frequency variant rs4803217T is high (T: 55%, G: 45%) is much lower than in the Asian populations (T: 7%, G: 93%)[16]. In other functional analysis of the rs4803217 SNP it was shown that it is associated with remodeling of *IFNL3* mRNA structure and that rs4803217T allele mRNA forms more dynamic 3'UTR structure[18]. In *in vitro* studies using Raji and peripheral blood mononuclear cells it was demonstrated that in the case of the rs4803217G allele expression of *IFNL3* is higher[19]. However, in a subsequent study in which liver biopsy specimens were analyzed, no differences in the level of *IFNL3* expression between CHC patients with GG and GT/TT genotypes were found[20].

In the present study the frequency of rs4803217 genotypes is different from that observed in European (CEU) population (www.1000genomes.org). We found that the unfavorable A allele is significantly more frequent, and that CC genotype is significantly less frequent in patients enrolled in the study as compared to CEU population (C/A: 54.85%/45.15% *vs* C/A$_{CEU}$: 72.2%/27.8%, $P < 0.0001$; CC/CA + AA: 27.55%/72.45% *vs* CC/CA + AA$_{CEU}$: 54.5%/45.5%, $P < 0.0001$). This discrepancy may be explained by the fact that the group enrolled in the study consists only of individuals who did not eliminate the virus spontaneously and develop CHC, therefore higher frequency of unfavorable allele associated with HCV persistence is observed.

In the first part of our study the association between rs4803217 and severity of CHC was evaluated. It was found that, similarly to other analyzed *IFNL3* SNPs, there is no significant relationship between this SNP and grade of inflammation, stage of fibrosis as well as baseline level of HCV RNA and biochemical parameters (ALT, AST and TBIL). The results obtained can be explained by the small number of patients enrolled

in each group. Further study with larger groups are needed to confirm that this SNP is not related with CHC severity. Hitherto, only in one study rs4803217 was analyzed in relation with risk of HCV-related HCC, but no significant association was demonstrated[21].

In the second part of the study, we analyzed the association between the rs4803217 SNP and CHC treatment outcome. It was found that rs4803217C allele is favorable and is significantly associated with SVR as well as non-relapse after antiviral treatment. Moreover, it was demonstrated that patients with CC genotype have more then 8-times higher chance of achieving SVR and more than 5-times higher chance of non-relapse compared with A allele carriers. To our knowledge there is the first study to analyze the association between rs4803217 and relapse after PEG-IFN-α and RBV therapy in CHC patients. In turn, the relation between rs4803217 and SVR in HCV-infected individuals was analyzed only in several studies. All of them confirmed that rs4803217C allele is strongly associated with SVR after IFN-based therapy[22-25]. Only in one study this association was weak, due to a small number of patients enrolled (n = 23, 7 *vs* 16) and the fact that no individual possessed the favorable homozygous genotype[24]. Moreover, in several studies association between rs4803217 and spontaneous HCV clearance was analyzed[21,25-28]. All of them confirmed that favorable rs4803217 allele promotes HCV elimination.

In the present study, when association with SVR was compared between analyzed SNPs, it was found that relation observed for rs4803217 is the most similar to rs12979860, and stronger than for rs12980275 and rs8099917. Moreover, it was indicated that among analyzed SNPs, rs4803217 is the most noticeable linked with relapse. The results obtained for comparison between rs4803217 and rs12979860 for association with SVR are consistent with those reported in HCV/HIV-1 co-infected patients from Barcelona. In that study the authors found that association with response to PEG-IFN-α plus RBV treatment was comparable for rs4803217 and rs12979860 genotypes[22]. Another study, analyzing *IFNL3* variants involved in spontaneous HCV clearance in an Egyptian population, reported slightly weaker genotype association for rs4803217 than for rs12979860[27]. Furthermore, rs4803217 was compared with the *IFNL4*-ΔG/TT frameshift variant (rs368234815, originally designated as ss469415590), which seems to be associated with HCV clearance more strongly than rs12979860. The authors found that *IFNL4*-ΔG/TT is the primary SNP for impaired spontaneous and treatment-induced HCV clearance[25].

Additionally, in the present study combined genotype analysis including the rs8099917 SNP, which is in moderate LD with rs4803217, was performed. It was found that genotyping of rs8099917 had no added benefit for response prediction. Moreover, it should be highlighted that a multivariate analysis revealed that among analyzed SNPs only rs4803217 is an independent predictor of SVR and relapse. It would seem that due to the strong correlation with rs12979860, which is well-established predictor of response to IFN-based anti-HCV treatment, there is no reason for additional testing for rs4803217. Nevertheless, taking into account the fact that rs4803217 is a functional SNP and an independent predictor of SVR and relapse, then it make sense to genotyping of this SNP as superior in predicting IFN-based treatment outcome.

It should be mentioned, that our study was carried out on patients receiving dual therapy before starting the use of direct antiviral agents (DAAs), which recently revolutionized treatment of CHC[29]. There is still need for characterization of the rs4803217 SNP in the context of this anti-HCV therapy. Hitherto, in some studies it was found that *IFNL3* variants with known significance in predicting the outcome of IFN-based treatment may still influence the response to IFN-free DAA treatment[30]. It cannot be excluded that rs4803217, as a causal variant, could have some superior effect. It should be noted, however, that high efficacy of new anti-HCV therapies results in diminished interest in prediction of response.

In summary, in the preset study association between *IFNL3* SNP rs4803217, compared with rs12979860, rs8099917 and rs12980275, and the severity of CHC as well as the outcome of PEG-IFN-α and RBV treatment in 196 Polish patients infected with HCV genotype 1, was analyzed. It was demonstrated that rs4803217, similar to other analyzed SNPs, is not related with severity of disease, yet, it is the only one, among studied SNPs, which is independently associated with SVR as well as relapse. Moreover, the association of rs4803217 with SVR was comparable as observed for rs12979860 and stronger as observed for rs12980275 and rs8099917, whereas association of rs4803217 with relapse, was the strongest as compared with the other SNPs. Based on the results obtained, it can be concluded that in HCV genotype 1 infected CHC patients the functional rs4803217 SNP is a strong and independent predictor of SVR and relapse after PEG-IFN-α and RBV treatment and seems to be superior to the well-established *IFNL3* (*IL28B*) SNPs.

COMMENTS

Background

McFarland *et al* reported the identification of a functional single nucleotide polymorphism (SNP) rs4803217, located in the 3' untranslated region of *IFNL3* (*IL28B*) gene, which alter transcript stability and expression of *IFNL3* mRNA during hepatitis C virus (HCV) infection. The authors indicated that favorable rs4803217 allele is associated with decreased degradation of *IFNL3* mRNA and in this way with enhanced HCV elimination. Based on results obtained, rs4803217 SNP has been proposed as a causal variant that may affect HCV clearance.

Research frontiers

It was repeatedly found that SNPs near the *IFNL3* (*IL28B*) gene (rs12979860,

rs8099917, rs12980275) are good predictors of sustained virological response (SVR) after pegylated interferon alpha (PEG-IFN-α) and ribavirin (RBV) treatment in chronic hepatitis C (CHC) patients. The exact mechanism underlying association between these SNPs and HCV clearance has, however, remained unclear. rs4803217, which is in strong linkage disequilibrium with rs12979860, as a causal SNP, seems to have some importance in this area.

Innovations and breakthroughs

The study aims at analyzing the association between *IFNL3* SNP rs4803217, compared with well-established SNPs (rs12979860, rs8099917 and rs12980275), and the severity of CHC as well as antiviral treatment outcome. Number of such studies in CHC patients is limited. Moreover, to our knowledge it is the first study to analyze the rs4803217 SNP as a predictor of relapse after PEG-IFN-α and RBV treatment in CHC patients.

Applications

The results obtained indicate that the rs4803217 SNP is a strong and independent predictor of SVR and relapse in HCV genotype 1 infected CHC patients treated with PEG-IFN-α and RBV. Moreover, we found that rs4803217 seems to be superior to well-establish *IFNL3* SNPs (rs12979860, rs8099917, rs12980275) in predicting SVR and relapse.

Terminology

SVR - undetectable serum HCV RNA six month after completion of 48-wk therapy; end of treatment response (ETR) - undetectable serum HCV RNA at the end of treatment; relapse - achieving of ETR but not SVR; grade - degree of inflammation that reflects ongoing liver disease injury; stage - amount of currently established fibrosis; PEG-IFN-α - modification of interferon by conjugation with polyethylene glycol, which changes the physical and chemical properties of interferon, and results in an improvement in the pharmacokinetics of the drug.

Peer-review

The reviewers of this paper have classified the manuscript as very good. One of them has emphasized that the authors reported the association of several SNPs of *IFNL3* (*IL28B*) gene with clinical outcome of IFN and RBV treatment in Polish patients infected with HCV genotype 1 and found that rs4803217 is the most relevant SNP to prediction of SVR as well as relapse in CHC patients.

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