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Expression of hepatitis C virus hypervariable region 1 and its clinical significance

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Abstract

AIM: To explore the properties of hypervariable region 1 (HVR1) in the envelope 2 gene of hepatitis C virus by analyzing the reactivity of HVR1 fusion proteins from different Chinese HCV strains with sera of patients with chronic hepatitis C and by comparing their reactivity between interferon therapy responders and non-responders.

METHODS: Gene fragments of HVR1 of four HCV strains (three genotype 1b and one genotype 2a) were amplified from pGEMT-E2 plasmids and sub-cloned into pQE40 vectors respectively to construct recombinant expression plasmids which expressed HVR1 fused downstream to DHFR in Escherichia coli strain TG1. The purified DHFR-HVR1 proteins were then used to detect the anti-HVR1 antibodies in 70 serum samples of patients with chronic hepatitis C.

RESULTS: Four DHFR- HVR1 fusion proteins were successfully expressed in E.coli (320-800 ug fusion proteins per 100 ml culture). Each fusion protein (SH1b, BJ1b, SD1b and SD2a) reacted with 72.8 % (51/70), 60 % (42/70), 48.6 % (34/70), and 58.6 % (41/70) of the anti-HCV positive patients' sera respectively by ELISA. 57.1 % (4/7) of non responders reacted with all four HVR1 fusion proteins, while only 15.3 % (2/13) of responders reacted with all of them. The O.D. values of sera from IFN therapy responders were significantly higher than those of non responders ($P<0.05$).

CONCLUSION: The selected HVR1 fusion proteins expressed in E. coli can broadly react with HCV-infected patients' sera. The intensity and/or quality of the immune response against HCV may be a critical factor determining the response to interferon treatment. With the evolution of virus strains, anti-HVR1 antibodies can not neutralize all the quasispecies. A polyvalent and high immunogenic vaccine comprising a mixture of several HVR1 sequences that cover the reactivity of most HCV isolates may be useful.

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INTRODUCTION

Hepatitis C virus (HCV) is the major etiologic agent of blood transfusion-associated and sporadic non-A non-B hepatitis worldwide. About 70 % of the infections become chronic, among which a significant proportion eventually develops cirrhosis and hepatocellular carcinoma. Despite recent success after the combination therapy with Interferon-α and ribavirin[1-3], about 60 % of patients still fail to respond. Thus, the development of HCV vaccine is especially important, but it remains an urgent challenge due to the high mutation rate of HCV.

Multiple lines of evidence indicate that one of the principal neutralization determinants corresponds to the hypervariable region 1 (HVR1), which is located in the amino-terminus of E2 of HCV (nt1150-1230). Zibert *et al*[4] found that an early appearance of antibodies directed to HVR1 is associated with acute self-limiting infection of HCV, while the persistence of HVR1 antibodies is associated with chronic HCV infection. Antibodies against HVR1 have been shown to block adsorption to susceptible cells in vitro[5,6]. Animal antibodies raised against this region have provided effective prophylaxis in chimpanzee challenge experiments[7-9], but attempts to develop a HVR1 vaccine against HCV were hampered by the frequent mutations of HVR1. Although anti-HVR1 antibodies react with HVR1 proteins specifically, a single fraction of antibodies has potentiality to react with more than one HVR1 protein sharing a similar amino acid sequence[10]. Theses findings suggest that HVR1 may play an important role in the prevention of HCV infection.

Our previous study[11] analyzed the variability of HCV envelope region in 12 dominant strains from different cities of China and predicted the immunogenicity with computer programs, demonstrating that genotypes and epidemic areas should be considered when identifying the cross-reactive epitopes for vaccine design. In this study, we selected four HCV strains of two genotypes from three regions (Shanghai, Beijing and Shandong) of China according to the results of the variant analysis and immunogenicity prediction of the envelope region in Chinese HCV strains. The gene fragments of HVR1 were amplified from four corresponding pGEMT-E2 plasmids and sub-cloned into pQE 40 vectors respectively to construct four recombinant prokaryotic expression plasmids that can express HVR1 as fusion proteins with DHFR. The purified DHFR- HVR1 proteins were then used to detect the anti-HVR1 antibodies in sera of patients with chronic hepatitis C to further explore their antigenicity and analyze the different reactivity between IFN therapy responders and non-responders.

MATERIALS AND METHODS

Patients

Fifty HCV-infected patients were studied, who were all anti-HCV positive by commercial anti-HCV assays (third generation of enzyme-linked immunosorbent assay [ELISA], Abbott, North Chicago, IL). Of these, 20 patients received interferon (IFN) therapy, Roferon (Roche, Switherland), 3 million units three times a week for 6 months. 13 patients who showed negativity of serum HCV RNA and normalization of

alanine aminotransferase (ALT) level after cessation of IFN were considered as responders, while the other 7 patients who remained HCV RNA positive and/or presented fluctuation of ALT were designed non responders (Table 1). Quantitation of serum HCV RNA was performed using commercial kit from Fu Hua Gene Company, Shanghai. Informed consent was obtained from all the patients, and study protocol was approved by the committee on human ethics. 20 healthy blood donors who were anti-HCV negative were also analyzed as negative control.

Table 1 Characteristics of chronic hepatitis C patients treated with IFN-α

	Responders	Non responders
Number	13	7
Age (Yr)	41	40
Sex (F / M)	5 / 8	2 / 5
Known duration of infection (Yr)	10.6	9.9
Baseline ALT (IU / ml)	185	126 (P=0.1660)
Baseline viral load (copies / ml)	4.37×10^6	2.92×10^6 (P=0.6908)

Methods

Construction of recombinant expression plasmids Part of E2/NS1 regions was cloned from sera of the patients and sequenced as described before[11]. From these plasmids pGEMT-E2, DNA fragments containing HVR1 (nt1150-1233, aa384-411) were amplified by PCR. The primers for HCV strain Shanghai 1b (SH1b), sense: nt1150-1161, 5' TTAGATCTGCAACCTACACG3' , anti-sense: nt1225-1233, 5' CCCAAGCTTAGATTTTCTG3' ; the primers for strain Beijing 1b (BJ1b), sense: nt1150-1161, 5' TTAGATCTGGCACCTATACG3' , anti-sense: same as SH1b; the primers for strain Shandong 1b (SD1b), sense: nt1150-1159, 5' TTAGATCTGAGACCCGTG3' , anti-sense: same as SH1b, and the primers for Shandong 2a (SD2a), sense: nt1150-1159, 5' TTAGATCTAGCACCCACG 3' , anti-sense: nt1225-1233, 5' CCCAAGCTTAGATGTTCTG3' . The PCR products were purified and ligated into the Hind III, Bgl II sites of the expression vector pQE40 which allows fusion of HVR1 encoding sequences downstream to the murine dihydrofolate reductase (DHFR) with a N-terminal 6×His tag.

The recombinant plasmids were identified by digestion with Hind III and Bgl II. The inserts were then sequenced to ensure that the DNA encoded the authentic HCV sequence. The identified plasmids were named as pQE40-HVR1-SH1b, BJ1b, SD1b and SD2a respectively.

Expression and purification of the fusion proteins The recombinant plasmids and pQE40 vector were transformed to E.coli strain TG1.DHFR-HVR1 fusion proteins were expressed by induction with 1 mmol/l isopropyl-β-D-thiogalactotyranoside (IPTG) in 100 ml of LB/ampicillin media cultured at 37 ℃ with vigorous shaking. After 6 h of induction, cells were harvested by centrifugation at 4 ℃ and 5 000 g for 30 min.

Harvested cells were re-suspended in 10 ml of 8M urea/ 20mM β-ME/PBS pH8.0, and cell disruption was performed using an ultrasound sonication method. After centrifugation at 20 000 g and 4 ℃ for 30 min, the supernatant was saved for purification on Ni^{2+}-nitrilotriacetate (NTA)-agarose (Qiagen) according to manufacture' s instructions at room temperatures. Denatured crude extract was allowed to bind to Ni^{2+}-NTA-agarose pre-equilibrated in 8M urea/20mM β-ME/PBS pH8.0 for 2 h. The gel matrice were then washed with the same solution pH6.3 and gel-bound proteins were eluted with that of pH4.3.

The purified fusion proteins were run on 12 % sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) for identification. For protein visualization and quantification, gels were stained with Coomassie brilliant blue (Sigma). The purity and yield of recombinant proteins were calculated from densitometric scanning results by comparing with known quantity of BSA (Bio-Rad) run on the same gel.

ELISA The plates were coated with four purified fusion proteins respectively or combined at of 0.2 μg/well for 1 hour at 37 ℃ and then overnight at 4 ℃ in carbonate buffer pH 9.5. After blocking with 1 % BSA for 1 hour at 37 ℃, sera were dispended in wells at a dilution of 1:20 and incubated for 45 min at 37 ℃, followed by washing. HRP conjugated goat anti-human IgG (Sino-American Biological Company) diluted 1:8 000 was then added and plates were incubated for 45 min at 37 ℃. After washing, the color was developed using TMB according to standard procedures, and the optical density values were measured at 450nm (OD_{450}) in automatic photometer (Wellscan K3, Labsystem Company).

Statistical analysis

The results were analyzed by the t test. In all analyses, a P value less than 0.05 was considered statistically significant.

RESULTS

Construction of the recombinant plasmids

Four recombinant plasmids expressing HVR1 fused with DHFR were constructed as described in methods. Obtained clones were digested with Hind III and Bgl II, 95bp fragments of HVR1 coding sequences could be detected from each of them (Figure 1). Automatic sequencing confirmed that the inserted HVR1 fragments corresponded to reported data (Figure 2). The reading frames of the recombinant plasmids were correct.

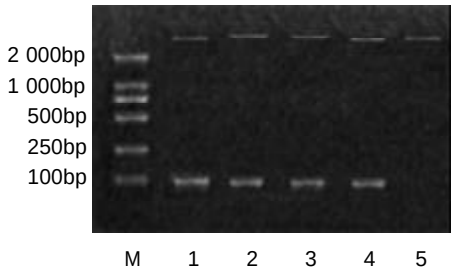

Figure 1 Digestion of the recombinant plasmids with HindIII and Bgl II. M is DNA marker (DL 2000). Lane 1-lane 4 show four recombinant plasmid digestion results, SH1b, BJ1b, SD1b and SD2a respectively. Lane 5 is the control (pQE40).

```
HVR1-SH1b  GCAACCTACA  CGACGGGGGG  GGCGGCTTCC  CACAACACCC
HVR1-BJ1b  -GC-----T-  ----------  ----CAGGG-  -GTGC-----
HVR1-SD1b  -AG---CGTG  T---------  --T-CAAAG-  T-T-C-CT--
HVR1-SD2a  AGC---C--G  TC--T--T--  CATT---G-G  -G-GC-G--A

HVR1-SH1b  GGGGGTTTAC  GTCCCTCTTT  AGTTCTGGGT  CGCAGCAGAA
HVR1-BJ1b  A---CC-C--  ----------  TCACG-----  --GCT-----
HVR1-SD1b  ----CC-C--  ----------  -CG------C  -AGCT-----
HVR1-SD2a  -CA-T--CGT  CAG-T-GC-C  -CCC---ACG  -TA-A-----

HVR1-SH1b  A
HVR1-BJ1b  -
HVR1-SD1b  -
HVR1-SD2a  C
```

Figure 2A The nucleotide sequences of the four HVR1 frag-

ments in recombinant pQE40-HVR1 palsmids. Dashes represent nucleotides identical to those of HVR1-SH1b.

```
          384                                410
CONSENSUS ETHVTGGVAG HTTSGFTSLF TSGPSQK
HVR1-SH1b A-YT---A-S -N-R------ S--SQ--
HVR1-BJ1b G-YT---AQ- RA-Q-L---- -R-SA--
HVR1-SD1b --R-----QS Y-LA-L---- ----A--
HVR1-SD2a G------I-A RAA-S-V--L -PDAK--
```

Figure 2B Deduced amino acid sequence of the HVR1 fragments in pQE40-HVR1. Sequences are shown with a single-letter amino acid code where residue is different from the consensus sequence of genotype 1b defined by Hattori *et al*, and with a dash where residue is identical.

Expression and purification of the fusion proteins

The proteins expressed in transformed E. coli were analyzed by SDS-PAGE. The fusion proteins migrated as an approximately 28 kDa band, approximately 3 kDa larger than DHFR (Figure 3A), in accordance with the fusion of 28 aa HVR1. Figure 3B shows the SDS-PAGE result of four purified fusion proteins and DHFR. The concentrations estimated by BSA grades are 0.4-1.0 μg/μl, so about 320-800 μg of purified protein can be obtained from every 100 ml of bacteria culture.

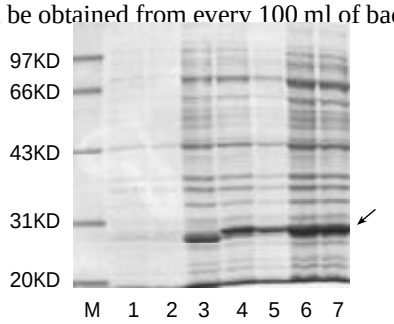

Figure 3A SDS-PAGE of the fusion proteins expressed in E. coli strain TG1. M: protein molecular marker. Lane 1 is vector pQE40 before induction, Lane 2 is one of the recombinant plasmids before induction. Lane 3~7, are recombinant plasmids pQE40-SH1b, BJ1b, SD1b, and SD2a after 4 hours of induction with 1 mmol/l IPTG respectively.

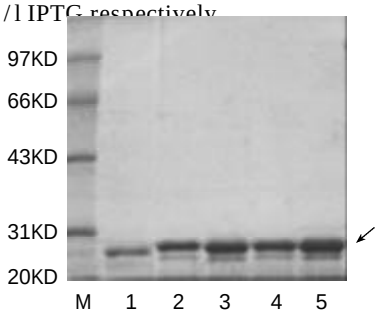

Figure 3B Results of the purified proteins. M is protein marker. Lane 1 is plain DHFR. Lane 2-5 are the fusion proteins SH1b, BJ1b, SD1b and SD2a serially.

Detection of anti-HVR1 Ab in HCV infected patients' sera

Anti-HVR1 antibodies were detected in 70 serum samples from 50 patients with chronic hepatitis C (sera were tested before and after IFN therapy for 20 patients) using the four DHFR-HVR1 fusion proteins respectively. None of the healthy blood donors, who were anti-HCV negative, was anti-HVR1 positive. Anti-HCV seronegative healthy donors had mean OD_{450} value of *0.06*. Sera were scored positive showing OD value >0.2 (*cut off = 3 time mean neg* + 10 %) in at least two experiments. Each fusion protein (SH1b, BJ1b, SD1b and SD2a) reacted with 72.8 % (51/70), 60 % (42/70), 48.6 % (34/70), and 58.6 % (41/70) of the anti-HCV positive patients respectively. SH1b was the most broadly reactive fusion protein. 91.4 % (64/70) of the tested sera reacted positively with one or more fusion proteins, and among these, 89.1 % (57/64) can react with more than one fusion proteins, 20.3 % (13/64) samples of these sera were shown to react with all four fusion proteins.

The reactivity of sera was compared between responders and non-responders in 20 patients who received interferon therapy (Table 2). The reactive rates of sera with the four fusion proteins were higher in non-responders than in responders before interferon therapy, but there was no statistical significance. 57.1 % (4/7) of non-responders reacted with all four DHFR-HVR1 fusion proteins, while only 15.3 % (2/13) of responders react with all of them (Figure 4). With the three fusion proteins (SH1b, BJ1b and SD2a), the ODs of the serum reactivity of the non-responders were higher than those of responders, and the difference had statistical significances for the four mixed proteins between the two groups of patients (P<0.05) (Figure 5).

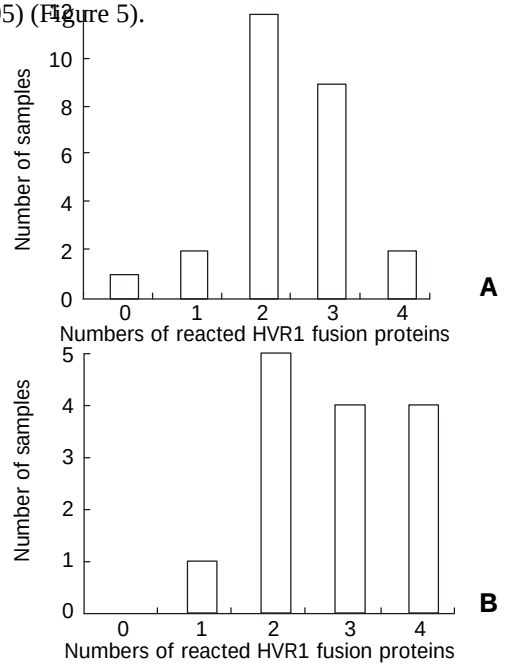

Figure 4 The serum of hepatitis C patients reacted with different numbers of HVR1 fusion proteins. A shows that the sera of IFN responders reacted with 0-4 kinds of HVR1sequence; B shows that the sera of IFN non-responders reacted with 1-4 kinds of HVR1sequence.

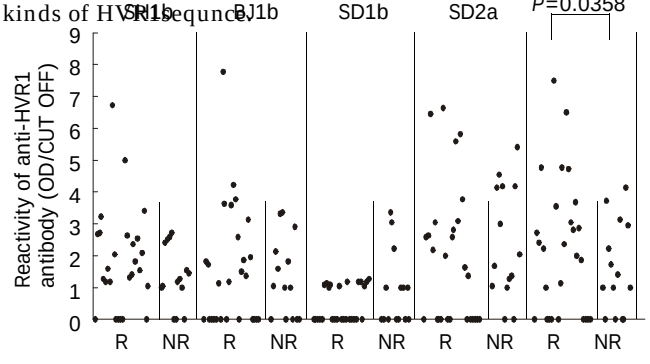

Figure 5 The OD values of 40 HCV-infected patients' sera reacted with the four HVR1 fusion proteins respectively or totally, which were compared between R (IFN-responders) and NR

(IFN-non responders). For the mixed fusion proteins, the values (OD / CUT OFF) of the responders' sera were significantly higher than those of non-responders (P=0.0358).

Table 2 The serum reactive rates of chronic hepatitis C patients with the four HVR1 fusion proteins before IFN therapy

Proteins	Responders(13)	Non responders(7)	Total(20)
SH1b	69.2 %(9 / 13)	85.7 %(6 / 7)	75 %(15 / 20)
BJ1b	53.8 %(7 / 13)	57.1 %(4 / 7)	55 %(11 / 20)
SD1b	30.8 %(4 / 13)	71.4 %(5 / 7)	45 %(9 / 20)
SD2a	69.2 %(9 / 13)	71.4 %(5 / 7)	70 %(14 / 20)
MIX	69.2 %(9 / 13)	71.4 %(5 / 7)	70 %(14 / 20)

DISCUSSION

Antibodies against HVR1 of the main envelope protein of HCV are hypothesized to be neutralizing, but frequent mutation in HVR1 is driven by the host' s humoral immune response[12] and is a major mechanism of viral persistence by escaping host immune recognition[13,14]. The relationship between the severity of liver diseases and the molecular evolution of HCV during chronic infection remains unclear and controversial[15]. HVR1 has the potential to provide a viral antigen for vaccine development, and the cross-reactivity of HVR1 sequences is an essential consideration in the development of a broadly protective vaccine to prevent HCV infection. Cerino *et al*[16] reported that the appearance of anti-HVR1 is earlier than anti-E2, and the early appearance of anti-HVR1 antibodies may be predictive of the later clearance of HCV. It has also been found that HVR1 sequences could induce anti-HVR1 antibodies capable of reacting with many HVR1 sequences in vitro other than the original immunogen sequence[17]. It might be the conserved sub-regions in HVR1 sequences that determined the observed immunological cross-reactivity, by which it may eventually be possible to develop a polyvalent vaccine using a mixture of several HVR1 sequences that cover the reactivity of most HCV isolates.

Synthetic HVR1 peptides have been used in many studies, but this method is too expensive to be used widely. Hattori *et al*[10] successfully expressed HVR1 (aa383-410) as fusion proteins with GST (about 32kDa) in E.coli DH5α, and each fusion protein reacted with 36.1 % to 59.3 % of HCV infected patients' sera. Others reported different reactivity of chronic hepatitis C patients' sera with HVR1 proteins from 15 % to 67 %[18,19]. In this study, we selected four HCV strains of two genotypes from our previous reported clones (SH1b, BJ1b, SD1b and SD2a), because the HVR1 fragments in these four strains was predicted to have higher hydrophilicity and possess 2-3 immunogenic epitopes in each of them[11]. We used pQE40 vector to express HVR1 fused with DHFR in E.coli. pQE40 is constructed for expression of N-terminally 6×his-tagged DHFR-fusion proteins and is recommended for expression of poorly expressed proteins or short peptides. DHFR could enhance both stability and antigenicity of the fusion protein, while DHFR itself displays little immunogenicity[20].

The reactivity of the fusion proteins with HCV-infected patients' sera, especially SH1b was higher than most reports. We selected the HVR1 sequences according to computer prediction. The reason that the selected HVR1 sequence reacted with a considerable proportion of HCV-infected patients' sera may be that cross-reactive antibodies react with the different HVR1 proteins through common epitopes, as suggested by Scarselli *et al*[21]. It might also be the consequence of exposure to multiple strains of HCV.

HCV genotype and the baseline level of viremia have been pointed out as the most important predictive factors of responsiveness to IFN therapy[22]. However, several studies suggested that other factors, such as the heterogeneity of virus population[23] and replication in PBMC[24], might also influence the effectiveness of therapy. Like most RNA viruses, HCV circulates in the human host as a complex population of different but closely related viral variants, commonly referred to as quasispecies[25]. It has been suggested that a reduction in genetic diversity leading to an increasingly homogeneous viral population in the envelope genes, and especially in the HVR1 of the E2 gene, is likely to be the result of a more successful and balanced cellular and humoral immune response[26], which can be observed in IFN therapy responders with viral clearance[27,28]. It was also reported that the broad reactivity of serum anti-HVR1 antibodies correlated with viral loads and response to IFN in genotype-1b-infected patients[10]. But Del porto *et al*[29] found that the frequency of anti-HVR1 T cell response was significantly higher in patients who recovered after IFN therapy than that in those who did not, while no difference in the anti-HVR1 antibody reactivities were detected. In our study, the reactive rates of the four HVR1 fusion proteins with patients' sera were higher in non-responders than those in responders, although there was no statistical difference, which might be due to insufficient number of patients. Meanwhile, 57.1 % (4/7) of non responders reacted with all four HVR1 fusion proteins, while only 15.3 % (2/13) of responders reacted with all of them. These facts suggested that the genetic diversity of HCV was greater in non responders than that in responders. The broad cross-reactivity of anti-HVR1 anti-bodies causes the inefficiency of neutralizing activity as proposed by the theory of "viral antigenic sin"[21,30]. According to this theory, after the exposure to the first immunodominant and cross-reactive virus strain, patients produce not a new antibody to the second related virus strain, but an antibody to the original antigen, which is inefficient to neutralize the new variant. The findings that the serum reactive rates with the four HVR1 fusion proteins were higher in non responders than in responders may be interpreted by this theory. On the other hand, the O.D. values of anti-HVR1 antibodies were higher in responders than those in non responders. This reflected the immune status of these patients and implied that the pre-therapy immune response is a major factor determining eventual virus elimination as suggested by others[31,32].

In conclusion, the selected HVR1 fusion proteins expressed in E. coli can broadly react with HCV-infected patients' sera. The intensity and/or quality of the immune response against HCV could be a critical factor determining the response to treatment. With the evolution of virus strains, anti-HVR1 antibodies could not neutralize all the quasispecies. A polyvalent and high immungenic vaccine combining a mixture of several HVR1 sequences that cover the reactivity of most HCV isolates might be useful.

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