CONCISE COMMUNICATION

The Amino Acid Sequence of the PKR-eIF2α Phosphorylation Homology Domain of Hepatitis C Virus Envelope 2 Protein and Response to Interferon-α

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A region of the hepatitis C virus (HCV) envelope 2 protein, the protein kinase, PKR and early initiation factor 2α phosphorylation homology domain (PePHD), may be important in interferon (IFN)–α resistance. The PePHD was amplified by polymerase chain reaction and sequenced, and the amino acid sequence derived from pretreatment serum of 14 genotype 3–infected patients with a range of responses to IFN-α therapy. Only 1 patient had a PePHD variant. IFN-resistant PePHD variants present at low titers in pretreatment serum should be selected by therapy; therefore, the PePHD amino acid sequence was also obtained from serum collected during or after treatment in 5 patients with breakthrough or relapse of HCV RNA positivity. No difference was found between the pre- and posttreatment PePHD sequences. Thus, it appears that pretreatment sequencing of the PePHD would not enable clinicians to predict the treatment response. There was no evidence that IFN therapy exerts selection pressure in this region.

Hepatitis C virus (HCV) is a major cause of chronic liver disease; 80% of those infected become long-term carriers, with attendant risks of cirrhosis and hepatocellular carcinoma. The mainstay of treatment is interferon (IFN)–α, which, when combined with ribavirin, results in sustained viral clearance in 38%–43% of patients treated [1]. This poor response rate, combined with the high cost of IFN, has fueled research into the mechanism of HCV IFN resistance.

IFN-α is a cellular protein that acts by inducing the transcription of several antiviral genes. One such gene expresses the protein kinase PKR (double-stranded RNA–activated protein kinase), which phosphorylates the translation initiation factor eIF2α, thereby blocking protein synthesis. Recently, a 12-aa sequence was identified within the HCV envelope 2 (E2) protein (aa 659–671 of the HCV polyprotein), which shows homology to both the PKR and eIF2α phosphorylation sites. The sequence was called the PKR-eIF2α phosphorylation homology domain (PePHD) [2]. The degree of homology is greater in genotype 1 than in genotype 2 or 3. Constructs containing the E2 gene of HCV genotype 1 can block the action of PKR in yeast and human cell lines, and this is abrogated by mutation of the PePHD, to resemble that of HCV genotypes 2 and 3. It was suggested that the PePHD-PKR interaction could explain the relative IFN resistance of genotype 1, compared with that of other genotypes.

Although clinical data confirm that HCV genotype 3 is more IFN sensitive than genotype 1 [3], a significant proportion of genotype 3–infected patients do not have a sustained response [1]. We hypothesized that within genotype 3, IFN-resistant strains may carry a PePHD variant similar to that of HCV genotype 1. If such a variant were identified in pretreatment samples, it would provide a powerful prognostic tool for clinicians. To investigate this, we sequenced the PePHD from 14 genotype 3–infected patients with a range of treatment outcomes. Since HCV exists as a quasi species [4], failure to demonstrate a PePHD variant in pretreatment samples could simply reflect its presence as a minority. If so, the resistant variant would be selected by treatment and would be present at higher titers in samples collected during or after treatment. To capitalize on this selection pressure, we also sequenced the PePHD in serum samples of 5 patients collected during or after treatment.

Patients and Methods

Patients. Eighty patients infected with HCV were offered IFN-α monotherapy if liver biopsy showed changes typical of chronic infection. Exclusion criteria included age >70 years, other cause for chronic hepatitis, decompensated cirrhosis, human immuno-

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The study was approved by the ethics committee of the West Glasgow University Hospital National Health Service Trust, and informed consent was obtained from all patients.

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deficiency virus infection, significant comorbidity, injection drug use within the last year, or contraindication to IFN-α. Each patient was prescribed IFN-α–2a (Roferon; Roche Products, Lewes, UK) at 6 million units (MU) three times weekly for 12 weeks. Patients negative for HCV after 12 weeks were continued on 3 MU thrice weekly for another 36 weeks (total dose, 540 MU over 48 weeks). Serum samples were collected before treatment and after 2, 4, 8, 12, 48, and 72 weeks and were stored at −70°C.

Only 3 of 36 patients with genotype 1 infections had sustained responses to treatment, whereas 8 of 35 with genotype 3 had sustained responses. We studied serum samples from 16 genotype 3–infected patients with sufficient remaining sera. Definition of outcome was as follows: sustained responder, HCV RNA negative at 48 and 72 weeks; relapser, HCV RNA negative at 48 weeks and positive by 72 weeks; breakthrough, HCV RNA negative at 12 weeks and positive by 48 weeks; and nonresponder, HCV RNA positive at 12 weeks. Detection of HCV RNA was by in-house nested reverse transcription–polymerase chain reaction (RT-PCR) [5] or by Amplicor (Roche Diagnostics, Lewes, UK). Among patients with sustained responses and in those who did not respond, only pretreatment serum samples were studied. Among 5 patients who experienced a relapse or breakthrough, a serum sample taken during or after treatment was also analyzed (table 1). For simplicity, these serum samples are referred to as posttreatment samples.

Amplification of HCV E2 RNA by RT-PCR. To minimize the introduction of sequence error during processing, serum samples were separated and were frozen within 4 h of sampling, and precautions to avoid contamination were adopted throughout PCR experiments [6]. Complementary DNA was synthesized using Superscript II reverse transcriptase (Gibco BRL, Paisley, UK), according to the manufacturer's instructions. The initial primer used was MS2 (5′-TGAYAAGGTAAAGAAGCCG-3′). Two samples were successfully reverse transcribed (patients 2 and 14), but owing to a high failure rate, a new primer was designed: AC5 (5′-CRATCCARTGCTTATACCA-3′). After RT, 1 U of ribonuclease H (RNase H; Roche Molecular Biochemicals, Lewes, UK) was added on ice and then incubated at 37°C for 20 min. The 2 samples reverse transcribed with MS2 were amplified using *Taq* polymerase (Gibco BRL) with MS2 and JM3E2TPAHCV (5′-CCGGGAATTCTTGGATCCCACACATATACCACCGG-3′), followed by MS4 (5′-GCAGGCATGGGCGTGAA-3′) and MSQ7 (5′-GTCTGCGGCCCTGTGTACTG-3′). The rest were amplified with a PCR kit (Advantage plus cDNA) for first-round PCR and then with *Taq* polymerase with Taq Start antibody (both from Clontech, Basingstoke, UK) for the second round. The following primers were used: AC6 (5′-CAGCTGYAAGCCCATCAC-3′) and AC5 (see above), followed by AC7 (5′-ACCRTAYTGCTGGCACTA-3′) and AC8 (5′-GCMACRCACACGCGTGCGTC-3′). (Full details of reactions, including thermal cycling conditions, are available by request.)

Gel purification and silica column extraction. We subjected 95 μL of PCR product to electrophoresis on an 0.8% agarose TAE gel. DNA was extracted from gel slices with silica gel columns (Recovery DNA purification kit II; Hybaid, Ashford, UK).

Nucleotide sequencing and analysis. Sequencing was performed with an ABI prism 377 automated sequencer (Perkin-Elmer, Beaconsfield, UK). Sense and antisense primers used were located, respectively, at nucleotide positions 2038–2061, ACSQ4 (5′-CCCYTGYAACATCTAGGGG-3′), 2503–2522, and ACSQ3 (5′-AYGAGGAYRACGAACTCCCA-3′) of HCV genotype 3a [7]. Sequences were translated and were aligned by use of TRANSLATE, PILEUP, and PRETTY programs in the GCG package (Genetics Computer Group, Madison, WI) [8].

Table 1. Time of withdrawal (during or after treatment) of serum in relation to interferon (IFN)–α therapy.

Patient	Response to IFN-α	Time of posttreatment failure sample
4	Breakthrough	16 Weeks after end of treatment
5	Breakthrough	While receiving IFN-α (week 36)
6	Breakthrough	While receiving IFN-α (week 20)
8	Relapse	6 Weeks after end of treatment
9	Relapse	18 Weeks after end of treatment

NOTE. Breakthrough was defined as hepatitis C virus (HCV) RNA negative at 12 weeks and positive by 48 weeks; relapse was defined as HCV RNA negative at 48 weeks and positive by 72 weeks.

Results

Treatment outcome was assigned according to the results of viral RNA testing by a PCR method sensitive to 2000 RNA copies/mL [5]. A satisfactory nucleotide sequence equivalent to aa 638–704 was obtained from 14 of the 16 patients identified. The amplification system used (Advantage plus) contains a polymerase that provides 3′→5′ proofreading, and the Taq Start antibody allows for automatic "hot start" PCR. These measures minimize the production of inaccurate sequences and reduce nonspecific amplification products. The response to therapy and the derived amino acid sequences for each patient are aligned in figure 1. The PePHD in genotype 3 is from aa 665–677. The Genbank accession numbers for the nucleotide sequences are AF289520–AF289538. Only 1 patient (a nonresponder) had a PePHD variant, a single amino acid change toward the genotype 1b sequence. Therefore, there was no consistent difference between sustained virological responders and nonresponders, and there was no difference in the PePHD sequence between pretreatment and posttreatment samples. The 30 amino acids downstream of the PePHD were identical in all study samples; the upstream sequence showed some variation but did not correlate with treatment response or with time of sample collection.

Discussion

In this study, only 1 of 14 patients infected with HCV genotype 3 had a PePHD variant. The pretreatment and posttreatment failure PePHD sequences were identical in all patients tested. This study was primarily designed to assess the value of the PePHD sequence as a prognostic marker for clinical use. For this reason, the HCV RNA was extracted from serum samples, a source readily available to clinicians, and attention was paid to the reliability of patient data and the accuracy of the sequencing. We are confident that patient compliance was

			617					665	677			707
Consensus Sequence this study			YPYRLWHYPC	TVNFTLFKVR	MFVGGFEHRF	TAACNWTRGE	RCDIEDRD**RS**	**EQHPLLHSTT**	ELAILPCSFT	PMPALSTGLI	HLHQNIVDVQ	Y
Genotype 1b (HCV-J)			----------	-----V----	-Y--------	-LN-------	----I-----	**-LS---L---**	-WQ-------	TL--------	---R------	-
Genotype 3a (NZL1)			----------	--D-R-----	----------	----------	----------	----------	----------	----------	----------	-
Patient	Response	Sample										
1	SR	Pre	----------	----K-----	----------	----------	----------	----------	----------	----------	----------	-
2	SR	Pre	----------	--------M-	----------	----------	----------	----------	----------	----------	----------	-
3	SR	Pre	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
4	BT	Pre	----------	----------	----------	N---------	----------	----------	----------	----------	----------	-
4	BT	Post	----------	----------	----------	N---------	----------	----------	----------	----------	----------	-
5	BT	Pre	----------	-I--------	----------	----------	----------	----------	----------	----------	----------	-
5	BT	Post	----------	-I--------	----------	----------	----------	----------	----------	----------	----------	-
6	BT	Pre	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
6	BT	Post	----------	------Y---	----------	----------	----------	----------	----------	----------	----------	-
7	BT	Pre	----------	-I--------	----------	----------	----------	----------	----------	----------	----------	-
8	REL	Pre	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
8	REL	Post	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
9	REL	Pre	----------	----------	----------	A---------	---L------	----------	----------	----------	----------	-
9	REL	Post	----------	-I--------	----------	----------	----------	----------	----------	----------	----------	-
10	REL	Pre	----------	-I--------	----------	----------	----------	----------	----------	----------	----------	-
11	NR	Pre	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
12	NR	Pre	----------	----------	----------	----------	----------	----------	----------	----------	----------	-
13	NR	Pre	----------	-I--------	----------	----------	----------	----------	----------	----------	----------	-
14	NR	Pre	----------	----------	----------	----------	----------	**-L**--------	----------	----------	----------	-

Figure 1. Alignment of derived amino acid (aa) sequences of part of envelope 2 (E2) protein for 14 hepatitis C virus (HCV) genotype 3–infected patients. Pre- and posttreatment failure sequences are shown for 5 patients. Protein kinase, PKR and early initiation factor 2α phosphorylation homology domain (aa 665–667), denoted in boldface, is compared with published sequences for genotypes 1b [9] and 3a [7]. Numbering of aas is as for genotype 3a. Dashes indicate aa identical to consensus sequence. BT, breakthrough; NR, nonresponder as defined in Patients and Methods; Pre, pretreatment sample; Post, after or during treatment sample (exact timing given in table 1); REL, relapser; SR, sustained responder.

good, since treatment was supervised by a single specialist nurse whose records agree with those of the hospital pharmacy.

In the second part of the study, we probed for evidence of PePHD involvement in IFN resistance by looking for selection of PePHD variants during therapy. To maximize the likelihood of finding a difference between the pretreatment and posttreatment sequences, we elected to study patients who had HCV disease breakthrough or relapse. In these patients, the initial virological response to therapy suggests that the dominant pretreatment species is IFN sensitive. Subsequent virological rebound may represent replication of an IFN-resistant variant that was previously present as a minority. The posttreatment serum samples were drawn after an average of 40 weeks of IFN therapy. Given the short turnover time of HCV (half-life, 100–182 min) [10], this should have allowed time for selection of IFN-resistant variants. It is possible that these variants may be less "fit" than other strains and therefore could rapidly lose dominance after withdrawal of the IFN selection pressure. Thus, the lack of PePHD change in patients 5 and 6 is particularly important, since the posttreatment failure sample was drawn while IFN therapy was ongoing.

We derived PePHD sequences for each serum sample from a single PCR product. Therefore, our sequence represents a consensus of all the variants that make up the quasi species in the serum samples. Support for the use of a consensus sequence, as representative of the dominant species, comes from previous studies in our laboratory, in which change in the dominant species, demonstrated by single-stranded conformation polymorphism, corresponded with change in the consensus sequence [5].

In a study of 7 genotype 3–infected patients, there was no correlation between PePHD sequence and response to IFN [11]. Of interest, 1 "responder" had a PePHD variant that resembled genotype 1. Assuming that this patient had a sustained response, this shows that the variant alone is insufficient to confer IFN resistance. Unfortunately, the term "responder" was not defined, nor were the times of serum sample withdrawal related to IFN therapy. The PePHD was sequenced from hepatocytes rather than from serum samples. This difference is unlikely to be important because, although there is often a difference in quasi-species complexity between HCV isolates from these 2 tissues, the consensus sequence is usually identical [12]. Another study [13] of the PePHD in 33 genotype 3a–infected patients treated with IFN alone or in combination with ribavirin showed that the majority who achieved a sustained response had no amino acid differences from the consensus. Hydrophobic mutations occurred only in 4 of 16 sustained responders, but 1 of these differences (Q668L) was found in a nonresponder in the current study. This and the small numbers of differences seen overall suggest a random finding.

The low frequency of PePHD variants, combined with the

lack of correlation with response, indicates that pretreatment sequencing of the PePHD is unlikely to give any useful information to clinicians. IFN-α resistance in any individual patient is probably the result of a combination of host and viral factors. Our results do not preclude a role for the PePHD-PKR interaction, but the lack of selection of a PePHD variant during IFN-α therapy suggests that it was not the primary resistance mechanism in the 5 patients tested. Similar studies of the NS5a IFN sensitivity–determining region (ISDR) have produced conflicting results, and there is some evidence that a region downstream of the original ISDR may be important [14]. We sequenced the E2 gene 50 aa upstream and 30 aa downstream of the PePHD without discovering any correlation with IFN-α response.

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