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World J Gastroenterol 2003;9(6):1256-1260
World Journal of Gastroenterology
 ISSN 1007-9327

• VIRAL HEPATITIS •

Cross-reactivity of hypervariable region 1 chimera of hepatitis C virus

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Supported by National 10th Five-Year Plan of Science and Technology Brainstorm Project, No. 2001BA708B06 and a grant from Natural Science Foundation of Beijing, No.7002031
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Received: 2003-01-04 **Accepted:** 2003-02-16

Abstract

AIM: To analyze the amino acid sequences of hypervariable region 1 (HVR1) of HCV isolates in China and to construct a combinatorial chimeric HVR1 protein having a very broad high cross-reactivity.

METHODS: All of the published HVR1 sequences from China were collected and processed with a computer program. Several representative HVR1's sequences were formulated based on a consensus profile and homology within certain subdivision. A few reported HVR1 mimotope sequences were also included for a broader representation. All of them were cloned and expressed in *E.coli.* The cross-reactivity of the purified recombinant HVR1 antigens was tested by ELISA with a panel of sera from HCV infected patients in China. Some of them were further ligated together to form a combinatorial HVR1 chimera.

RESULTS: Altogether 12 HVR1$_s$ were selected and expressed in *E.coli* and purified to homogeneity. All of these purified antigens showed some cross-reactivity with sera in a 27 HCV positive panel. Recombinant HVR1s of No. 1, 2, 4, and 8# showing broad cross-reactivities and complementarity with each other, were selected for the ligation elements. The chimera containing these 4 HVR1s was highly expressed in *E.coli*. The purified chimeric antigen could react not only with all the HCV antibody positive sera in the panel but also with 90/91 sera of HCV -infected patients.

CONCLUSION: The chimeric antigen was shown to have a broad cross-reactivity. It may be helpful for solving the problem caused by high variability of HCV, and in the efforts for a novel vaccine against the virus.

Xiu BS, Ling SG, Song XG, Zhang HQ, Chen K, Zhu CX. Cross-reactivity of hypervariable region 1 chimera of hepatitis C virus. *World J Gastroenterol* 2003; 9(6): 1256-1260
http://www.wjgnet.com/1007-9327/9/1256.asp

INTRODUCTION

Hepatitis C virus (HCV) is a major etiological agent of non-A, non-B hepatitis worldwide[1-3], and HVR1, the N-terminal 27 amino acid residues of the putative HCV envelope protein E2, is known as the principal neutralization epitopes up to date[4-7]. Antibodies to HVR1 in human sera have been shown to block viral attachment to human cell lines *in vitro* and to protect chimpanzees from HCV infection *in vivo*[8-10]. The HVR1 sequence is highly variable, and is the greatest obstacle for the vaccine development and immune therapy[11,12]. However, the highly variable HVR1s have been shown to have some cross-reactivities with each other, indicating that a broadly cross-reactive HVR1 peptide or their cocktails are helpful to solving the problem[13]. Data were accumulated in this study all over the world[14-17].

In China, HVR1 sequences of different HCV isolates have been reported by many authors, but few studies were on HVR1 cross-reactivity. Integrating the HVR1 sequences reported in China together with some published mimotopes, 12 representative HVR1 sequences were selected using bioinformatics technology. All of the representative HVR1 sequences were cloned and expressed, and their cross-reactivity was studied with a panel of 27 HCV positive sera. Finally we obtained an HVR1 fusion antigen broadly cross-reactive with the HCV-infected sera.

MATERIALS AND METHODS

Human sera

Samples of HCV-infected sera were obtained from blood donor applicants in Beijing Red Cross Blood Center and from chronic HCV-infected patients from 302 Hospital of PLA. All were positive for HCV antibodies using the 2nd-generation ELISA kit. (Ortho Diagnostics, Raritan N.J).

Selection of representative HVR1 sequences

All of the HVR1 sequences published in China were loaded into database and their consensus sequence was obtained by BASIC program according to the frequency of amino acid residues. All of these HVR1 sequences were divided into several groups according to their alignment, and one sequence was chosen as the representative from each group. All of the work above was operated on Goldkey (a molecular biology software developed by our institute). Some HVR1 sequences or mimotopes published were chosen as representative ones for their high cross-reactivity with sera of HCV infected patients from other countries.

Construction of expression plasmid HVR1-1 # to 12

The representative HVR1 sequences were modified considering the Escherischia coli's favorable codon usage. The coding genes were synthesized chemically and to facilitate further ligation, two linkers with a specially designed restriction endonuclease site were incorporated into their N- and C-terminals respectively. The N terminal arm is F1 (5'- gc ctc gag ggt ggt gga tct -3'), The C terminal arm is R1 (5'-gc tct aga acc tcc acc act -3'). The fragments were digested with XhoI and XbaI enzymes and inserted into the expressing plasmid pBVIL1 digested by the same restrictive enzymes. In

the same way, 12 different pBVIL1-HVR1 constructs were prepared and the HVR1 genes were expressed as fusion protein with IL1β in *E. coli*.

Construction of the chimeric plasmid

According to the cross-reactivity with the HCV antibodies positive sera panel, several HVR1s were chosen to ligate together one by one as illustrated in Figure 1. The plasmid pBVIL1-HVR1-1# (pHVR1#) was chosen as a vector digested by Xba I and BamH I, while the plasmid pBVIL1-HVR1-2#, was chosen as the donor of pattern, amplified using constant primer F2 (5' gc act agt ggt ggt gga tct 3') and R2 (5'cg gga tcc tta gga aga cac aaa 3') which annealed to C-terminal of IL1β. The PCR product was digested with *Spe* I and *BamH* I, and inserted into the digested vector, pBVIL1-HVR1-1#. Owe to the same cohesive end of the endonuclease *Xba* I and *Spe* I, the digested PCR fragment could accurately linked to the digested plasmid and the new ligated site could be digested by neither of them.

The pBVIL1-HVR1-1+2# had the same enzyme sites with pHVR1# and so it could be used as a new vector and connected with other HVR1 gene fragments. In this way, the pBVIL1-chimeric-HVR1 was constructed to contain four HVR1 genes, HVR1-1#, HVR1-4#, HVR1-6# and HVR1-8#.

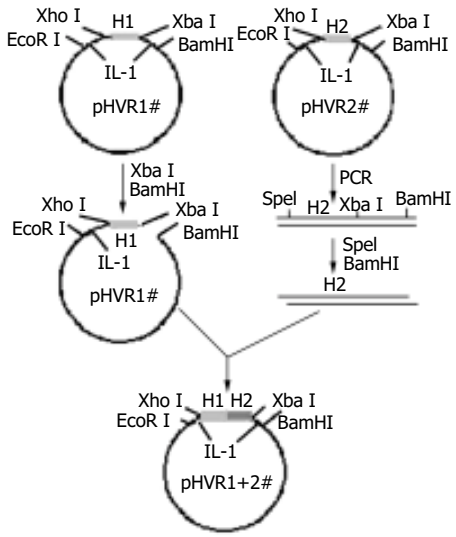

Figure 1 Four different HVR1 gene fragments were cloned on pBVIL-1. HVR1-2 gene fragment was ligated with pBVIL-1-HVR1-1 (pHVR1#). The new pHVR1+2 had the same site with pHVR1#, and the HVR1-4# gene fragments could be ligated by the same way. After 3 cycles, the chimera HVR1 plamid was constructed.

Purification of representative HVR1-1-12# and the chimeric antigen

The plasmids carrying HVR1 fragments were transformed into HB101 as routine, and were examined for their orientation and nucleic acid sequences. The transformed HB101 was grown overnight, diluted 1:20 with fresh LB-medium and further incubated at 37 ¡æto an OD_{600} of 0.6. After induction for 4 h at 42 ¡æ, the bacteria were harvested by centrifugation, and lysed by sonication. All of the recombinant proteins existed in inclusion bodies, and could be dissolved in a solution containing 8 M urea. The recombinant proteins were isolated and purified consecutively by Q-Sepharose-FF and Sephadex G50 chromatography.

ELISA

Microplates were coated with 0.3 μg recombinant HVR1 peptide in 100 mM phosphate buffer (pH7.4) by incubation overnight at 4 ¡æ The plates were then blocked with the phosphate buffer containing 0.2 % BSA at 4 ¡æfor 3 h, and then incubated with 100 μl of the serum sample 1:10 diluted with a sample buffer (100 mM sodium phosphate buffer, pH7.5 containing 10 % goat serum and 0.05 % Tween) at 37 ¡æfor 1 h. After being washed for five times with 100 mM phosphate buffer (pH7.5) containing 0.05 % Tween, the plates were then incubated for 30 min at 37 ¡æwith 1:25 000 diluted HRP-conjugated monoclonal antibody against human IgG. After washing, the reaction was visualized in the substrate buffer (50 mM sodium phosphate-citric acid buffer, pH5.0 containing 0.4 mg/ml TMB and 0.4 μl/ml of 30 % hydrogen peroxide). The reaction was stopped by adding 50 μl of 2 M sulfuric acid, and the absorbance was measured in a microplates ELISA reader at 450 nm.

RESULTS

Determination of 12 representative peptides

A total number of 123 sequences on HVR1 were reported in China, and the derived consensus profile of them is shown in Figure 2A. Some amino acid residues of HVR1 were shown to be hypervariable, while those at position 385, 389, 406, 409 were conserved. The sequence on first line was defined as CCS (Chinese consensus sequence), whose amino acid residues emerged most frequently. CCS was chosen as the first representative sequence, named HVR1-1#, being different at some positions from Puntoriero's consensus sequence[13] (Figure 2B). The homology of the 123 sequences was analyzed using the Goldkey software, and divided into 6 groups, HVR1-2 to 7# according to their alignment to CCS. In this way 6 sequences named were obtained. HVR1-8# 9# were from GenBank (L19383, S24080), both being broadly cross-reactive with mice sera induced by mimotopes. HVR1-10# and 11# were sequences for the mimotope R9 and M122 respectively (Puntoriero *et al*, 1998), and HVR1-12# reported by Watanabe[14].

A

```
1     5     10    15    20    25  27
STHVTGGAAGRTTSGFTSLFTPGPSQK
GHYTSAAVQAHSLRSLAGFLSSAAAKN
DSRAVTRTTSQAIQAIVNISALSSK N
N QII SSVHYNAHRVMRMKNF R  I
E TLM  KSTAGVYTKGTVTRR Q  T
Q KSH  QEKNVMGIMIA YQT T
R V L  DHQIDHAHYKK  KH N
T F W  EGRDIPLK S   MA
A L    MR KK N Y   N
H S       FQ T     Q
I C       GR F     V
K D       SY K     Y
L I         W
Y
```

B

```
STHVTGGAAGRTTSGFTSLFTPGPSQK
E------S-A-----L----S--A---
```

Figure 2 Derivation of the Chinese consensus sequence. (A) Consensus pattern of the 123 natural variants of the HCV HVR1 sequence used in this work. Shaded residues accounted alone for 80 % of the observed frequency. Residues were listed in decreasing order of observed frequency from top to bottom. The first line was Chinese consensus sequence (CCS). (B) The Chinese consensus sequence (upper) was different with Puntoriero's (lower). Dashes indicate residues identical to the upper line.

Reactivity of representative peptide with sera of panel I

Twelve representative HVR1 gene as shown in Figure 3, were expressed in *E.coil* fused with human IL-1β. The HVR1/IL-1β fusion protein migrated at the expected position of about 21 kD (Figure 4). Twenty-seven HCV patients' sera were used as panel I to show the cross-reactivities of 12 representative HVR1 by ELISA. As shown in Figure 5, all of the HVR1 peptide reacted with more than one serum. No reactivity was detected to IL-1β in sera of panel I, and none of the anti-HCV negative sera reacted with the 12 recombinant peptides. The most broadly cross-reactive HVR1 was HVR1-11#, and the Chinese consensus sequence (CCS) which showed a higher cross-reactivity too.

We took HVR1-1,2,4,8# as components for the best cocktail, because these 4 HVR1 peptides showed complementary reactivities to the sera in panel I, as showed in Figure 5. There overall cross-reactivity was found to be 25/27.

1#	STHVTGGAAGRTTSGFTSLFTPGPSQK
2#	STHVTGGVQGHSLRGLTSLFTSGPAQK
3#	ITRVTGGVQGHSLRSLTSLFTPGPAQK
4#	STHVTGAVQGRSLQSFTSFLSPGPSQK
5#	DTHVTGGAAARGASGLANLFTSGPAQK
6#	GTYVTGGATAHTASGFASLFTTGSKQN
7#	TTHVTAGTAAHATSSFTKLFAPGAKQN
8#	NTYVTGGSAAHTTSRFTSLFSPGPQQN
9#	ETHTSGGSVARAAFGLTSIFSPGKSQN
10#	TTTTTGGVQGHTTRGLVRLFSLGSKQN
11#	DTTVTGGQAARTTQSFTSLFPPGPSQK
12#	DTTVTGGQAARTTQSFTSLFTPGPSQK

Figure 3 The amino acid sequence of 12 representative HVR1 sequences. Amino acid residues were indicated by standard single-letter codes.

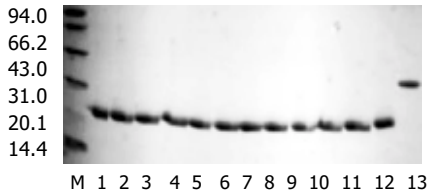

Figure 4 The Coomassic stain after SDS-PAGE of 12 purified representative and chimera HVR1 antigen. M. marker; 1-12. 12 purified representative HVR1 antigen; 13. chimera HVR1 antigen.

Reactivity of chimera HVR1 antigen with panel I and panel II

HVR1-1#, 2#, 4# and 8# were ligated one by one in tandem within plasmid pBVIL1. The chimeric protein was expressed in HB101 and further purified (Figure 4).

As expected, a broader reactive spectrum was observed for the chimeric HVR1 antigen. It was shown to be reactive with all of the sera of panel I, including sera 73# and 39# which were not reactive with any single HVR1 (Figure 5). For more data 91 sera from HCV-infected patients were also used for the assay as panel II, with 90 reactive with chimera antigen (Table 1). The data indicated that application of the chimera protein helped to acquire a higher cross-reactivity.

Table 1 Reactivity of F4HVR1 with another panel (91 sera of HCV infected patient)

OD difference between sera and co	No. of reactive sera	The adding up percent of total sera
>2.0	56	61.5
1.5~2.0	14	76.9
1.0~1.5	13	91.2
0.5~1.0	6	97.8
0.371	1	98
0.065	1	

The cutoff of the ELISA was as defined in Figure 5.

DISCUSSION

HVR1, which contains a principal neutralization epitope in HCV, is important for the development of HCV vaccine[4-7]. Due to the high mutation rate of HVR1, there are now hundreds of HVR1 isolates reported, presenting a great obstacle for HCV vaccine development[18-21]. It was suggested that to select a highly cross-reactive HVR1 antigen could solve the variability problem[22-27], thus highlighted the importance to study the cross-reactivity of HVR1.

Most of the work about the cross-reactivity of HVR1 focused on single HVR1 antigen. However we think the cross-reactivity of single HVR1 is limited. Recently, multi-epitope chimeric antigen was used to improve the sensitivity of HCV immunoassay reagents[28,29]. Here we provided evidence for enhancing the cross-reactivity by constructing a chimeric antigen that incorporates several representative HVR1 peptides.

Considering geographical variation of HVR1[30-33], we gave

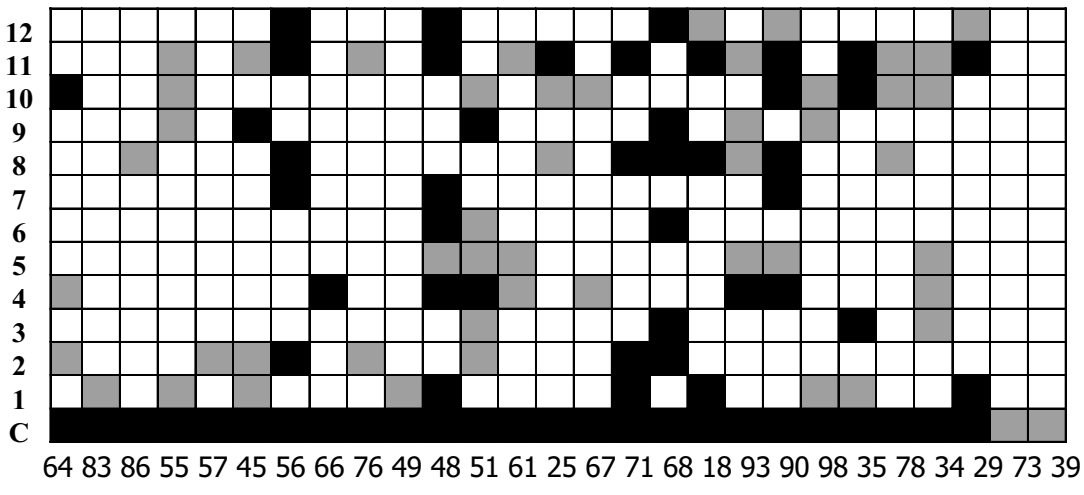

Figure 5 Reaction of the 12 representative HVR1$_s$ and chimera HVR1antigen (C) with 27 sera from HCV-infected patients. HVR1 names are indicated at the left of each column. For each serum (indicated on the bottom of each column) average values (A450) have been determined from two independent experiments. The mean of 10 sera from non-infected individuals plus 4SD defined the cutoff (co). Results were expressed as the difference between the average value of the HCV antibody positive sera and co. Strong positive values (>0.5) are indicated in black. And weak positive values are indicated in grey (0.15-0.5).

priority to Chinese sequence when we selected representative HVR1 sequences. The differences between CCS and Puntoriero's suggest the HVR1 variant found in China differs to a certain extent from what occurred elsewhere[14]. The chimeric antigen contains 3 representative HVR1 sequences coming from China, and showed broad cross-reactivity with sera of the HCV-infected patients.

The reported HVR1 antigen or mimotope could cross-react with no more than 80 % of sera containing HCV antibodies[22-27]. Chimeric HVR1 antigen could cross-react with 90/91 (98 %) of tested sera. The results also suggested that most of HCV infected patients could generate some antibodies against HVR1. The possible association between HVR1 antibody and the self-limiting course of HCV infection and a more favorable response to interferon[34-40], remains to be evaluated in the following study.

Evidently, the reaction spectrum of the chimera HVR1 antigen include the total cross-reactivity of the representative HVR1 antigen contained. Interestingly, the chimera HVR1 antigen could react with sera 73# and 39#, which are not definitely reactive with any of the four representative HVR1. In our consideration, those samples might react with some of the representative HVR1 used for ligation, but reactions are too weak to be detected. The OD value would be elevated when 4 HVR1 is added up together.

In this study, we used a prepared chimeric antigen instead of synthetic peptides[41-43]. The antigen may also be used in the study for the HCV vaccine. In addition, the chimeric antigen is fused with IL-1β. The latter part contains a nano-peptide sequence. It may act as an immune adjuvant[44-46], promoting a strong immune response when injected.

In summary, the chimeric HVR1 antigen, containing several representative HVR1 fragments, can show very high cross-reactivity, which may be helpful to overcome the variability of HCV. The chimeric HVR1 antigen is of potential application for HCV vaccination and immune therapy.

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Edited by Su Q and Wang XL