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Original Article

Mutations within Protein Kinase R-Binding Domain of NS5A Protein of Hepatitis C Virus (HCV) and Specificity of HCV Antibodies in Pretreatment Sera of HCV-Chronically Infected Patients and Their Effect on the Result of Treatment

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SUMMARY: We analyzed protein kinase R (PKR)-binding domain sequences of hepatitis C virus (HCV) NS5A protein and the profile of HCV-specific antibodies from pretreatment sera of HCV-chronically infected patients. Results were compared with clinical data to verify their influence on the course and result of therapy. Of 9 patients enrolled in a 12-month treatment with pegylated interferon alpha (PEG-IFN-α) plus ribavirin (RBV), 6 patients responded to therapy, as assessed by the lack of HCV RNA in their sera, and 3 did not. Among 8 HCV-1b-infected patients, those who responded did not have significantly more mutations in the IFN sensitivity determining region (ISDR) compared to non-responders ($P = 0.637$). Similarly, in the remaining 26-amino acid region of the PKR-binding domain, behind ISDR, the number of mutations did not differ significantly between the two groups ($P = 0.796$). A correlation was found between the presence of envelope 2 (E2)-specific antibodies and the result of treatment ($P = 0.048$). This pilot study indicates that mutations in the PKR-binding domain of HCV genotype 1b do not correlate with outcome of PEG-IFN-α/RBV therapy. However, the presence of E2-specific antibodies in the pretreatment sera of HCV-chronically infected individuals could serve as a prognostic marker predicting the result of treatment, before its initiation.

INTRODUCTION

In a majority of individuals, hepatitis C virus (HCV) infection progresses into a chronic phase, during which a spontaneous recovery is very rare. This state causes chronic inflammation of and damage to the liver, leading to viral cirrhosis and ultimately, in some cases, to hepatocellular carcinoma (1). According to the World Health Organization, about 170 - 200 million people worldwide have been infected with HCV (2). This presents a serious medical problem, since the first available monotherapy, with the use of interferon alpha (IFN-α), was effective only in a fraction of treated patients (approximately 20%) (3). The introduction of specially formulated interferon, pegylated (PEG)-IFN-α, in combination with ribavirin (RBV), allowed improvement in the overall effect of treatment for up to 54 - 56% of infected patients (4,5).

During infection, the effectiveness of immune responses is counteracted by a high heterogeneity of the virus itself that allowed its classification into six major genotypes (6), and a heterogeneity within an infected individual, relying on emerging closely related but distinguishable variants of the virus, known as quasispecies (7). The latter mechanism is thought to be a way HCV evades the immune pressure (8-11). As a result, the virus not only goes through the acute phase but also establishes itself in a chronic phase, in which it persists because the neutralizing, viral envelope glycoprotein E2-specific antibodies (E2 antibodies) are no longer effective (12). Recent findings have suggested that the ineffectiveness of those antibodies might not only be due to the heterogeneity of E2, particularly in hypervariable region 1 (HVR1), but also to different receptors HCV uses for its entry to a cell (13). In this way, antibodies directed against one part of E2, responsible for binding to one receptor, might not necessarily block the binding of the virus to another receptor.

In addition to heterogeneity, HCV has developed other molecular mechanisms that prevent the action of IFN-α. The viral nonstructural protein 5A (NS5A), a phosphoprotein of 56 - 58 kDa, has been reported to play a role in viral replication (14,15). The interest in this protein arose when in vitro studies indicated that NS5A binds to and inhibits IFN-α-inducible double-stranded RNA-activated protein kinase (PKR) (16,17). The C terminus of NS5A contains a PKR-binding domain, a region of 66 amino acids, of which the first 40 constitute an IFN sensitivity determining region (ISDR) (aa 2209 - 2248) (18,19). Japanese and some European studies have showed that mutations within the ISDR are associated with response to IFN-α treatment in patients infected with HCV-1b (18,20-24). Most of the results from Europe and North America, concerning treatment of patients infected with HCV-1a or -1b, have not confirmed those findings (25-32,44). Authors of a report from Japan had tried to solve this discrepancy by pointing out that this ISDR monitoring system is useful in patients infected with the Japanese-specific (J-type) subtype of HCV-1b, while in the worldwide-specific (W-type) subtype of HCV-1b, it does not apply (33). A meta-analysis focused on geographical differences has shown that distribution of wild- (no mutation), intermediate- (1-3 muta-

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tions), and mutant- (≥4 mutations) type ISDR sequences differed significantly between Japanese and European patients and that at pretreatment viremia of <6.6 log copies/ml, ISDR mutant-type infection was associated with a sustained virologic response rate of 97.1% in Japanese patients but only 52.5% in European patients (34). Other studies have implied that mutations within the 26-amino acid section of the PKR-binding domain, lying behind the ISDR (31,35), and in some variable regions of the C terminus of NS5A, might be involved in response to IFN-α in patients infected with HCV-1a or -1b (31,35-37).

In this pilot Polish study, we investigated mutations in the PKR-binding domain of NS5A from pretreatment sera of HCV-chronically infected patients and analyzed their effect on the result of treatment. The analysis included both the ISDR and the remaining region of the PKR-binding domain. The data were combined with a profile of HCV-specific antibodies in pretreatment sera to obtain a more complex view, on the level of preexisting heterogeneity of HCV and activity of humoral immune response, of the factors influencing the possible outcome of the therapy.

MATERIALS AND METHODS

Patients and treatment: Nine patients from the Department of Infectious Diseases of the University of Medical Sciences in Poznań were enrolled to undergo a combined treatment. Their sera taken before treatment were used in the present study. The Ethical Committee of the University approved the study, and a written consent was obtained from each patient. The histopathological examinations (liver biopsy) of the level of inflammation (grading) and fibrosis (staging) confirmed chronic hepatitis. Other causes of liver damage, such as infection with hepatitis B virus (HBV) or other hepatotropic viruses and alcohol abuse, were excluded. During a 12-month treatment, patients received 180 μg PEG-IFN-α-2a (Pegasys; Hoffmann-La Roche, Nutley, N. J., USA) given as a once-weekly subcutaneous injection plus daily RBV (Hoffmann-La Roche), taken orally at a dose of 600-1,200 mg (according to body weight).

Virological assessment: Viral load was determined quantitatively by using reverse transcription (RT)-PCR (Amplicor Monitor v. 2.0; Roche Diagnostics, Branchburg, N. J., USA, linear range <600->700,000 IU/ml) at the baseline and 3 months from the start of therapy. A qualitative RT-PCR assay (Cobas AmpliScreen v. 2.0 HCV Test; Roche Diagnostics, detection limit 100 IU/ml) was carried out at the end of treatment and 6 months after stopping therapy to assess a sustained virologic response (SVR).

In parallel, total HCV Core antigen (Ag) was quantified by using an Ortho trak-C enzyme-linked immunosorbent assay (Ortho-Clinical Diagnostics, Raritan, N. J., USA), at baseline and 3 months from the start of therapy. According to the manufacturer, the lower limit of detection for this assay is 1.5 pg/ml and the upper limit is 100 pg/ml. Samples with HCV Core Ag higher than 100 pg/ml were retested after dilution.

A line probe assay (INNO-LiPA; Innogenetics, Antwerp, Belgium) was used to genotype HCV.

All assays were performed according to manufacturers' instructions.

The levels of alanine aminotransferase (ALT) were determined 2 weeks before the start of therapy, at baseline, and then during late periods of therapy.

Cloning and sequencing: A total RNA from pretreatment sera was extracted according to Chomczynski and Sacchi (38), using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, Calif., USA) as indicated by the manufacturer. RT was performed in a 30- μl reaction mixture containing 10 μl RNA, 6 μl 5 x buffer (Invitrogen Life Technologies), 1 μl (28 U) RNase inhibitor (RNAzin; Promega, Madison, Wis., USA), 3 μl 0.1 M dithiothreitol, 1.5 μl 2.5-mM deoxynucleoside triphosphates (dNTPs) (Sigma-Aldrich, St. Louis, Mo., USA), 6.5 μl H_2O, 1 μl of (200 U) Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies), and 1 μl (50 pmol) HCV-specific degenerate antisense primer A: 5′-TwGTrGCGAGCTCCGCCAAG-3′, nucleotide (nt) position 7377-7358, as in reference HCV-1b sequence (HCV-J), GenBank accession number D90208 (39). The mixture was incubated for 1.5 h at 37°C, followed by 5 min boiling and a subsequent chilling on ice. A semi-nested PCR was then used to amplify PKR-binding domain sequence. A set of degenerate primers for the first round of amplification consisted of sense primer B: 5′-AGATCGATTGCGAGC CyGAACCGGAyGTG-3′, *Cla*I restriction site underlined, nt position: 6813-6833, and antisense primer A, used in RT. A set of degenerate primers for the second round of amplification consisted of sense primer B and antisense primer C: 5′-CTCTCGAGACmACCGTCCkCTTCyTCCG-3′, *Xho*I restriction site underlined, nt position: 7330-7311. In the first round of amplification, 4 μl of cDNA was added to 16 μl PCR mixture containing: 1.6 μl 2.5 mM dNTPs (Sigma-Aldrich), 0.4 μl each 20 μM primer, 2 μl 10 x buffer, and 0.2 μl 3 U/μl high-fidelity pfu polymerase (Promega). The PCR mixture for the second round of amplification contained the same reagents, except one primer and a template, which was 4 μl of 20-fold diluted DNA from the first-round PCR. In both amplifications, the cycling parameters were as follows: 1 min at 94°C; then 40 cycles of 1 min at 94°C, 2 min at 45°C, 3 min at 72°C; and a single final extension step for 7 min at 72°C. DNA products, 517 bases long, were purified, digested with restriction enzymes *Cla*I and *Xho*I (Roche Diagnostics GmbH, Mannheim, Germany), ligated into a plasmid pBluescript SK+/− (Stratagene, La Jolla, Calif., USA) digested in the same way, and used to transform the competent *Escherichia coli* cells (strain XL1-Blue; Stratagene). After the recombinant colonies had been detected, they were picked up and amplified in 2 ml of LB broth (Invitrogen Life Technologies), followed by the isolation of plasmids. The recombinant plasmids, corresponding to each patient, were subjected to sequencing with the use of fmol Sequencing System (Promega) and the CY5 labeled standard T7 primer, according to manufacturer's protocol. The electrophoretic separation and determination of the labeled fragments was carried out in 6% polyacrylamide gel containing 8 M urea, at 1,500 V, 60 mA, and 25 W, using ALFexpress sequencer and software (Amersham-Pharmacia Biotech, Uppsala, Sweden). The data were further analyzed for an amino acid sequence by using the OMIGA 2.0 program (Genetic Computer Group, Oxford Molecular Company 1999, Madison, Wis., USA).

Enzyme immunoassay: The presence of HCV-specific antibodies in pretreatment sera was determined by a commercial LiaTek HCV III test (Organon Teknika N.V., Turnhout, Belgium), according to the instruction of the manufacturer. Briefly, nylon strips, covered with HCV antigens specific for structural and nonstructural parts of HCV genome, C1, C2, HVR1 E2 1b, NS3 1b, NS4 (NS4A + NS4B), and NS5A in the form of separate bands, were incubated with

1:100 dilutions of patients' sera, followed by washing to remove the unbound material. Next, the strips were incubated with alkaline phosphatase-conjugated anti-human Ig antibody, and the reaction was visualized by adding a chromogen, 5-bromo-4-chloro-3-indolyl-1-phosphate/nitro blue tetrazolium (BCIP/NBT). The intensity of brown color measured against negative and positive controls was proportional to the amount of specific antibody present in the sample.

Statistical analysis: Comparison of viremia at baseline between responders and non-responders was determined by using a *t* test. Fisher's exact test was used to compare qualitative variables between responders and non-responders. A *P* value of less than 0.05 was considered significant.

RESULTS

Characteristics of the patients and responses to PEG-IFN-α/RBV combination therapy: All the clinical data and viral factors discussed below are summarized in Table 1. Four out of the 9 patients studied (44.44%; Patients 1 - 4) achieved SVR. Of the 5 non-responders, 2 (Patients 5 and 6) were end-of-treatment complete responders, as assessed by lack of detectable HCV RNA in their sera, but relapsed into HCV RNA-positive status 6 months after stopping the therapy. In sustained responders and relapsers, the quantitative analyses of HCV RNA levels at baseline and after 3 months of therapy (at week 12) showed at least 2-log reduction of HCV RNA. However, at baseline, there was not statistically significant difference in viral load between sustained responders and non-responders, including relapsers ($P = 0.128$). Taking into account a prognostic value of the HCV Core Ag level as the additional diagnostic marker of viremia, we observed, as in the work of Buti et al. (40), its higher baseline values for non-responders, including relapsers, compared to sustained responders. We could not establish whether this difference was statistically significant, because the values of HCV Core Ag for some patients were above the linear range of the Ortho trak-C assay (Patients 4, 6, 7). The levels of HCV Core Ag measured 3 months after the start of treatment (at week 12) were not as predictive in regard to the result of therapy as the recent findings suggested (40,41). In sustained responders, except for patient 4, we did not observe that the HCV Core Ag value dropped below the assay threshold level of 1.5 pg/ml at week 12, a value indicative of a good response to treatment (41). However, when considering the small number of patients analyzed and the low values of viremia at week 12, beyond the linear range of the method for assaying the viral load quantitatively, the concomitant HCV Core Ag values must be treated with criticism.

In addition to the influence of viral factors i.e., viremia and HCV Core Ag levels, an advancement of fibrosis (staging, S), as histopathological examinations revealed, seems to affect treatment because non-responders showed higher S scores at baseline compared to the scores of responders (Table 1).

HCV genotyping with the use of a line probe assay disclosed subtype HCV-1b in all patients except for Patient 4, in whom HCV-1a was confirmed.

Cloning, sequencing, and analysis of mutations in the PKR-binding domain in relation to the result of treatment: The sequences coding for the PKR-binding domain were amplified from pretreatment sera of studied patients and cloned into plasmid pBluescript SK+/–. For every patient, 3 to 6 clones were sequenced and analyzed using the OMIGA 2.0 program. The data are presented as 66-amino acid sequences, aligned to a reference sequence which is HCV-J (GenBank accession number D90208) (39) for subtype 1b and HCV-1 for subtype 1a (GenBank accession number M62321) (42) (Fig. 1). Some sequences, together with 5′- and 3′- flanking regions, were submitted to a GenBank Database, and the following accession numbers are for Patients 1-9, respectively: AY861448, AY861449, AY861451, AY861454, AY861450, AY861452, AY861446, AY861447, and AY861453.

We next analyzed the relationship between variability of

Table 1. Patients' and viral parameters at baseline ("0"), after 3 months of therapy ("3"), at the end of 12-month therapy ("12") and 6 months after stopping therapy ("18") with PEG-IFN-α-2a/RBV

Patient	Sex	Age	ALT (IU/l)[1)]	Biopsy	Viremia "0", HCV Core Ag "0"	Viremia "3", HCV Core Ag "3"	HCV-RNA "12"	HCV-RNA "18"
1	M	23	78, 153, 30, 28	G1, S2	3.71×10^5 IU/ml 76 pg/ml	$<6.0 \times 10^2$ IU/ml 78* pg/ml	–, R	–, SVR
2	M	19	37, 28, 18, 15	G1, S1	2.64×10^5 IU/ml 37 pg/ml	$<6.0 \times 10^2$ IU/ml 40* pg/ml	–, R	–, SVR
3	F	47	105, 99, 106, 46, 33, 28	G2, S1	8.30×10^5 IU/ml 191* pg/ml	– 8* pg/ml	–, R	–, SVR
4	F	28	106, 86, 42, 28, 26	G2, S2	2.84×10^5 IU/ml beyond sensitivity +[2)]	– 1* pg/ml	–, R	–, SVR
5	M	34	45, 49, 56, 37, 32	G2, S2	4.97×10^5 IU/ml 97 pg/ml	$<6.0 \times 10^2$ IU/ml 10* pg/ml	–, R	+, RE
6	F	46	80, 37, 42	G2, S2	10.74×10^5 IU/ml beyond sensitivity +[2)]	$<6.0 \times 10^2$ IU/ml 3* pg/ml	–, R	+, RE
7	M	52	116, 88, 76, 78, 116, 96	G2, S3	8.64×10^5 IU/ml beyond sensitivity +[2)]	6.48×10^5 IU/ml beyond sensitivity +[2)]	+, NR	+
8	M	29	105, 127, 77, 82, 41, 30	G2, S3	7.79×10^5 IU/ml 225 pg/ml	8.37×10^5 IU/ml 37 pg/ml	+, NR	+
9	M	50	68, 66, 44, 28	G2, S3	4.74×10^5 IU/ml 316 pg/ml	– 11* pg/ml	+, NR	+

[1)]: ALT, alanine aminotransferase; first measurement 2 weeks before therapy, then at baseline, and then late during therapy.
[2)]: beyond sensitivity +, very high HCV Core Ag levels, beyond linear range of the assay.
*: HCV Core Ag values that must be treated with criticism due to too low or too high viremia values (beyond linear range of the assay).
– and +, HCV RNA undetected and detected, respectively; R, end-of-treatment response; NR, non-response; SVR, sustained virologic response; RE, relapse.

the PKR-binding domain and responsiveness to therapy. After 12-month treatment with PEG-IFN-α plus RBV, 6 patients were responders as assessed by lack of HCV RNA in their sera, and 3 were non-responders. Among HCV-1b patients, the ones who responded to therapy did not have significantly more mutations in the ISDR isolated from pretreatment sera, compared to non-responders (31 total mutations relative to HCV-1b prototype sequence HCV-J in the 27 clones versus 14 total mutations relative to HCV-J in 15 non-responder clones, $P = 0.637$) (Fig. 1). Similarly, there was not a significant correlation between the number of mutations in the 26-amino acid region of the PKR-binding domain, lying behind the ISDR (113 total mutations relative to HCV-1b prototype sequence HCV-J in the 27 clones versus 60 total mutations relative to HCV-J in 15 non-responder clones, $P = 0.796$) (Fig. 1).

Analysis of HCV-specific antibodies: A commercial LiaTek HCV III test was used to determine the amount and specificity of antibodies directed against HCV antigens in pretreatment sera of patients. Antibodies directed against nonstructural proteins 3 and 4 (NS3 and NS4) were present in all patients except Patient 2, in whom antibodies directed against NS4 were not detected (Table 2). Antibodies directed against NS5 were present in sera of Patients 3, 4, and 7-9. Also, almost all patients had antibodies specific to C1 and C2 antigens derived from structural core (C) protein. Analysis of E2-specific antibodies in pretreatment sera of patients showed a correlation between their presence and the result of treatment. They were present in pretreatment sera of 5 out of 6 end-of-treatment responders (Patients 1-5), whereas they were not

(A) HCV-1b end-of-treatment responders

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     237                                                                302
HCVJ PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESENKVVILDSFDPIRAVEDEREISVPAEI
   1 ........I.....................................LC.E.....V.L.... 2
   1 ........I......G..............................LC.E.....V.L.... 1
   1 ........V.....................................L..E....RG.....N 1
   1 .........Y.G..................................L..E.....V.L.... 1
   1 ........I.....................................L..E.....V...... 1

   2 .........R.................................V....E.L..E.....V...... 5
   2 .........R...QN............................V....E.L..E.....V...... 1

   3 .........R.....................................L..E.....V...... 5
   3 ...R.....R.....................................L..E.....V...... 1

   5 .........R.................................V......L..E.....V.I.... 4
   5 .........R..............L..................V.G....L..E.....V.I.... 1
   5 .........R.....................................V......L..E.....VFI.... 1

   6 ..................................................L..E..... 2
   6 ........................R.....................L..E..... 1
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(B) HCV-1b end-of-treatment non-responders

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     237                                                                302
HCVJ PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESENKVVILDSFDPIRAVEDEREISVPAEI
   7 ..................................................LH.E.....V...... 1
   7 ..............V.....................................L..E.....V...... 1
   7 ..................................................L..E..D..V...... 1
   7 ..............G.....................................L..E..D..V...... 1

   8 .........N.....................................L..E...G........ 1
   8 ........IN.....................................L..E...G........ 1
   8 ........IN.....................................L..E...G...L.... 1
   8 ........IN.....................D...............L..E...G...L.... 1
   8 .........N.....................................L..E...G...L.... 1
   8 .........N.....................................L..E.......L.... 1

   9 ..................................................L..E.....V.I.... 3
   9 ..........................................Y..SL..E.....V.I.... 1
   9 ...........P.............R.................Y..SL..E.....V.I.... 1
```

(C) HCV-1a end-of-treatment responder

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     237                                                                302
HCV1 PSLKATCTANHDSPDAELIEANLLWRQEMGGNITRVESENKVVILDSFDPLVAEEDEREISVPAEI
   4 ..........................................................V...... 5
   4 ..............G...........................................V...... 1
```

Fig. 1. Sequence analysis of pretreatment PKR-binding domain-containing isolates from HCV-1b-infected patients and an HCV-1a-infected patient, aligned to a prototype genotype 1b (HCVJ) and 1a (HCV1) isolate, respectively. In prototype sequences, the ISDR is shown as normally typed, while the remaining 26-aa region of the PKR-binding domain is highlighted in bold letters and underlined. The positions of the first and last amino acid of the PKR-binding domain, with regard to the whole NS5A protein, are shown above prototype sequences. Dots indicate amino acids identical with prototype sequence. The changed amino acids are shown. The number to the left of each sequence indicates a patient, while the number of clones comprising each sequence is listed to the right. (A) Clones from HCV-1b-infected patients who were end-of-treatment responders. (B) Clones from HCV-1b-infected patients who were end-of-treatment non-responders. (C) Clones from HCV-1a-infected patient who was end-of-treatment responder.

Table 2. HCV antibody profile in pretreatment sera of patients

Patient	Antigen					
	C1	C2	E2	NS3	NS4	NS5
1	+++	+++	+++	+++	+++	−
2	++	++	++	+++	−	−
3	+++	+++	++	+++	++	+
4	+++	+++	+	+++	+++	+++
5	+++	+++	++	+++	++	+
6	++	++	−	+++	+++	−
7	−	++	−	+++	+++	+++
8	+++	+++	−	+++	+++	+++
9	++	++	−	+++	+++	++

+, ++, +++, different levels of detected HCV-specific antibodies.
–, undetected.

detected in the 3 non-responders (Patients 7-9) ($P = 0.048$) (Table 2). As Patient 4 was infected with HCV genotype 1a and the test utilizes HVR1 fragments of E2 1b as antigen to capture the E2-specific antibody, the detection of a weak positive signal for the presence of E2 antibody in Patient's 4 serum might be the result of cross-reaction and/or dual infection. Similarly, the test contains a recombinant NS3 1b protein as an antigen, so the presence of NS3-specific antibodies in this patient might also be explained this way (Table 2).

DISCUSSION

In this study, 9 chronically HCV-infected patients, 8 of whom were HCV-1b-infected and 1 HCV-1a-infected, underwent a combination PEG-IFN-α-2a/RBV therapy. The rate of end-of-treatment response was 66.66%, and the SVR rate was 44.44%. Although the number of patients was low, the rate of SVR was comparable to the rate of SVR for HCV genotype 1-infected patients reported in a large study with the use of PEG-IFN-α-2a plus RBV (46%) (5). This finding allowed us to assume that other viral and patient parameters measured in this study might be as representative as those studied in a large cohort of patients, unless otherwise indicated.

Upon the quantitative analyses of HCV RNA levels, performed at baseline and after 3 months of therapy (at week 12), we confirmed the accepted view of the prognostic value of a 2-log reduction in plasma HCV RNA level characteristic for responders, who were most likely to achieve SVR (43). Therefore, the quantitative measurement of HCV RNA seems to be important in monitoring the progression of therapy in order to predict its outcome and in justified cases to cease it when the level of viremia is persistently high. The lack of a statistically significant difference in viral load at baseline between sustained responders and non-responders, including relapsers, which is in contrast to the report of Buti et al. (40), could be accounted for by the small number of patients included in this study. We also noticed that the pretreatment fibrosis score was higher in non-responders, thus perhaps reflecting the longer duration of the infection in those patients resulting in insensitivity to the IFN/RBV treatment. This was in agreement with the trend observed by Chambers et al., who reported that the pretreatment fibrosis score appeared to be a predictor of antiviral response (44).

In the next part of this study, we attempted to determine whether the number of mutations in the PKR-binding domain isolated from pretreatment sera of chronically infected Polish patients influenced PEG-IFN-α plus RBV therapy and its final outcome. We reverse transcribed HCV RNA and then amplified, cloned, and sequenced the PKR-binding domains from 9 patients. Those sequences were presented together with clinical data showing levels of viremia at baseline, at week 12, at the end of therapy, and 6 months after the cessation of therapy. Due to the fact that only 1 patient was infected with HCV genotype 1a, all subsequent comparative studies regarding mutations in the PKR-binding domain and response to treatment were performed on HCV-1b-infected patients. For this analysis, end-of-treatment responses were taken. In HCV-1b-infected patients, consistent with other European and North American studies (25-32,44), we found no evidence that substitutions in amino acid sequence of the ISDR in relation to HCV-J consensus sequence were associated with a response to antiviral therapy. Responders and non-responders did not differ significantly in number of substitutions in this region, which was isolated from pretreatment sera. The majority of ISDR HCV 1b sequences analyzed in this study were of intermediate type, that is, they contained 1 - 3 mutations compared to the HCV-J prototype sequence, except for two sequences of Patient 6, two sequences of Patient 7, and four sequences of Patient 9, which were wild-type ISDR (Fig. 1). It has been shown that different distribution of intermediate-type ISDR sequences between Japanese and European patients (37.6 versus 63.4%, respectively) generally does not affect the rate of SVR (17.5 versus 18.9%, respectively) in both groups with intermediate-type ISDR (34). However, the relationship between the number of mutations in the ISDR and the result of treatment becomes more prominent between groups of Japanese and European patients with mutant-type ISDR (4 or more mutations) (34). Despite rather small difference in size of the two groups (18.3% Japanese versus 11.8% European patients with mutant-type ISDR), their comparison revealed that the SVR rate in the Japanese cohort was nearly twice as high as that in the European cohort (79.2 versus 43.5%, respectively) (34). These data may explain why Japanese studies generally show a correlation between the number of mutations in ISDR HCV-1b and the result of treatment. As a reason for that, the data support the existence of geographical differences in HCV genotype 1b infection which can be attributed to a genetic factor, for example, the existence of a Japanese-specific type of HCV-1b, and/or to an as yet unidentified host or racial factor (33,34).

Some reports have suggested a role played by the remaining portion of the PKR-binding domain, located downstream from the ISDR, in the resistance to IFN. Because reports on this portion of the domain were not as numerous as reports on the ISDR, we decided to sequence this region too. When we analyzed it, we found that the number of substitutions in this 26-amino acid sequence was substantially higher compared to the ISDR. However, this occurred in all patients infected with HCV-1b, and statistical analysis of this region also allowed the excluding of the relationship between variability of this sequence and the result of treatment. This is in contrast to previous reports, where such relationships have been found, both for HCV-1a (31,35) and -1b infections (31), but in line with a more recent report (37). It seems our data would require a further confirmation drawn from the studies of a larger sampling of patients. However, results of our and other studies suggest that it might be necessary to investigate the entire region of the NS5A protein or at least the extended C-terminus down the PKR-binding domain to elucidate whether the quasispecies nature of HCV in this region has important implications in viral resistance to IFN (31,36,37). For example, a study of the variable 3 (V3) domain, a region located

at the C terminus of the NS5A protein, has revealed that responsiveness to IFN/RBV therapy was correlated with mutations there. In other words, NS5A might interfere with IFN-induced antiviral response using sequences outside the ISDR and the entire PKR-binding domain (31,36).

Another aspect of our study addressed the profile of HCV-specific antibodies in pretreatment sera of patients with chronic hepatitis C and their influence on the result of therapy. The antibodies specific to C, NS3, NS4, and NS5 antigens were present in different quantities in virtually all patients treated, both responders and non-responders, suggesting only overall activity of the immune system without any predictable effect on the course and outcome of therapy. Only the presence of antibodies directed against E2 glycoprotein, which plays a role in viral attachment and entry to a cell (13,45), correlated with the result of IFN/RBV treatment. E2 antibodies have been reported as important in natural clearing of HCV during acute and chronic infection (9,46,47). However, in the majority of cases, E2 antibodies, even if persist during the chronic phase, are not able to eradicate the virus itself (48). This might be due to the quasispecies nature of HCV, enabling it to escape from immune system pressure, and/or not enough support from the HCV-specific T-cell response. However, a combination IFN/RBV treatment seems to overcome this obstacle and, as a result, the presence of E2 antibodies in pretreatment sera of chronically infected HCV genotype 1b patients appears to be an important factor influencing the course and response to therapy. Our data are in agreement with data of other groups who have established the same relationship between pretreatment E2 antibody levels and a response to therapy (49-51). For example, Chu et al. (50) reported that among chronically HCV-1b-infected patients, those with a pretreatment level of HCV RNA below 10^6 copies/ml responded to therapy significantly better than did patients with a higher number of HCV RNA copies, and among responders, the presence of pretreatment E2 antibodies correlated with sustained response. Results of work by Zampino et al. (51) have shown that higher baseline levels of E2 antibodies and their decrease or even disappearance during IFN treatment were associated with a sustained response, whereas no reduction in the level of E1/E2 antibodies was observed in non-responders.

In conclusion, by studying HCV genotype 1b-infected patients we established that mutations in the ISDR region, as well as mutations in the remaining portion of the PKR-binding domain of NS5A protein from pretreatment sera, did not correlate with the response to IFN/RBV therapy. Results concerning the ISDR are generally in line with those reported in European studies. The ones referring to the remaining sequence of the PKR-binding domain need still more data to elucidate the role of this region in HCV resistance to IFN/RBV treatment. As this was a pilot study on a Polish population of HCV-infected patients, it is clear that a larger cohort, divided into groups with wild-, intermediate-, and mutant-type ISDR should be analyzed to gain a deeper insight into the relationship between variability of the ISDR and the result of therapy. The data we obtained about the relationship between pretreatment E2 antibody levels and a response to therapy, although significant and in agreement with that of other reports (49-51), also require confirmation from extended study. If confirmed, they could have potent meaning, together with low pretreatment viral plasma load, in the future design and monitoring of antiviral therapy.

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