Interleukin 28B Gene Polymorphisms in Hepatitis C Virus-related Cryoglobulinemic Vasculitis

Domenico Sansonno, Sabino Russi, Gaetano Serviddio, Vincenza Conteduca, Giovanna D'Andrea, Loredana Sansonno, Fabio Pavone, Gianfranco Lauletta, Maria Addolorata Mariggiò, and Franco Dammacco

ABSTRACT. ***Objective.*** Single-nucleotide polymorphisms (SNP) in the interleukin 28B (IL-28B) gene region are strongly predictive of the response of infected patients to antiviral therapy for hepatitis C virus (HCV). We sought to determine the prevalence of SNP IL-28B rs12979860 C/C and non-C/C (C/T plus T/T) genotypes in HCV-related cryoglobulinemic vasculitis (CV), as compared with HCV-positive patients without CV. We also searched for their association with peculiar clinical manifestations of CV and potential influence on the complete response (virological, molecular, and immunological) to the therapy.

Methods. The study cohort comprised 159 and 172 HCV-infected patients with and without CV, respectively, prospectively followed starting from 1990. SNP rs12979860 genotyping was performed by Taq-Man allelic discrimination. In 106 patients (66.6%) with CV, the profile of circulating B cell clonalities was determined as well. All patients with CV were treated with pegylated interferon-α/ribavirin-based antiviral therapy.

Results. The T/T IL-28B genotype was more common in patients with CV than in those without (17% vs 8.1%, p = 0.02). In patients with CV, compared with non-C/C variants, the IL-28B C/C genotype was associated with a higher rate of complete response (52.6% vs 39.2%, p = 0.13), whereas a treatment response of 61.4% was demonstrated when solely virological response was considered (p = 0.008). A higher frequency of expanded B cell clonalities in the circulation (84.2% vs 55.9%; p = 0.005), kidney involvement (21% vs 2.9%; p = 0.003), and B cell non-Hodgkin lymphoma (17.5% vs 6.8%; p = 0.048), were also observed.

Conclusion. In HCV-positive patients with CV, the IL-28B C/C genotype is distinguished biologically by a higher frequency of restriction of B cell response and clinically by a higher risk of cryoglobulinemic nephropathy and B cell malignancies, while acting as an independent predictor of a sustained virological response to antiviral therapy. In addition, we found that IL-28B T/T variant was more prevalent in patients with CV than in those without. (First Release Dec 1 2013; J Rheumatol 2014;41:91–8; doi:10.3899/jrheum.130527)

Key Indexing Terms:
HCV INFECTION ANTIVIRAL THERAPIES CRYOGLOBULINEMIC VASCULITIS
INTERLEUKIN 28B SINGLE-NUCLEOTIDE POLYMORPHISM

Hepatitis C virus (HCV) plays a key role in generating B cell immunity[1]. Specifically, B cell stimulation by HCV and microenvironmental cosignals may result in the expansion of autoreactive B cells, in turn leading to the production of large amounts of immunoglobulin M (IgM) with rheumatoid factor (RF) activity and to the formation of

From the Section of Internal Medicine and Clinical Oncology, Laboratory of General Pathology and Experimental Oncology, Department of Biomedical Sciences and Human Oncology, University of Bari Medical School, Bari; Section of Internal Medicine, Department of Medical Sciences, and Section of Medical Genetics, Department of Biomedical Sciences, University of Foggia, Foggia, Italy.

Supported by grants from the Italian Medicines Agency, funds for independent studies 2007, contract no. FARM7SJX (DS); Cassa di Risparmio di Puglia Foundation; Italian Association for Cancer Research, and the University of Bari. The funders had no role in the design, conduct, or analysis of the study.

D. Sansonno, MD, PhD; S. Russi, PhD, Section of Internal Medicine and Clinical Oncology, Department of Biomedical Sciences and Human Oncology, University of Bari Medical School; G. Serviddio, MD, PhD; V. Conteduca, MD, Section of Internal Medicine, Department of Medical Sciences, University of Foggia; G. D'Andrea, PhD; L. Sansonno, MD, Section of Medical Genetics, Department of Biomedical Sciences, University of Foggia; F. Pavone, MSc; G. Lauletta, MD, PhD, Section of Internal Medicine and Clinical Oncology, Department of Biomedical Sciences and Human Oncology, University of Bari Medical School; M.A. Mariggiò, MD, Laboratory of General Pathology and Experimental Oncology, Department of Biomedical Sciences and Human Oncology, University of Bari Medical School; F. Dammacco, MD, Section of Internal Medicine and Clinical Oncology, Department of Biomedical Sciences and Human Oncology, University of Bari Medical School.

Address correspondence to Dr. D. Sansonno, Liver Unit, Division of Internal Medicine and Clinical Oncology, Department of Biomedical Sciences and Human Oncology, University of Bari Medical School, Piazza G. Cesare 11, 70124 Bari, Italy.
E-mail: domenicoettore.sansonno@uniba.it

Accepted for publication September 12, 2013.

circulating immune complexes (CIC)[2]. Almost one-third of these CIC, when exposed to low temperatures, become insoluble and thereby mediate a clinically overt cryoglobulinemic vasculitis (CV) that typically involves the small vessels[3].

Clinical improvement of CV is closely associated with the inhibition of HCV replication; the regression of expanded B cell clones in the circulation, bone marrow, and liver; and the disappearance of cryoproteins[4]. Pegylated interferon-α (pIFN-α) plus ribavirin (RBV) and, more recently, the addition of the anti-CD20 monoclonal antibody rituximab have remarkably improved the clinical response of patients with CV. This improvement, however, is usually short-lived and is frequently followed by disease relapse[5]. Although a virological response is invariably associated with the resolution of CV, it can be accompanied by no or only partial improvement of the glomerulonephritis and/or neuropathy, suggesting that factors other than infection adversely affect the clinical outcome[2].

Viral and host factors are predictive of the patient's ability to respond to therapy by achieving a sustained virological response (SVR). Among the former are HCV genotype, levels of HCV RNA, and amino acid substitutions in the HCV core and IFN-sensitivity determining regions[6]. The latter are patient age and sex, and the levels of liver fibrosis, liver steatosis, and insulin resistance[7].

Genome-wide association studies have described single-nucleotide polymorphisms (SNP) on chromosome 19, in the region of the interleukin 28B (IL-28B) gene, as highly reliable predictors of a spontaneous resolution of acute hepatitis C and a response to pIFN-α/RBV therapy, in both the general population[8,9,10,11] and immunocompromised individuals[12]. SNP in the IL-28B rs12979860 C/C genotype is predictive of a favorable response to therapy in genotype-1 HCV infection, and can be used to identify patients who are eligible for a shortened course of therapy[13]. IL-28B status also identifies treatment-naive patients who are likely to achieve an SVR[14].

Whether a clinical prediction model based on IL-28B genotype and clinical features can yield a personalized prediction of treatment success in HCV-related CV has not been established. In addition to SVR, evaluation of treatment response in this condition includes an assessment of the molecular response, inferred from the deletion of activated/expanded B cell clones, and of the immunological response, comprising disappearance of serum cryoglobulins[15]. Occurrence of B cell clonalities has a direct influence on the clinical spectrum of HCV infection, being invariably associated with extrahepatic manifestations[16,17]. In some patients with CV, cryoglobulins persist after successful eradication of HCV infection or else relapse occurs despite an SVR, suggesting that pIFN-α/RBV antiviral therapy only partially interferes with the B cell activities responsible for the persistent production of cold-precipitable CIC[18,19].

The pathogenetic mechanisms underlying the persistence of cryoglobulin production in spite of HCV clearance are unclear, but it is likely that pIFN-α/RBV combined therapy is unable to delete virus-independent B cell clonotypes[15].

Against this background, the aim of this study was to assess the possible significance of IL-28B polymorphisms in HCV-related CV. Specifically, the following issues were addressed: (1) whether the frequency of IL-28B C/C and non-C/C genotypes differed in patients with and without CV; (2) whether IL-28B C/C homozygosity was predictive of treatment response in HCV-related CV; and (3) whether IL-28B polymorphisms were associated with a higher frequency of B cell clonal expansions, as well as with peculiar clinical manifestations of CV disease.

MATERIALS AND METHODS

Patient selection. The present cohort of patients is part of a multiyear study aimed to assess the occurrence of CV in chronically HCV-infected patients, its natural history, and longterm outcome[20]. All HCV-positive patients referred to the Liver Unit of our department were prospectively followed starting from 1990.

Our study comprised 159 patients (106 females, 66.7%) with CV and 172 patients (80 females, 46.5%) without CV and other extrahepatic disorders. Our study was carried out according to the principles of the Declaration of Helsinki and Good Clinical Practice and was approved by the Ethical Review Board of the University of Bari. Informed consent for the collection and storage of their serum and peripheral blood mononuclear cells (PBMC) was obtained from all patients.

Clinical/laboratory evaluation. Baseline evaluation included disease history and stage, current signs and symptoms, and previous medications. Physical examination and laboratory values were recorded. The following inclusion criteria were applied: (1) positivity for anti-HCV antibodies and PCR-based assay to detect HCV RNA in serum of patients with/without palpable purpura; (2) detection of serum cryoglobulins; (3) liver biopsy showing chronic hepatitis performed within 3 months of enrollment; (4) negativity for hepatitis B surface antigen and human immunodeficiency virus antibodies; and (5) no previous administration of IFN or immunosuppressive drugs.

HCV status was assessed based on the presence of detectable serum HCV RNA, as determined by transcription-mediated amplification (Versant, HCV RNA Qualitative assay, Bayer Health Care, Diagnostic Division); the detection limit of this method is 5 IU/ml. HCV RNA levels were measured with a branched-chain DNA assay (bDNA 3.0, Bayer Health Care, Diagnostic Division); the detection threshold is 615 IU/ml. HCV genotypes were determined using the INNOLIPA HCV assay (Innogenetics).

Therapeutic schedules. Subcutaneous injections of 180 μg pIFN-α2a (Pegasys, Hoffmann-La Roche Ltd.) or 1.5 μg pIFN-α2b/kg (Peg-Intron, Merck) were administered to all patients with CV once a week together with a daily dose of 800–1200 mg RBV according to body weight. The median length of followup was 49 ± 11 months. HCV genotypes 1/4 and genotypes 2/3 were treated for 48 and 24 weeks, respectively. A complete response (CR) included SVR, defined as undetectable HCV RNA 24 weeks after the cessation of therapy, in addition to the disappearance of B cell expanded clones from the blood (molecular response), and the non-detectability of cryoproteins (immunological response). The primary endpoint of our study was the proportion of patients who achieved a CR at any time. Partial response was subdivided into type 1 (partial response #1) and type 2 (partial response #2). The former consisted of patients who

achieved SVR but not molecular response and immunological response, whereas the latter identified patients with a molecular response and an immunological response but not an SVR. Patients with none of these responses (SVR, molecular response, immunological response) were regarded as nonresponders.

Detection of cryoglobulins. Serum cryoglobulins were measured, isolated, and purified, as described elsewhere[21]. The monoclonal component of mixed cryoglobulins was characterized by immunofixation.

IL-28B locus rs12979860. Genomic DNA was extracted from the buffy coat of the patients' whole blood and IL-28B genotyped using the ABI Taq-Man allelic discrimination kit (Applied Biosystems) and the ABI7300 sequence detection system (Applied Biosystems). The primers used in the 25-µl Taq-man genotyping master mix were: forward 5′-GCC TGT CGT GTA CTG AAC CA-3′, reverse 5′-GCG CGG AGT GCA ATT CAA C-3′, probe (C allele) 5′-VIC-TGG TTC GCG CCT TC-3′, probe (T allele) 5′-FAM-CTG GTT CAC GCC TTC-3′. The PCR profile consisted of denaturation at 95°C for 10 min, 40 cycles at 92°C for 15 s, and termination at 60°C for 1 min[22].

B cell clonal expansions. DNA was purified from PBMC, as described elsewhere[16]. Twenty µl of reaction mixture were analyzed by electrophoresis on 5% agarose gel in tris-borate-EDTA buffer, stained with ethidium bromide and optically evaluated by UV transillumination. Control mixtures in the reaction were devoid of DNA or included DNA from a clonal cell line. A monoclonal B cell expansion was defined as 1 or 2 (if both alleles were rearranged) discrete narrow bands within the predicted size. Distinction of biclonal monoallelic rearrangements from monoclonal biallelic rearrangements was based on the results of the sequence analyses, in that a nonfunctional rearrangement of 1 of 2 alleles was detected in the case of monoclonal disorders, whereas both dominant bands were representative of a functional IgH rearrangement in biclonal disorders. Because DNA material was sometimes insufficient, IgH VDJ gene segment amplification was carried out in 106 of the 159 patients with CV.

Statistical analyses. Data were expressed as counts and percentages for qualitative variables and as a median with interquartile range for quantitative variables. The Mann-Whitney U test was carried out to compare groups. Fisher's exact test was used to explore associations between different qualitative measures. Pretreatment measures that were identified as significant in the logistic regression model were further assessed in stratified analyses across different categories. Variables were eligible for entry into a multiple logistic regression model if they were significantly associated with the admissions ($p < 0.25$). The κ statistic was used to assess reliability of variables. Given the few comparisons considered sensible, no formal corrections were done[23]. When statistically nonsignificant ($p > 0.05$), variables were eliminated from the multivariate model, and calibration was assessed using Hosmer-Lemeshow goodness-of-fit test[24]. All statistical tests were 2-tailed; the significance cutoff was $p < 0.05$. All the data were analyzed using a statistical package (SAS, version 6.04; SAS Institute).

RESULTS

The frequencies of IL-28B genotypes carrying the SNP rs12979860 were evaluated in all chronically HCV-infected patients, with and without CV. These data, summarized in Figure 1, indicated that the IL-28B rs12979860-T/T genotype occurred significantly more often in patients with CV than in those without (17% vs 8.1%; $p = 0.02$). We also found that C/C homozygosity was less often represented in the CV than in the non-CV group (35.8% vs 43.6%), although this difference was not significant.

All patients were white and of European ancestry. For further analyses, CV and non-CV patients were split into 2 subgroups depending on their C/C or non-C/C genotype; the latter subgroup comprised C/T as well as T/T variants. Univariate analysis consisted of variables that were compared with each other in patients with "favorable" C/C allele carriage and in those with "less favorable" T-alleles. As shown in Table 1, the median log viral load did not significantly differ between IL-28B genotype subgroups. HCV genotypes also had a similar distribution. Thus, in subgroups with and without CV, HCV genotype 1 occurred

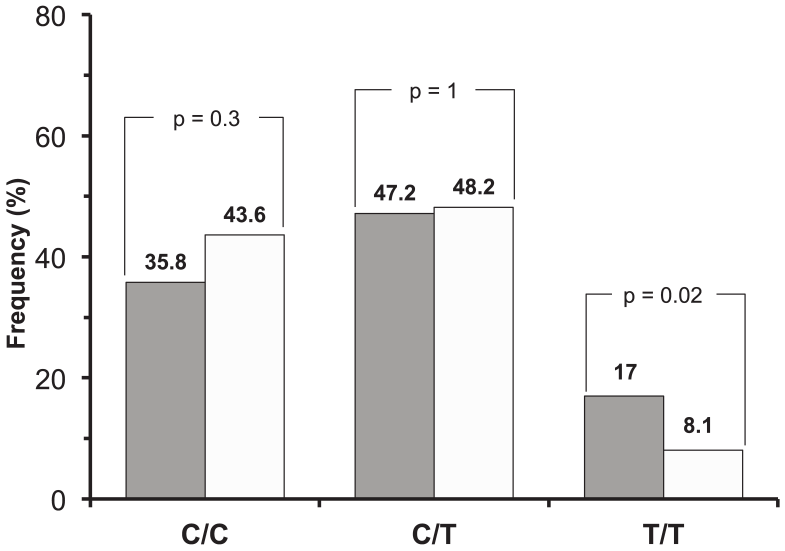

Figure 1. Frequency of interleukin 28B genotypes at the single-nucleotide polymorphism rs12979860 in chronically hepatitis C virus-infected patients with (dark gray shading) and without (light gray shading) cryoglobulinemic vasculitis.

Table 1. Epidemiological and virological variables in chronically hepatitis C virus (HCV)-infected patients with and without cryoglobulinemic vasculitis who were stratified for interleukin (IL)-28B rs12979860 C- and non-C alleles.

Variables	Cryoglobulinemic Vasculitis			
	With, n = 159		Without, n = 172	
	C/C	Non-C/C	C/C	Non-C/C
Patients, n (%)	57 (35.8)	102 (64.2)	75 (43.6)	97 (56.4)
Age, mean ± SD	67.2 ± 9.8	64.8 ± 11.1	66.3 ± 13.6	65.6 ± 14.2
Females, n (%)	41 (71.9)	65 (63.7)	33 (44)	47 (48.4)
Virology				
Serum HCV RNA log IU/ml, median (IQR)	5.9 (5.7–6.4)	5.5 (5.1–6.1)	5.9 (5.6–6.4)	5.9 (5.6–6.3)
HCV genotype, n (%)				
gt-1	28 (49.1)	51 (50)	30 (40)	50 (51.6)
gt-2	25 (43.9)	48 (47)	35 (46.7)	40 (41.2)
gt-3	4 (7)	3 (3)	8 (10.6)	5 (5.1)
gt-4	—	—	2 (2.7)	2 (2.1)
Mode of HCV transmission, n (%)				
Blood/blood products transfusion	10 (17.5)	22 (21.6)	18 (24)	26 (26.8)
Needle-stick puncture	6 (10.5)	28 (27.4)	12 (16)	32 (32.9)
Unidentified	41 (71.9)	52 (50.9)	45 (60)	39 (40.2)

IQR: interquartile range.

in 28 of 57 (49.1%) and in 30 of 75 (40%) patients with the IL-28B C/C variant, and in 51 of 102 (50%) and 50 of 97 (51.6%) patients with the non-C/C variant, respectively. Mean age did not differ among subgroups and the source of HCV infection remained unidentified in the majority of patients.

As shown in Table 2, there was no difference between the C/C and non-C/C subgroups of patients with CV regarding the mean levels and the immunochemical type of cryoglobulins. By contrast, chronic renal damage, including glomerulonephritis and nephrotic syndrome, were significantly more frequent in C/C than in non-C/C variants (21% vs 2.9%, p = 0.003). Similarly, B cell non-Hodgkin lymphoma (B-NHL) prevailed in the C/C variant (17.5% vs 6.8%, p = 0.048). Palpable purpura, weakness, arthralgias/nonerosive arthritis, or the occurrence of cutaneous torpid ulcers did not differ between the 2 groups, and the frequencies of liver diseases, including cirrhosis, and the mean duration of liver disease were roughly comparable. Among laboratory measures, no differences were detected for complement C3 and C4 and C1q concentrations. There were also no differences in serum RF and IgM levels, the distribution of peripheral lymphocyte subpopulations, and mean serum alanine aminotransferase (ALT) and gamma-glutamyl transferase levels.

IgH VDJ gene rearrangement was analyzed in circulating B cells and carried out in step with IL-28B genotyping in 106 of the 159 (66.6%) patients with CV. Our aim was to assess whether IL-28B polymorphisms were associated with profiles of circulating B cell clonalities. As shown in Table 3, C/C patients had a significantly higher frequency of expanded B cell clonalities than did their non-C/C counterparts. B cell monoclonality and oligoclonality were recorded in 32 of 38 (84.2%) C/C and in 38 of 68 (55.9%) non-C/C patients (p = 0.005), while a fully polyclonal pattern was detected in the remaining 6 (15.8%) C/C and 30 (44.1%) non-C/C patients.

The response to pIFN-α/RBV combined therapy is summarized in Table 4. A CR was achieved more frequently in patients with C/C (52.6%) than in those with non-C/C variants (39.2%). HCV genotype 1 was identified in 13 C/C (46.4%) and 18 non-C/C (35.3%) patients, and HCV genotypes 2/3 in 17 (58.6%) and 22 (43.1%) patients, respectively. Overall, a SVR occurred in 35/57 (61.4%) patients with the IL-28B C/C genotype and in 40/102 (39.2%) of those with the non-CC variant (p = 0.008).

A partial response #1 was confirmed in 5 of the 57 (8.8%) C/C patients, but was not detected in any of the 102 patients with the non-C/C variant (p = 0.006). Conversely, 17 (16.7%) patients with the non-C/C genotype, but none of the 57 patients with the C/C genotype, had a partial response #2 (p = 0.0004). In 22 of the 57 (38.6%) and in 45 of the 102 (44.1%) patients with the C/C and non-C/C variants, respectively, response to therapy was consistently absent.

By multivariate analysis, we found that age and cryocrit (but not complement levels, baseline viral load, ALT, RF, or IgM concentration) were significantly associated with a CR to pIFN-α/RBV therapy in patients belonging to either the C/C or the non-C/C variant group (Table 5).

DISCUSSION

Our data are consistent with a role for IL-28B in HCV-related CV, an immune complex disorder with substantial genetic implications and environmental influ-

Table 2. Clinical and immunological variables in 159 patients with hepatitis C virus (HCV)-related cryoglobulinemic vasculitis who were stratified for interleukin (IL)-28B rs12979860 C- and non-C alleles.

Variables	Cryoglobulinemic Vasculitis		
	C/C, n = 57	Non-C/C, n = 102	p
Cryoglobulins			
Cryocrit, median (IQR)	5 (1–45)	4 (1–56)	0.7
Cryoglobulins [immunochemical type, n (%)]			
II	51 (89.5)	90 (88.2)	1
III	6 (10.5)	12 (11.8)	1
Symptomatology, (%)			
Palpable purpura	44 (77.3)	85 (83.3)	0.4
Weakness	36 (63.1)	79 (77.4)	0.1
Arthralgias/nonerosive arthritis	28 (49.1)	68 (66.7)	0.04
Cutaneous ulcers	18 (31.5)	45 (44.1)	0.1
Immunological variables			
IgG (mg/dl, 700–1600), median (IQR)	1250 (698–1499)	1180 (947–1380)	0.7
IgM (mg/dl, 40–230), median (IQR)	220 (136–538)	264 (136–480)	0.8
IgA (mg/dl, 70–400), median (IQR)	110 (94–164)	126 (87–184)	1
Clq (mg/dl, 21–39), median (IQR)	38 (35–41.5)	36 (31–39)	0.6
C3 (mg/dl, 90–180), median (IQR)	102 (82–130)	96 (80–102)	0.2
C4 (mg/dl, 10–40), median (IQR)	4 (3–10)	3 (2–5)	0.3
RF (ng/ml, 10–120), median (IQR)	359 (127–806)	286 (69–511)	0.3
ß2-microglobulin (μg/ml, 0.8–2)	1.9 ± 1.3	1.2 ± 0.7	0.2
Peripheral lymphocytogram, mean ± SD			
CD3 (%, 72 ± 10.2)	66.6 ± 12.4	71.3 ± 13.9	0.1
CD4 (%, 46.1 ± 9.3)	48.3 ± 14	52 ± 12	0.9
CD8 (%, 32 ± 10.5)	42 ± 18	45 ± 10	0.7
CD19 (%, 10.2 ± 5.4)	28 ± 14	24 ± 5	0.9
CD20 (%, 10.2 ± 5.4)	26.9 ± 15.5	23.8 ± 5.5	0.8
Liver involvement			
ALT (IU/l, 30–65), mean ± SD	55.3 ± 27	57.8 ± 30.9	0.6
γGT (IU/l, 5–55), mean ± SD	57.9 ± 17.1	61.8 ± 24.7	0.7
Duration of liver disease (yrs, mean ± SD)	12.5 ± 4.3	14.1 ± 4.16	0.5
Cirrhosis (%)	10 (17.5)	14 (13.7)	0.6
B cell non-Hodgkin lymphoma, n (%)	10 (17.5)	7 (6.8)	0.048
Nodal	8 (80)	6 (86)	1
Follicular	2 (25)	1 (16)	1
Diffuse large B cells	5 (63)	4 (58)	1
Small lymphocyte	1 (12)	1 (16)	1
Extranodal	2 (20)	1 (14)	1
Stomach	1 (50)		—
Spleen		1 (100)	—
Liver	1 (50)		—
Renal disease, n (%)	12 (21)	3 (2.9)	0.0003
Membranoproliferative glomerulonephritis	8 (66.7)	2 (66.7)	1
Nephrotic syndrome	4 (33.3)	1 (33.3)	1

IQR: interquartile range; ALT: alanine aminotransferase; γGT: gamma-glutamyl transferase.

ences[25]. In patients with CV, a high percentage of clonally expanded peripheral B cells is prone to anergy and/or apoptosis, thus accounting for an altered B cell immune response[26]. However, the mechanisms by which causative genes influence the susceptibility and progression of HCV-related CV are poorly understood, because investigations are hampered by the heterogeneity of the clinical phenotypes and the complex interactions between genetic disposition and environment.

In our study, assessing the putative role of the IL-28B gene, stringent criteria were applied in the selection of patients with CV and in defining their response to antiviral therapy. The frequency of the T allele was higher in patients with than in those without CV. In this respect, the C/C genotype is distinguished biologically by a restriction of the B cell response and clinically by the high risk of cryoglobulinemic nephropathy and the higher frequency of B cell malignancies.

The production of IgM-RF molecules with high pathogenic potential is strictly associated with B cell clonal expansions[17]. It can be argued that activated and expanded B cell clonotypes contribute to the formation of harmful

Table 3. Frequencies and profiles of IgH VDJ gene rearrangements in the circulating B cells of 106 patients with hepatitis C virus (HCV)-related cryoglobulinemic vasculitis.

SNP rs12979860 genotype	Patients, n	Circulating B- cells IgH VDJ Gene Rearrangements		p
		Monoclonal Oligoclonal	Polyclonal	
CC, n (%)	38	32 (84.2)	6 (15.8)	0.005
Non-CC, n (%)	68	38 (55.9)	30 (44.1)	0.005
Total	106	70 (66)	36 (34)	—

SNP: single-nucleotide polymorphism.

Table 4. Response to antiviral treatment as a function of viral and host genotypes.

Type of Response	IL-28B SNP rs12979860						p*
	HCV Genotypes, C/C (n = 57)			HCV Genotypes, Non-C/C (n = 102)			
	gt-1, n = 28	gt-2/3, n = 29	Total	Total	gt-1, n = 51	gt-2/3, n = 51	
Complete, n (%)	13 (46.4)	17 (58.6)	30 (52.6)	40 (39.2)	18 (35.3)	22 (43.1)	0.13
Partial #1, n (%)	1 (3.6)	4 (13.8)	5 (8.8)	0	0	0	0.006
Partial #2, n (%)	0	0	0	17 (16.7)	7 (13.7)	10 (19.6)	0.0004
No response, n (%)	14 (50)	8 (27.6)	22 (38.6)	45 (44.1)	26 (51)	19 (37.2)	0.6

* Statistical significance between total C/C and total non-C/C. Complete response includes virological response, molecular response, and immunological response. Partial response #1 includes virological response. Partial response #2 includes molecular response and immunological response. SNP: single-nucleotide polymorphism; IL: interleukin; HCV: hepatitis C virus.

Table 5. Multivariate analysis of variables associated with a complete response to pIFNα/RBV-based therapy in 137 patients with cryoglobulinemic vasculitis.

Variables	IL-28B rs12979860 Genotypes Response to Therapy					
	C/C			Non-C/C (C/T plus T/T)		
	CR, n = 30	NR, n = 22	p	CR, n = 40	NR, n = 45	p
Complement C4, mg/dl, median (IQR)	4.6 (3.1–8.3)	3 (2.5–19)	0.06	4.6 (3–6.2)	3.3 (2–9.8)	0.3
Mean age, yrs, mean ± SD	61.2 ± 5.5	68.8 ± 10.1	0.02	60.1 ± 12.3	69.4 ± 6.8	< 0.0001
Cryocrit, %, mean ± SD	3.7 ± 2.0	8.5 ± 5.1	0.002	5.8 ± 4.6	9.9 ± 5.9	0.004
Serum HCV RNA, log IU/ml, median (IQR)	5.5 (5.2–5.8)	5.6 (5.3–5.8)	0.5	5.6 (5.1–6.3)	5.5 (4.9–5.8)	0.4
ALT, IU/l, mean ± SD	39.7 ± 9.6	62.4 ± 27.3	0.055	63.8 ± 29.9	59.1 ± 32.8	0.1
Rheumatoid factor, IU/ml, median (IQR)	381 (80–1230)	353 (141–1011)	0.5	97 (81–133)	177 (61–967)	0.8
IgM, mg/dl, median (IQR)	404 (266–471)	321 (131–363)	0.9	129 (40–317)	233 (149–455)	0.7

IQR: interquartile range; CR: complete response; NR: no response; pIFNα/RBV: pegylated interferon-α plus ribavirin; IL: interleukin; HCV: hepatitis C virus; ALT: alanine aminotransferase; IgM: immunoglobulin M.

CIC, capable of mediating tissue damage[3]. This may explain the disproportionate distribution of renal damage in patients with CV with the C/C but not the non-C/C variant. In a recent reappraisal of our cohort of patients with HCV-related CV, kidney involvement was present in 32.6% (46/141) of HCV-infected patients with and 3% (18/601) of those without CV (p = 0.0001)[20]. The very low prevalence of renal damage in the latter group provides strong support for the biological and clinical effects of underlying B cell clonal expansions, which are infrequent in these patients[16,17].

Combined pIFN-α plus RBV therapy has been reported as ineffective in achieving complete clinical response in cryoglobulinemic patients with renal function impairment, suggesting that nephropathy may represent an independent factor of resistance to antiviral treatment[27]. However, these findings need to be further clarified and cannot leave out consideration of the exact duration of such therapy, which mainly depends on the viral genotypes. The efficacy of antiviral therapy in HCV-related kidney disease may take longer than the observational period: a metaanalysis has indeed shown that antiviral therapy based on IFN-α can significantly decrease proteinuria and stabilize serum creatinine[28].

Similarly, C/C genotype failed to protect against B-NHL,

which was detected in 17.5% of these patients and in 6.8% of those with non-C/C variants (p = 0.048). A prerequisite for the development of B-NHL is an HCV-driven B cell derangement that interrupts crucial cell regulatory mechanisms[21].

In an Italian study[29], IL-28B polymorphisms were comparatively assessed in 2 groups of HCV-positive patients, with or without mixed cryoglobulinemia, respectively. In terms of SNP distribution and both virological and clinical responses, no significant differences were revealed between the 2 groups. We are unable to explain this discrepancy. Our more stringent diagnostic criteria, longer followup of the patients and assessment of B cell clonal expansion may possibly account for the observed differences.

Our data provide further evidence for the protective nature of the IL-28B rs12979860 C/C genotype as a significant independent predictor of a virological response to pIFN-α/RBV combination therapy. We also found that the C/C genotype was inaccurate in predicting treatment response in almost 9% of the present series of patients with CV who, despite achieving a virological response, had persistent B cell expanded clones, cryoglobulin production, and vasculitic complications. Rather, HCV seems to act as an independent factor capable of activating the immune system, thereby leading to clonal B cell expansions, which become autonomous because of the ongoing infection[30]. The persistence of B cell clonalities implies that these patients are refractory to the direct effect of antiviral therapy, and it accounts for the ongoing cryoprotein production. Previous studies showed that HCV-related CV-dominant B cell clonotypes displayed identical rearrangements and remarkable stability of the IgH VDJ gene, a finding consistent with the antigen-independent nature of these cells[31], likely occurring in an environment favorable to cell immortalization[2].

Conversely, 16.7% of our patient series with non-C/C genotypes were unable to achieve an SVR but did develop a molecular response and an immunological response; thus, the CV outcome was favorable despite ongoing HCV infection. In this subgroup, there was a decline or even a full regression of B cell clonal expansions, although the patients remained viremic. These findings suggest that rs12979860 C and T alleles function as independent genetic protective factors; consequently, they provide additional discriminatory power in the identification of molecular responders to treatment. The biological rationale for the separation of these 2 genetically related effects is unclear. However, the rs12979860 T allele may have a role in virus containment in addition to conferring protection against hepatic necroinflammatory processes and the progression of liver fibrosis[32].

Multivariate analysis identified patients' age and cryocrit levels as responsible for the discordance between IL-28B genotype and the response to therapy. Younger age and lower cryocrit values were independently associated with the response to pIFN-α/RBV. It may be that these factors influence disease duration, which in turn would highlight the importance of treating patients as soon as they are diagnosed.

Although the IL-28B variant was more frequently predictive of response in HCV genotype 1 than in HCV genotypes of 2/3 infected patients, the importance of this distinction would be expected to decrease in patients treated with the new direct-acting antiviral agents[33,34]. Regardless of the use of direct-acting antiviral agents in patients with HCV-related CV, differences between IL-28B genotypes will likely influence response-guided therapeutic decisions in clinical practice. The clinical implications of these genetic associations include the selection of patients with CV most likely to respond to antiviral therapy. They also support the potential utility of determining rs12979860 haplotypes, to predict the clearance of B cell clonal expansions and the regression of HCV-related CV.

Our data show, paradoxically, that the minor (T) allele of IL-28B rs12979860 can be unfavorable to the host by reducing the chances of viral clearance and favorable by reducing the B cell dysregulation at the same time. Although the importance of these findings is not yet clear, they may support the notion that the IL-28B T allele is superior in patients with CV in specific situations where cellular immunity is outstanding, as in the regulation of the adaptive immune response, such as blunting the Treg response[35,36]. It can be speculated that the IL-28B T allele may be in linkage disequilibrium with other polymorphisms with functional effects. This may hint at a potential heterogeneity of the response outcome as demonstrated in the present CV series.

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