Genetically Engineered Single-Chain Fvs of Human Immunoglobulin against Hepatitis C Virus Nucleocapsid Protein Derived from Universal Phage Display Library

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Hepatitis C virus (HCV) is the major causative agent of post-transfusion non-A, non-B hepatitis.[1] This virus is of clinical importance because more than half of HCV-infected patients subsequently develop chronic hepatitis which may progress to cirrhosis and liver cancer. HCV is transmitted mainly via blood transfusion and other parenteral contacts. The majority of patients with chronic hepatitis C infection, and can transmit the virus, are however asymptomatic. Therefore, the screening of blood and blood products for antibodies to HCV is currently the most effective method for prevention of HCV in the population.[2] HCV can be found worldwide, with the prevalence in Thai blood donors of around 1-5%. The northeastern part of Thailand has a relatively high prevalence of this viral infection, and up to 8.3% of male blood donors aged between 31-40 years have antibodies to HCV.[3]

HCV is a single stranded RNA virus and belongs to the

SUMMARY Specific single-chain Fvs (scFvs) of human immunoglobulin that specifically recognized the recombinant hepatitis C virus (HCV) nucleocapsid protein were isolated from a large phage display antibody library. This universal library of genetically engineered filamentous phagemids displayed random pairings of the variable regions of both human heavy and light chain immunoglobulin in the scFv format. Specific clones were isolated by affinity selection with purified recombinant HCV protein fused to glutathione-S-transferase (GST). The GST-specific clones were excluded by blocking the phagemid library with GST prior to the selection. After 4 rounds of selection, the HCV-reactive clones were enriched by a factor of 100,000. About 4% and 9% of the clones from rounds 4 and 5, respectively, specifically reacted to the HCV portion of the fusion protein in an enzyme immunoassay. The specificity was confirmed by specific binding inhibition with plasma from an HCV-infected individual. Nucleotide sequence analysis of 3 HCV-specific clones indicated that all 3 clones contained an almost identical VH gene sequence which was derived from the VH3 germline gene family. These clones had different VL gene sequences of the lambda type. There were some differences between nucleotide and amino acid sequences of the HCV-specific scFv genes and those of the closest matched germline genes, indicating the presence of somatic mutation. This study illustrated the feasibility of using antibody engineering technology with the universal phage display library to isolate human antibodies with predefined specificity to important microbial pathogen which may be useful for future therapeutic purpose.

family *Flaviviridae*. The viral genome encodes a single large polyprotein which is subsequently cleaved into a series of structural and nonstructural proteins. The analysis of nucleotide sequences and deduced amino acid sequences of HCV isolates from different parts of the world showed high degrees of heterogeneity of the viral genome. HCV can be classified into at least 6 major genotypes and a

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number of subtypes on the basis of nucleotide sequence similarity.[4] The degree of sequence variability is not uniformly distributed throughout the genome. The gene encoding nucleocapsid protein (viral core protein) is the most conserved coding region with approximately 90% sequence identity between different HCV genotypes. The antibody to this protein can be found in most HCV patients regardless of the infected genotypes, and is therefore very important in the diagnosis of the virus.[5] It may also be useful in studying the pathogenesis and in the treatment of this infection.

In recent years, there have been rapid developments in the area of antibody engineering by combining the knowledge on the functional activity and specificity of the antibody molecules and the ability to genetically modify the molecules to perform desired functions. Since it is fundamentally difficult to produce useful human monoclonal antibodies by hybridoma technique, the antibodies can be obtained by replacing the constant domain and most of the framework region of the variable domain of the mouse immunoglobulin genes with their human counterparts.[6] Such technique is very powerful but it is time-consuming and requires expensive procedures. Recently, large repertoires of the variable region of human immunoglobulin genes can be cloned into bacteriophage and expressed on the surface of the phage as a fusion protein with phage coat proteins.[7] This approach enables the isolation of human monoclonal antibodies with the desired specificity *in vitro* by selecting the "phage display" antibody libraries with the appropriate antigen. It overcomes the problems associated with the production of human monoclonal antibodies by

hybridoma technique. The libraries used for selection have been constructed from immunized or naive individuals or can be synthesized *in vitro*.[8,9] Powerful and rapid selection techniques have been developed. Since these antibodies are of human origin, they can be used for both diagnostic and therapeutic purposes.[10,11] The quality of the libraries have been markedly improved and they can be used as a source for selecting antibodies to a vast number of antigens.[9]

The objective of the present study is to isolate the phagemid clones expressing human antibodies specific to hepatitis C virus nucleocapsid protein by using a universal phage display library. The usage of germline gene segments in the phagemids producing scFvs to HCV nucleocapsid protein is also illustrated.

MATERIALS AND METHODS

Purification of recombinant HCV nucleocapsid and GST proteins

Purified HCV nucleocapsid protein was obtained by expressing a recombinant *Escherichia coli* transfected with plasmid pCGBB51-g which contained the HCV nucleocapsid gene. The protocols for protein expression and affinity purification have been described.[12] In brief, the gene encoding the first 123 amino acids of HCV nucleocapsid protein of HCV isolated from a Thai patient was cloned into the 3' end of a gene encoding glutathione-S-transferase (GST). Protein expression was induced by the addition of isopropyl b-D-thiogalactoside (IPTG). The protein was expressed as a fusion protein containing GST at the amino terminal and HCV nucleocapsid protein at the carboxyl

terminal. This fusion protein was purified by affinity chromatography using glutathione-Sepharose 4B beads (Pharmacia Biotech, U.S.A.). The purified protein was dialyzed extensively with phosphate-buffered saline (PBS) in a microdialyzer (Pierce, U.S.A.). Protein concentration was quantified by a Bradford Protein Assay Kit (Bio-Rad, U.S.A.). The protein was stored at -70°C. Purified GST protein was obtained by expressing plasmid pGEX-3X (Pharmacia Biotech, U.S.A.), containing the gene for GST protein. The protein was expressed, affinity purified and dialyzed as described above.

Plasma samples

An anti-HCV antibody-positive plasma sample was obtained from a Thai blood donor who was infected with HCV. The viral genome from this sample (BB51) was used for constructing the recombinant plasmid containing the viral recombinant nucleocapsid protein as described above. In addition, an anti-HCV antibody-negative sample was obtained from a healthy blood donor. Antibody to HCV was detected by enzyme immunoassay and confirmed by an immunoblot assay (HCV ELISA and HCV Blot 3.0, Diagnostic Biotechnology, Singapore). The plasma samples were thawed once, aliquoted and immediately stored at -70°C.

Phage display antibody library of scFv

The phage display library used in this study contained the variable regions of the human heavy and light chain immunoglobulin genes (VH and VL, respectively), arranged in the single

chain Fv format (scFv). The VH and VL genes were randomly cloned into a phagemid (phage plus plasmid), allowing the expression of scFv as a fusion protein with the minor coat protein (pIII) of the filamentous phage. The construct contained an amber codon for selective expression as a bacteriophage pIII surface protein or as a soluble protein.[8] The library size was estimated to be around 5×10^8-5×10^9 diverse clones. This phage display antibody library was a gift from Dr. G. Winter, MRC Centre for Protein Engineering, Cambridge, U.K.

Selection of scFvs reactive to recombinant HCV nucleocapsid protein

The scFvs were selected from the library using affinity selection technique with the recombinant antigen on solid surface. To avoid selecting the scFvs reactive to the GST component of the recombinant fusion protein, purified GST was added as a competing antigen during the selection process. This markedly reduced the frequency of GST-reactive clones. The selection process consisted of 4-5 rounds of repeated selection. In brief, a Maxisorp immunotube (Nunc, U.S.A.) was coated overnight with 10 μg/ml of the recombinant HCV-GST fusion protein at room temperature. The tube was washed three times with PBS and then blocked with 3% (w/v) skimmed milk powder in PBS (3% MPBS), followed by another 3 rounds of washing. In the first round of selection, the original universal phage display scFv antibody library was added to the fusion protein-coated immunotube at the titre of 2×10^{13} colony forming units of the

phagemids. The tube was incubated at 37°C for 120 minutes, with gentle rotation in the first 30 minutes followed by 90 minutes of static incubation. The unbound phagemids were removed by extensive washing for 20 times with PBS containing 0.1% Tween 20, and then 20 times in PBS without Tween 20. The bound phagemids were eluted from the immunotube using freshly made 100 mM Triethylamine (BDH Chemicals, U.S.A.). The reaction was neutralized by adding 1/2 volume of 1 M Tris-Cl pH 7.4. The eluted phagemids were used for infecting the mid-log phase E. coli strain TG1. The infected TG1 were then plated onto a large culture disk containing TYE agar[13] with 100 μg/ml ampicillin and 1% glucose. The plate was incubated overnight at 37°C. After the incubation, bacterial colonies containing phagemids including those reactive to recombinant HCV-GST fusion protein were grown on the plate. The recombinant bacteria were then collected from the plate and grown in 2xTY media[13] containing 100 μg/ml ampicillin and 2% glucose, to log phase. The phagemids were rescued by adding 10-fold excess of VCS-M13 helper phage (Stratagene, U.S.A.). The tube was incubated at 37°C without shaking for 20 minutes and then with shaking for 40 minutes. The culture was centrifuged and the cell pellet was resuspended in 2xTY media containing 100 μg/ml ampicillin and 50 μg/ml kanamycin, followed by incubation at 30°C with shaking. The phagemids were recovered from the culture supernatant by precipitation with 20% polyethylene glycol 6000 in high salt solution, and finally resuspended in PBS. The phagemids recovered after the first round of selection

were then offered for selection in the second round. The selection process was repeated at least 4 times. After the first round, the selection was carried out in the presence of GST as competitor. The phagemids were preblocked in a buffer containing 50 μg/ml of purified GST, before incubating with the immunotubes coated with purified recombinant HCV-GST fusion protein.

Quantitation of phagemids

The phagemids offered and recovered from each round of selection were quantitated by serial dilutions. One microlitre of the phagemid suspension was used for infecting 1 ml of mid log-phase E. coli TG1. The resulting infected bacteria were diluted serially from 10^{-1} to 10^{-7} and plated onto TYE plates containing 100 μg/ml ampicillin and 1% glucose. The plates were incubated overnight at 37°C. The concentration of phagemids was calculated from the number of colonies grown on each plate.

Induction of expression of soluble scFv by the phagemids

A small aliquot of eluted phagemid after 3 to 5 rounds of selection was used for infecting E. coli strain HB2151 which allowed the expression of soluble scFv. The infected bacteria were randomly selected and then grew overnight by incubating at 37°C with shaking, in 100 μl of 2xTY media containing 100 μg/ml ampicillin and 1% glucose, in a 96-well culture plate (Corning, U.S.A.). Small innoculum of each clone was transferred to another 96-well plate and regrown to log phase and then induced with IPTG

to the final concentration of 1 mM. Soluble scFvs were recovered from the supernatant and used in the ELISA.

Detection of antibody activity to recombinant HCV nucleocapsid protein by ELISA

Microtitre assay plates (Falcon, U.S.A.) were coated overnight at room temperature with 100 μl per well of 10 μg/ml of either the purified recombinant HCV-GST fusion protein or purified GST. The wells were blocked with 200 μl per well of 2% MPBS, for 2 hours at 37°C. Soluble scFvs from the phagemid culture were added to each well in a buffer containing 2% MPBS. The plate was incubated at room temperature for 1 hour and then washed 3 times with PBS containing 0.05% Tween 20, and 3 times with PBS without Tween 20. The bound scFv was detected using a mouse monoclonal antibody specific to c-myc peptide which was genetically engineered as tagged peptide at the carboxyl terminal of the scFv phage display library (antibody 9E10, a gift from Dr. G. Winter, Cambridge). The signal was amplified by using peroxidase-conjugated anti-mouse IgG, Fc-specific (Sigma, U.S.A.), and detected with TMB (3,3',5,5'-tetra-methylbenzidine) as substrate. The optical density was read at 450 nm, using 650 nm as standard. For the inhibition ELISA, the soluble scFv was incubated with the recombinant HCV-GST fusion protein-coated plate in the presence of serial twofold dilution of anti-HCV-positive and anti-HCV-negative human plasma, started from 1:2 up to 1:64. The subsequent steps were similar to those described above.

DNA amplification and nucleotide sequencing

The VH and VL genes from HCV-reactive phagemid clones were amplified by polymerase chain reaction.[9] The PCR product was purified using Wizard PCR Prep system (Promega, U.S.A.). Nucleotide sequence of the PCR product was obtained using PRISM dye terminator FS sequencing system (Perkin-Elmer, U.S.A.) running on an automated DNA sequencer (Applied Biosystems, Inc., U.S.A.). The sequence obtained was analyzed using Sequence Navigator and MacVector programs (Applied Biosystems and Oxford Molecular Group, respectively) and compared with nucleotide and amino acid sequences of other VH and VL isolates deposited in the GenBank database via World Wide Web server (National Center for Biotechnology Information, U.S.A.). The usage of germline V and J gene segments were analyzed by comparing with those catalogued in the V BASE GOLD directory of human V gene segments and alleles using DNAPLOT package.[14]

RESULTS

Isolation of HCV-reactive scFvs

A total of 5 rounds of selection was performed for isolating scFvs reactive to the recombinant HCV-GST fusion protein from the universal phage display antibody library. The results are summarized in Table 1. After 4 and 5 rounds of selection, the frequency of the phagemids that bound to the recombinant HCV-GST fusion protein-coated immunotube was enriched by more than 10^5 fold from the original phage display antibody library.

scFvs reactive to HCV nucleocapsid protein and to GST

A total of 96 colonies each from rounds 3, 4 and 5 of the selection were randomly selected. The phagemids were then recloned from *E. coli* strain TG1 to strain HB2151, to allow the expression of

Table 1   Number of phagemids offered and recovered from each round of selection with the recombinant HCV-GST fusion protein-coated immunotube. The frequencies of the reactive phagemids in the library are also shown.

Selection rounds	Numbers of phagemid		Frequency of reactive /total phagemids
	Offered	Recovered	(recovered/offered)
Round 1	2×10^{13}	6×10^5	3×10^{-8}
Round 2	2×10^{12}	1×10^7	5×10^{-6}
Round 3	2×10^{12}	7×10^6	3.5×10^{-6}
Round 4	1×10^{12}	3.6×10^9	3.6×10^{-3}
Round 5	2×10^{12}	6.2×10^9	3.6×10^{-3}

Table 2   The frequency of the phagemids from each round of
.selection that were reactive to recombinant HCV-GST
fusion protein and to purified GST in ELISA test.

Rounds	Numbers of phagemid reactive to	
	HCV-GST fusion protein	GST
Round 1	nd	nd
Round 2	nd	nd
Round 3	1/96	1/96
Round 4	4/96	0/96
Round 5	24/96	15/96

nd, not determined.

soluble scFvs because of the presence of an amber stop codon in the construct. This construct also contained the gene encoding parts of c-myc peptide, enabling the detection of scFv by murine monoclonal antibody (9E10) to the tagged c-myc peptide. The results are summarized in Table 2. After round 3, only 1 in 96 clones was reactive to the recombinant HCV-GST fusion protein. This clone was reactive to GST portion, but not the HCV portion of the fusion protein. After round 4, all 4 positive clones obtained (4%) were reactive to the fusion protein but not to GST, indicating the reactivity to the HCV
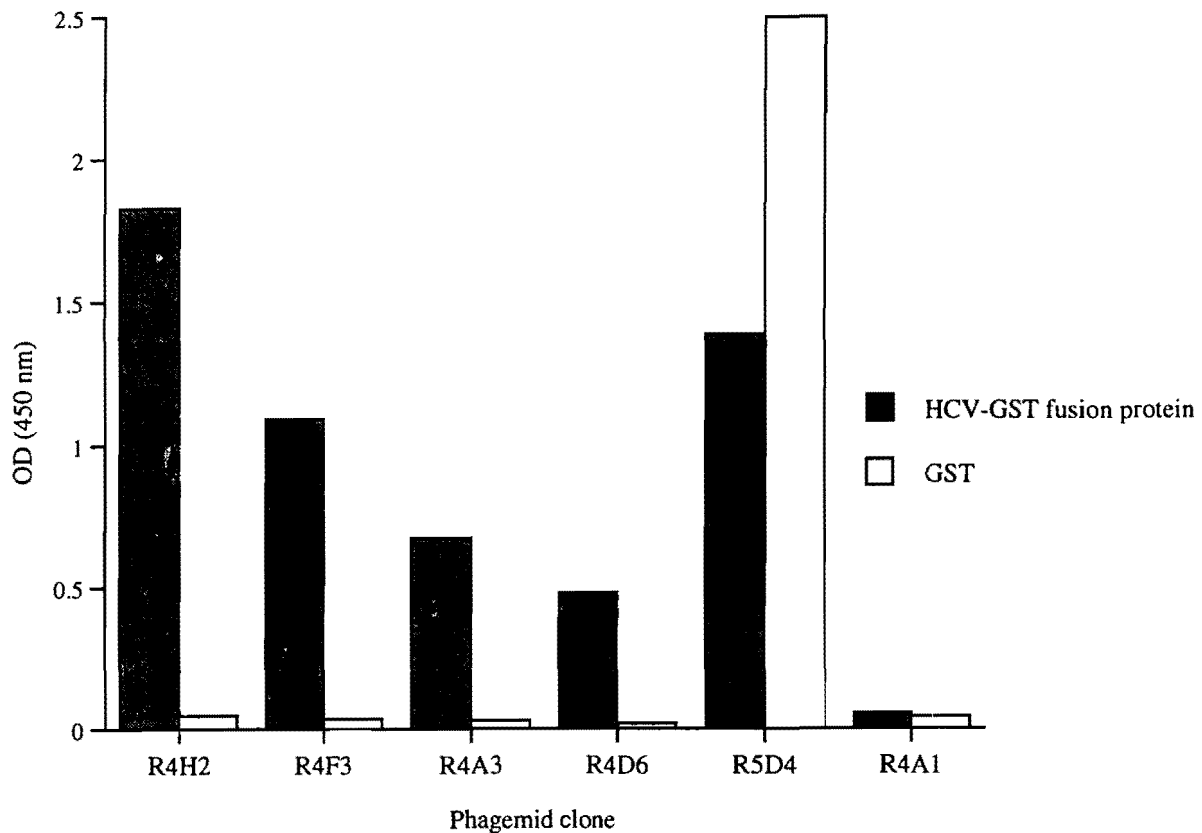

Fig. 1   Enzyme immunoassay for scFv antibody activity to recombinant HCV-GST fusion protein and to purified GST protein of the phagemids reactive to HCV (R4H2, R4F3, R4A3 and R4D6), and to GST (R5D4). Phagemid R4A1 reactive to neither of the two proteins were used as a negative control.
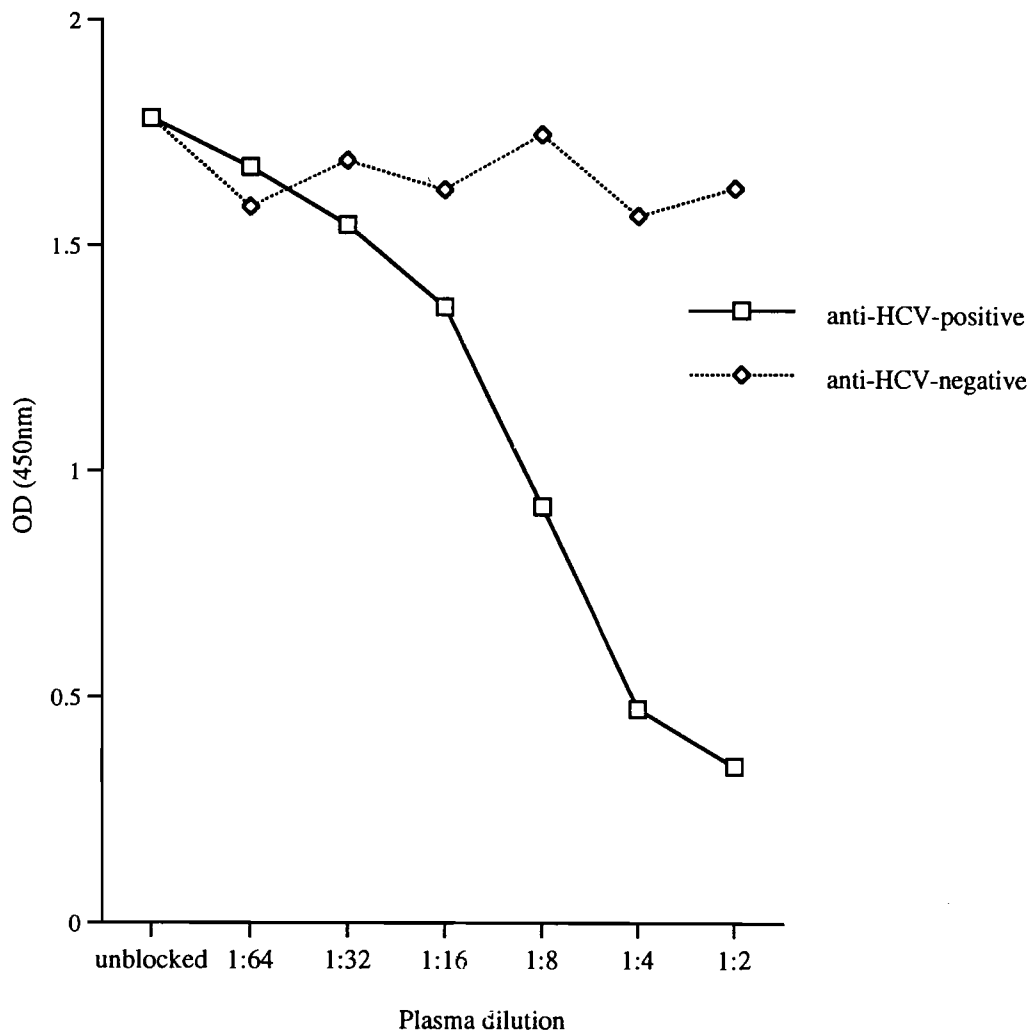
Fig. 2 Inhibition of scFv R4H2 binding to the recombinant HCV-GST fusion protein by plasma samples from anti-HCV-positive and anti-HCV-negative blood donors.

protein. The percentage of reactive clones increased to 24% after round 5, 9 clones (37.5%) of which were reactive to the HCV portion while the other 15 clones (62.5%) were reactive to GST.

Five HCV-reactive or GST-reactive phagemids were selected and recloned twice, then assayed by ELISA. Four clones (R4H2, R4F3, R4A3 and R4D6) were specific to HCV portion, and one (R5D4) was specific to the GST portion of the fusion protein

(Fig. 1). Clone R4H2 was further used in the inhibition ELISA. The binding of R4H2 scFv were inhibited, in a dose-dependent manner, by plasma containing human antibody to HCV confirming the specificity of this scFv (Fig. 2).

Nucleotide sequence analysis of HCV-specific phagemids

The VH and VL genes from 3 HCV-specific phagemids (R4H2, R4F3, and R4A3) were sequenced (Fig. 3 and Fig. 4). The

VH gene segment of all 3 phage-mids were almost identical to each other and were derived from the same germline gene segment in the VH3 gene family, with the closest similarity being to the DP-31 and DP-32 germline genes.[14] The J regions were derived from JH4 germline segment. However, there were some differences from the closest matched germline genes, indicating the presence of somatic mutation. The putative amino acid sequences of VH were identical to each other (Fig. 4).

(a) VH gene

```
                                                                    60
R4H2-VH   CAGGTGCAGCTGGTGCAGTCTGGGGGAGGCTTGGTACAGCCTGGCAGGTCCCTGAGACTC
R4F3-VH   _____
R4A3-VH   _____


                                            CDR1
                                            _ _ _ _ _ _ _ _ _ _ _ _ _ _ _
                                                                   120
R4H2-VH   TCCTGTGCAGCCTCTGGATTCACCTTTGATGATTATGCCATGCACTGGGTCCGGCAAGTT
R4F3-VH   _____
R4A3-VH   _____


                                                         CDR2
                                  _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _
                                                                   180
R4H2-VH   CCAGGGAAGGGCCTGGAGTGGGTCTCAGGTATTAGTTGGAATAGTGGTTACATAAGTTAT
R4F3-VH   _____
R4A3-VH   _____


          CDR2 (cont)
          _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _
                                                                   240
R4H2-VH   GCGGACTCTGTGAAGGGCCGATTCACCACTTCCAGAGACAACGCCAAGAACTCACTGTAT
R4F3-VH   _____
R4A3-VH   _____


                                                              CDR3
                                                              _ _ _ _ _ _
                                                                   300
R4H2-VH   CTGCAACTGAACAGCCTGAGAGCCGAAGACACGGCCGTTTATTATTGTGCGAGAGATCGG
R4F3-VH   _____
R4A3-VH   _____


          CDR3 (cont)
          _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _
                                    325
R4H2-VH   TACGGTCTCTTCGACTACTGGGGCC>
R4F3-VH   _____C_____>
R4A3-VH   _____C_____>
```

Fig. 3 Alignment of nucleotide sequences of (a) VH and (b) VL genes of 3 HCV-specific scFv phagemids.

(b) VL gene

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                                                                        60
R4H2-VL   CAGTCTGCCCTGACTCAGCCTGCCTCCGTGTCTGGATCTCGTGGACAGTCGATCACCATC
R4F3-VL   _____g_____a__g_____
R4A3-VL   _____


                              CDR1
          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                                                                       120
R4H2-VL   TCCTGCACTGGAACCACTGGTGACGTTGGTGGTTATGACTATGTCTCCTGGTACCAACAG
R4F3-VL   _____a_____t_____
R4A3-VL   _____gct_____a_____g___a


                                            CDR2
                                -  - - - - - - - - - - - - - - - -
                                                                       180
R4H2-VL   CACCCAGGC AAAGCCGCCAAACTCCTCATCTATGGTAACAGCAATCGGCCCTCAGGGGTC
R4F3-VL   _____t_____t_____
R4A3-VL   _____tt_____


                                                                       240
R4H2-VL   CCTGATCGCTTCTCTGCCTCCAAGTCCGGCAATTCGGCCTCCCTGACCATCTCTGGACTC
R4F3-VL   _____
R4A3-VL   _____g___t_____g___


                                            CDR3
                                - - - - - - - - - - - - - - - - - - - - -
                                                                       300
R4H2-VL   CAGGCTGAGGATGAGGCTGATTATTTCTGCAGCACATATGCACCCCCCGGTATTATTATG
R4F3-VL   _____g_g_g_____g_____g____
R4A3-VL   _____g_____


                              330
R4H2-VL   TTCGGCGGAGGGACCAAGCTGGAGATCAGA
R4F3-VL   _____
R4A3-VL   _____g_a_____
```

(a)   VH putative amino acid sequence

```
                                      CDR1                    CDR2
                                      -----              ----------
                                                                    60
R4H2-VH    QVQLVQSGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQVPGKGLEWVSGISWNSGYISY
R4F3-VH    _____
R4A3-VH    _____


           CDR2(cont)                          CDR3
           ------                              --------
                        80                    100
R4H2-VH    ADSVKGRFTTSRDNAKNSLYLQLNSLRAEDTAVYYCARDRYGLFDYWG>
R4F3-VH    _____>
R4A3-VH    _____>
```


(b) VL putative amino acid sequence

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                                 CDR1                      CDR2
                           --------------              -------
                                                                60
R4H2-VL    QSALTQPASVSGSRGQSITISCTGTTGDVGGYDYVSWYQQHPGKAAKLLIYGNSNRPSGV
R4F3-VL    _____a_____ka_____i_____f_____f_i_____
R4A3-VL    _____sc__d_____r_____f_____


                                 CDR3
                           -----------
                        80                  100
R4H2-VL    PDRFSASKSGNSASLTISGLCAEDEADYFCSTYAPPGIIMFGGGTKLEIR
R4F3-VL    _____rrd__a__s_____
R4A3-VL    _____y_____a_____e_____
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Fig. 4   Alignment of putative amino acid sequences of (a) VH and (b) VL genes of 3 HCV-specific scFv phagemids.

The VL gene segment of all phagemids were of the lambda type. Each of the 3 phagemids had different VL sequences including those in the CDR regions, although their VL segments were derived from the VL2 germline gene with closest similarity to the 2a2.272A12 gene.[15] The J genes of R4H2 and R4F3 were derived from the JL2/JL3a and JL3b segments, respectively.[16] The germline origin

of the R4A3 J segment could not be identified. The VL genes of these 3 phagemids were also different from the closest matched germline genes, indicating the presence of somatic mutation.

DISCUSSION

This study demonstrated the isolation of phagemids producing single-chain Fvs specific to

recombinant HCV nucleocapsid protein. The competing strategy was successfully employed to minimize the selection of scFv specific to GST, by blocking the scFv phage display antibody library with GST prior to offering to the recombinant HCV-GST fusion protein-coated immunotube. After 4 rounds of selection, the frequency of bound phagemids was increased by 100,000 fold, from 3×10^{-8} to

3.6×10^{-3}. This demonstrated the efficiency of multiple rounds of selection. More than 10^9 phagemids were obtained after eluting the bound phagemids from the antigen-coated immunotubes, about 5% and 9% of which were reactive to the HCV portion of the recombinant HCV-GST fusion protein, after round 4 and round 5, respectively.

The specificity of the scFvs to HCV nucleocapsid protein was illustrated by their specific interaction with the recombinant HCV-GST fusion protein but not with purified GST in ELISA test, and, using R4H2 scFv as a model, by the ability of antibody in human plasma from HCV-infected blood donor to block the binding of scFv to recombinant HCV-GST fusion protein, in a dose-dependent manner. Normal human plasma did not block this binding. This also demonstrated that this specific scFv recognized the same epitope as that which occurred during natural infection in humans.

Phage display antibody library selection is a very powerful technique for producing human antibody against several antigens. Instead of having to construct the library from immunized or infected individuals, the recent availability of universal libraries containing a large repertoire of human immunoglobulin genes has enabled us to isolate HCV-specific scFv from this unimmunized library. Although the selection method is still complicated and requires experienced investigators, scFv with good specificity can be isolated by trained scientists within weeks as compared to months by using hybridoma technique. Our group has recently reported the production of murine hybridoma clones reactive to the recombinant HCV-

GST fusion protein.[12] Both the scFvs and murine monoclonal antibodies were specific to the same antigen. The hybridoma-derived antibodies were of murine origin. Murine monoclonal antibodies are complete antibody molecules with intact effector function, and are good reagents for diagnostic purposes. It is expected that the phage display antibody technology will not replace hybridoma technology for producing specific diagnostic reagents. However, murine monoclonal antibodies have limited value for *in vivo* treatment.[6] On the other hand, the variable regions of both heavy and light chain immunoglobulin of the scFvs are of human origin which enabled them to be used in humans. However, scFvs are small molecule without the effector functions of intact antibody molecule, and further cloning is needed to add effector function domains to this molecule. Complete antibody molecules can be constructed from the scFvs and the "cassette-type" cloning system is now available.[9] In its present format, scFv can be used as a diagnostic reagent and the specific binding between scFv and antigen can be detected by utilizing the epitope tagged to scFv, such as c-myc peptide, as illustrated in this study. It should be noted that the scFvs isolated from the universal unimmunized phage display antibody do not have natural somatic mutations which occur after natural infection *in vivo* and therefore lack affinity maturation process which may be important if high-affinity antibodies are required. The changes in the nucleotide sequences of the scFv clones from the closest germline V gene sequences, as seen in this study, indicated mutations that occurred prior to the construction of the library and prior to the

encounter with the antigens. However, affinity maturation, if required, can be genetically mimicked *in vitro* by several molecular biological techniques such as chain shuffling, point mutagenesis and affinity-driven selection.[17,18]

There were two previous reports on the isolation of phage antibodies to HCV proteins, one in the Fab format and the other in the scFv format.[19,20] In contrast to this study, in which the universal library was used, in those two reports the phage display libraries were constructed from HCV-infected individuals. The obtained phage antibodies, two scFv fragments and one Fab format, were reactive to the HCV synthetic nucleocapsid peptides. Interestingly, the VH of all phage antibodies specific to HCV nucleocapsid protein were derived from the VH3 segment of the germline immunoglobulin gene, similar to that demonstrated in this study. The analysis of V segment usage of a hybridoma-derived monoclonal antibody to HCV nucleocapsid protein also showed that VH3 gene segment was used.[21] However, the nucleotide and deduced amino acid sequences of the phage antibodies isolated by ours and other groups were different from one another. All three scFvs sequenced in this study had almost identical VH sequences with only one change in the CDR3 segment of one scFv, which did not affect the amino acid sequence. It is possible that if more specific scFv clones are sequenced, those with different nucleotide and deduced amino acid sequences in the VH gene might be obtained. The VL of all three scFv were of lambda type but had different sequences. This indicated that the VH component may contribute more than VL in the

specificity to HCV nucleocapsid protein.

In summary, this study demonstrated the feasibility of isolating genetically engineered scFv antibodies to hepatitis C virus nucleocapsid protein by using universal phage display antibody library. The scFvs have been isolated and confirmed to be specific to the viral antigen. The analysis of nucleotide sequences showed that these scFvs contained identical VH amino acid sequence, but utilized different VL sequences.

ACKNOWLEDGEMENT

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