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Identification of two novel anti-HCV E2 $_{412\text{-}423}$ epitope antibodies by screening a Chinese-specific phage library

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Summary. – The hepatitis C virus (HCV) E2 $_{412\text{-}423}$ linear epitope has been found to be highly conserved across multiple HCV genotypes. The antibodies against this epitope have broadly neutralizing activity. Considering the poor immunogenicity of the epitope in humans and significant diversity in the global distribution of HCV genotypes, the aim of this study was to construct an anti-HCV phage library by using a series of optimal strategies to screen novel broadly neutralizing antibodies from Chinese donors. mRNA was isolated from peripheral blood samples of 39 patients who were anti-HCV positive. A phage library was constructed by inserting a single-chain variable fragment (scFv) gene repertoire into the T7Select10-3b vector. A synthetic peptide representing the HCV E2 N-terminal 412–423 region was used as "bait" for bio-panning. The binding affinities of phage clones to the synthetic peptide were evaluated through peptide-ELISA. Two scFv clones (R3-19 and R4-85) showing the strongest binding affinities were selected. The complementarity-determining regions (CDRs) of these clones were aligned with those of other previously reported broadly neutralizing anti-HCV antibodies, and multiple conserved amino acid sites were found. The optimized procedures ensured that two novel scFv antibodies were isolated from a constructed phage library and showed specific binding to the poorly immunogenic HCV E2 $_{412\text{-}423}$ linear epitope.

Keywords: phage antibody library; hepatitis C virus; broadly neutralizing antibody; synthetic peptide

Introduction

HCV infection is a highly complex process involving various hepatocyte receptors, including scavenger receptor class B type I (SR-BI), tetraspanin CD81, tight junction proteins, claudin1 and occludin (Colpitts and Baumert, 2016). Specific antibodies can block HCV particle attachment to cellular surface receptors, resulting in the inhibition of HCV endocytosis via neutralization. Several antibody drugs for HCV, including the fully human monoclonal antibodies (mAbs) HCV AB68, HCV AB65 and HCIG Civacir, are currently undergoing clinical trials (Tawar *et al*., 2016). Positive preliminary results indicate that the development of broadly neutralizing antibodies against highly conserved HCV epitopes is an effective strategy for preventing and treating HCV infection.

HCV envelope glycoprotein E2 is the major receptor-binding protein (Colpitts and Baumert, 2016) and the primary target of neutralizing antibodies. The region of E2 from amino acids 412 to 423 (HCV E2 $_{412\text{-}423}$, QLINTNG SWHIN) is recognized as a highly conserved linear epitope that plays a direct role in virus entry by mediating CD81 binding. The HCV E2 $_{412\text{-}423}$ epitope is conserved in over 5500 E2 sequences queried from the GenBank database and is not occluded by hypervariable region 1 (Bankwitz and Pietschmann, 2016). mAbs that bind to this epitope, such as mouse mAb AP33, have been reported to possess

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Abbreviations: CDR(s) = complementarity-determining region(s); HCV = hepatitis C virus; mAb = monoclonal antibody; scFv(s) = single-chain variable fragment(s)

broadly neutralizing characteristics (Sandomenico *et al.*, 2016; Sautto *et al.*, 2013). Furthermore, these antibodies effectively prevent and treat HCV infection in humanized mice and chimpanzees (Chung *et al.* 2013; Desombere *et al.*, 2016). Unfortunately, the most potent broadly neutralizing antibodies that target this epitope are murine antibodies, whereas similar human antibodies are rare. Moreover, although these antibodies were originally thought to recognize the same conserved epitope, research has shown that their key contact residues are different. Therefore, distinct antibodies exhibit different neutralizing potentials (Edwards *et al.*, 2012). HCV is a highly variable virus that is classified into 7 distinct genotypes with 67 confirmed subtypes and distinct geographical distributions. In China, conventional subtypes 1b and 2a have gradually decreased in prevalence, whereas subtypes 3a, 3b and 6a have increased; and more subtypes are still being identified (Gong *et al.*, 2016). Different HCV genotypes and hosts have their own unique immune response patterns. Therefore, antibodies derived from anti-HCV-positive patients in China are more likely to neutralize the predominant genotypes found in that region.

The purpose of this study was to construct a human single-chain variable fragment (scFv) phage display immune library derived from patients infected with predominant HCV genotypes in China. HCV E2 $_{412-423}$ synthetic peptides were subsequently used as bait for bio-panning to isolate novel scFvs against this highly conserved linear epitope.

Materials and Methods

Human peripheral blood samples. Peripheral blood samples from 39 anti-HCV-positive patients (3 ml each) were obtained from the Department of Infectious Diseases, The First Affiliated Hospital of the Third Military Medical University, Chongqing, China. These samples included the 5 most predominant HCV genotype subtypes in China – 1b (7 cases), 2a (2 cases), 3a (5 cases), 3b (5 cases), and 6a (4 cases) – and uncertain genotypes (16 cases, HCV RNA-negative after antiviral therapy). Each heparinized blood sample was immediately mixed with 33 ml of RNA/DNA stabilization reagent and stored at -20°C for further processing.

Reagents. RNA/DNA stabilization reagent for blood/bone marrow, the mRNA Isolation kit for blood/bone marrow and the High Pure PCR product purification kit were purchased from Roche (Mannheim, Germany). SuperScript™ III Reverse Transcriptase for RT-PCR, Dynabeads™ M-280 Streptavidin magnetic beads and NeutrAvidin™ Biotin-binding protein were purchased from Invitrogen (Carlsbad, USA). Q5® High-Fidelity DNA polymerase, a PCR amplification kit, T4 DNA ligase and restriction enzymes were purchased from NEB (Ipswich,USA). The T7Select® 10-3 cloning kit and the T7 Tag® Antibody HRP conjugate were purchased from Novagen (Darmstadt, Germany). All primers for cDNA synthesis and the amplification of human antibody variable regions were designed according to McCafferty *et al.* (Suppl. Table S1) (McCafferty and Johnson, 1996). A biotinylated HCV E2 $_{412-423}$ epitope polypeptide (QLINTNGSWHINGSGK-biotin) was synthesized by Sangon (Shanghai, China). MaxiSorp® 96-well polystyrene microtiter plates were purchased from Nunc (Roskilde, Denmark). Other reagents were purchased from local suppliers and were of the highest purity.

mRNA purification and cDNA synthesis. mRNA was directly extracted from blood lysates prepared with RNA/DNA stabilization reagent using an mRNA isolation kit according to the manufacturer's instructions. Briefly, following two-step procedure was performed: (1) the total nucleic acid fraction was obtained by adsorption to a glass surface, and (2) the mRNA fraction was purified by hybridization to biotin-labeled oligo-dT and captured with streptavidin-coated magnetic particles. First-strand cDNA from human antibody variable regions was synthesized using the human heavy/light-chain constant region-specific primers HuIgG1-4CH1AS, HuIgMAS, HuG_κAS, and HuC_λAS (Suppl. Table S1).

Generation of scFv gene repertoire. Based on the protocol of McCafferty (McCafferty and Johnson, 1996), equal molar amounts of VH, V_κ and V_λ reverse primers were pooled to a final concentration of 10 μmol. All 50 μl PCR mixtures contained 0.5 μmol of each forward and pooled reverse primers (Suppl. Table S1), 1 μl of cDNA, 10 μl of 5× Q5 reaction buffer, 200 μmol dNTPs, and 1 U of Q5® High-Fidelity DNA polymerase. The reactions were performed at 98°C for 30 s followed by 30 cycles of 98°C for 10 s, 60°C for 30 s and 72°C for 30 s and a final extension step of 72°C for 2 min. The amplified VL and VH genes were analyzed by 2% agarose gel electrophoresis and purified with the High Pure PCR product purification kit. Specifically, to amplify low-copy or single-copy human antibody variable regions, semi-nested PCR was performed sequentially using the HuVH2aSS, HuVH6aSS, HuV_κ5aSS, HuV_λ4SS and HuV_λ5SS primers with reverse transcription primers for the 1st round of amplification and with the corresponding pooled reverse primers for the 2nd round of amplification (data not shown) (Suppl. Table S1). Equal molar concentrations of the VH, V_κ and V_λ amplification products were then mixed separately to form VH mix, VL_κ mix and VL_λ mix. The VL_κ\VL_λ and VH fragments were each spliced separately to generate the VL_κ-$(G_4S)_3$-VH and VL_λ-$(G_4S)_3$-VH scFv genes by overhang extension PCR, which introduced a $(G_4S)_3$ flexible linker peptide. Each 50 μl PCR mixture contained 100 ng of purified VH mixed with 100 ng of purified VL_κ mix or VL_λ mix, 0.5 μmol scFvSS and scFvAS primers (Suppl. Table S1), 10 μl of 5× Q5 reaction buffer, 10 μl of 5× High GC enhancer, 200 μmol dNTPs and 1 U of Q5® High-Fidelity DNA polymerase. The reactions were performed at 98°C for 30 s followed by 30 cycles of 98°C for 10 s and 72°C for 20 s and a final extension step at 72°C for 1 min. The PCR products were separated on a 2% agarose gel and purified using the High Pure PCR product purification kit.

Construction of an scFv phage library. The human scFv gene products were digested with *Eco*RI and *Hind*III and purified. The VL_κ-$(G_4S)_3$-VH and VL_λ-$(G_4S)_3$-VH scFv fragments were mixed at a 2:1 molar ratio, based on the ratio of κ to λ light-chain antibodies in humans. In total, 0.12 pmol of each mixed scFv gene repertoire

was linked with 0.04 pmol T7Select10-3b *EcoR*I/*Hind*III vector arms in a working volume of 5 µl using T4 DNA ligase at 16°C for 16 h. The ligation products were added directly to 25 µl of T7 packaging extracts, and the mixtures were incubated at 22°C for 2 h for *in vitro* packaging. The reaction was stopped by the addition of 270 µl of sterile LB medium. The primary human scFv immune library against HCV was obtained from the packaged phages. This primary anti-HCV scFv library underwent liquid lysate amplification according to the manufacturer's instructions. The titers of the primary and amplified libraries were determined using plaque assay. Additionally, to ascertain the insertion efficiency and diversity of the scFv library, 12 plaques from the primary library plate were randomly selected. The inserted scFv genes were amplified by PCR using the primers T7Select UP and T7Select DOWN, which are included in the kit. All cloned PCR products were sequenced.

Bio-panning for specific phages with magnetic particles. The anti-HCV scFv library was bio-panned with the HCV E2 $_{412-423}$ biotinylated polypeptide QLINTNGSWHINGSGK-biotin. First, a total of 20 µg (2 mg/ml) of synthetic peptide was immobilized on 5 mg (500 µl) of Dynabeads™ M-280 streptavidin magnetic beads by incubation at 4°C for 16 h in a rotator. After unbound peptides were washed, the product was resuspended in 500 µl of PBS and stored at 4°C. To eliminate nonspecific phage binding, 200 µl (2×10^{10} PFU) of recombinant phage was preincubated with 2 mg (100 µl) of naïve streptavidin magnetic beads in 2 ml of PTM (PBS containing 1% Tween-20 and 2% nonfat dry milk powder) at room temperature (RT) for 2 h. The supernatant was carefully collected for further bio-panning. One hundred microliters of streptavidin magnetic beads bound to synthetic peptide were blocked with 1 ml of PTM at RT for 1 h. One ml of preprocessed recombinant phage was added to the magnetic beads, and the mixture was incubated at RT for 2 h. After several washes with 1.5 ml of PBS or PBST, the phage-bound magnetic beads were resuspended in 100 µl of PBS. The bound phages were eluted with 1% SDS from one-half of the beads for a plaque assay to determine the output titer, and the remaining half was added to early-log-phase *Escherichia coli* BLT5403 cultures for amplification. Four rounds of bio-panning were performed for specific phage screening (Table 1).

Peptide-ELISA assay. MaxiSorp 96-well polystyrene microtiter plates were first coated with NeutrAvidin™ biotin-binding protein at 5 µg/ml at 4°C for 12 h. After blocking with PTM at 37°C for 2 h, biotinylated HCV E2 $_{412-423}$ synthetic peptide at 1 µg/ml was added and incubated at RT for 1 h. Then, 100 µl of clonally amplified phages were added to the wells, and the plates were incubated at RT for 2 h. Phages bound to the coated peptide antigen were detected with anti-T7 Tag®-HRP. Wells without synthetic peptide antigen were used as negative controls, and the culture medium from *E. coli* BLT5403 was used as a blank. All clones with a sample OD_{450} value/negative control OD_{450} value greater than 10 (S/N>10) were considered positive (Konthur *et al.*, 2010).

DNA sequence analysis. The scFv genes inserted into each positive phage were amplified and sequenced as described above. The framework region (FR) and CDR of each sequenced VL and VH gene were analyzed using the MEGA 5 software program (Tamura *et al.*, 2011) and numbered using the Kabat numbering scheme. Each positive scFv gene sequence was homologous with the human germline according to the IMGT/V-QUEST tool (http://www.imgt.org/IMGT_vquest/vquest). The CDR sequences were aligned with those of 5 published anti-HCV E2 $_{412-423}$ antibodies.

Results and Discussion

Generation of scFv gene repertoire

To increase the percentage of functional antibody clones in the library and to acquire the scFv gene repertoire with maximum diversity, we first collected 39 peripheral blood samples from anti-HCV-positive patients. Conventional mRNA prepared from peripheral blood mononuclear cells (PBMCs) generally requires cell and total RNA isolation steps before mRNA can be purified from extracted total RNA. A typical mammalian cell contains approximately 10–30 pg of RNA, only 1–5% of which is mRNA (de Bekker *et al.*, 2011). Therefore, this laborious, time-consuming method tends to lead to the loss of rare mRNAs. To simplify the process of extracting mRNA from PBMCs and to decrease the degradation and loss of mRNA, we developed a simple experimental procedure that does not require prior cell separation and total RNA isolation steps. Instead, magnetic particles are used to directly isolate mRNA from full-blood sample lysates, and sample processing can be completed in approximately 1 h. The average mRNA yield was 115 ng/ml of peripheral blood, and the A_{260}/A_{280} ratio was ~2.0. Finally, to enrich human antibody genes, primers specific to human antibody variable regions were used for cDNA synthesis. The VL and VH genes were amplified using synthesized human IgG and IgM variable region cDNAs as templates. Specifically, low-copy or single-copy VL and VH genes were amplified by semi-nested PCR. The amplified products were connected using a flexible amino acid linker $(Gly_4Ser)_3$ via splicing overhang extension PCR, yielding the scFv gene repertoire (data not shown).

The HCV E2 $_{412-423}$ linear epitope is reportedly less immunogenic in humans, and less than 5% of individuals with chronic infections harbor antibodies against this epitope (Edwards *et al.*, 2012). Therefore, all of the above strategies have been important for the successful isolation of antibodies against the HCV E2 $_{412-423}$ epitope.

Construction of a phage display antibody library

A 300 µl primary scFv immune library specific to HCV was generated by cloning the scFv gene repertoire into the T7Select10-3b vector using *in vitro* packaging. Subsequently, the generated primary library was amplified using the liquid

Table 1. Bio-panning strategy and the results of phage display library

Rounds of bio-panning	Bio-panning		Input (PFU)	Output (PFU)	Number of selected clones	Number of positive clones	Positive rate
	1.5 ml PBST	1.5 ml PBS					
1st round	1×10 min	3×10 min	1.209×10^{10}	4.31×10^{6}	47	0	0
2nd round	5×10 min	1×10 min	1×10^{9}	6.8×10^{5}	46	1	2.17%
3rd round	10×10 min	1×10 min	1×10^{9}	1.44×10^{6}	47	11	23.40%
4th round	15×10 min	1×10 min	1×10^{9}	4.8×10^{6}	251	89	35.46%

Table 2. Sequence analysis of V genes of R3-19 and R4-85 scFv clones

	R3-19		R4-85	
	VH gene	VL gene	VH gene	VL gene
Family	VH3	VL2	VH3	VK1
Closest V region	Homsap IGHV3-49*05 F	Homsap IGLV2-14*01 F	Homsap IGHV3-49*05 F	Homsap IGKV1-5*03 F
Identity with germline	100.00% (294/294 nt)	92.71% (267/288 nt)	100.00% (294/294 nt)	92.83% (259/279 nt)

*Sequence analysis was performed by IMGT/V-QUEST (http://www.imgt.org/IMGT_vquest/vquest).

lysate method. The titers of the primary and amplified libraries were 4.03×10^{7} PFU/ml and 1.12×10^{11} PFU/ml, respectively. Cloning effects and the diversity of the primary library were analyzed via the PCR amplification and bidirectional DNA sequencing of 12 randomly selected phage clones. The expected scFv insertions were successfully amplified from all the clones. Sequence analysis revealed that the sequences of the 12 random scFv clones were clearly different from one another and the closest V region of the human antibody germline genes, with the exception of phage clone No. 3, which had a nonsense mutation in the VL region (Suppl. Table S2). These results demonstrate excellent clone effectivity and library diversity. In this study, the T7 bacteriophage was used as the scFv display vector. As a traditional M13 display system, the T7 phage is extremely robust, more stable under harsh conditions and can remain infective during bio-panning selection procedures. In addition, T7 display library diversity is higher than that of M13 due to *in vitro* packaging (Krumpe *et al.*, 2007). The T7Select10-3b vector can display an average of 5–15 copies of a protein molecule and is suitable for the stable expression of various genes up to approximately 3600 base pairs.

Immune libraries are generally modest in size (10^{7}–10^{8}) but sufficient for the isolation of specific and high-affinity antibodies (Moon *et al.*, 2011). Our scFv gene repertoire was isolated from anti-HCV-positive donors; therefore, the antibodies obtained were assembled via rearrangement and were affinity-matured *in vivo*. Thus, the constructed library had a strong bias toward the HCV antigen. The constructed scFv library, with a primary library size of 4.03×10^{7} and excellent diversity, qualifies for further bio-panning of anti-HCV E2 $_{412-423}$ epitope antibodies.

Bio-panning phage display library

To isolate specific anti-HCV E2 $_{412-423}$ epitope antibodies from the constructed library, we used a synthetic E2 $_{412-423}$ peptide as the "bait" antigen. However, direct binding of synthetic peptides to plastic surfaces often results in poor coating efficiency and can alter epitope availability. Therefore, we synthesized a biotinylated E2 $_{412-423}$ peptide for use in conjunction with magnetic streptavidin-coated microbeads to capture the specific phage-display scFvs. Importantly, interactions between phage particles and peptides occurred in solution, ensuring maintenance of the native peptide structure and exposing the maximum number of epitopes for binding. Similarly, the indirect coating of biotinylated E2 $_{412-423}$ peptides via streptavidin improves the sensitivity of peptide-ELISA analyses. To eliminate phages that bind naïve magnetic streptavidin-coated microbeads in a nonspecific manner, we performed a pre-absorption step. Four rounds of bio-panning with increasingly stringent washing conditions were performed to enrich the specific scFv phages. The output-to-input ratio and number of positive clones increased gradually with each round of bio-panning. The affinity reactivities of the 391 phage clones that bound the synthetic peptide antigen were evaluated by peptide-ELISA. The A_{450} values of the blank samples were <0.1; these values were substantially lower than the A_{450} values of the test samples. The positive clone rate was 35.46% after 4 rounds of bio-panning, whereas no positive phage clones were obtained after the first round. In total, we obtained 101 positive phage clones (Table 1, Fig. 1).

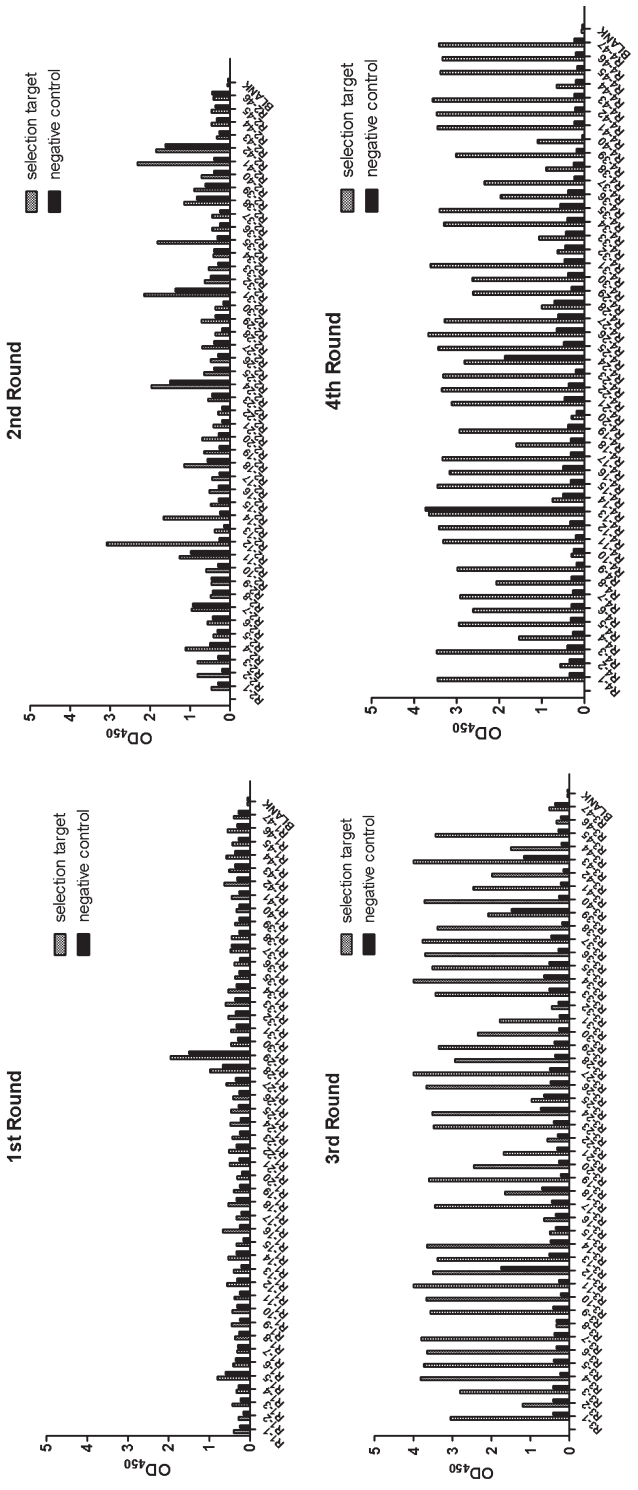

Fig. 1

Binding activities of the output phage clones with the HCV E2 $_{412\text{-}423}$ epitope synthetic peptide antigen were evaluated by peptide-ELISA

After every round of bio-panning, the binding activities of the selected phage clones were measured via peptide-ELISA. Biotinylated synthetic peptides captured via NeutrAvidin™ were used as selection antigens, and the negative controls contained no peptides. The natural culture supernatant of *E. coli* BLT5403 was used as a blank.

	HCDR1	HCDR2	HCDR3
AP33(mouse)	SGYWN	YIS---YSGSTYYNLSLRS	ITTTTYAM---------DY
3/11(rat)	DSYLA	SITN--SGGRFYYRDSVKG	M----------------DY
HCV1(human)	NYGMH	VIWF--DENNKYYADSVRG	DISLVRDAFIYF-----DF
Hc33.1(human)	NFAVS	AISS--SDGSTYYSDSVKG	AVVSSDITYTYWSKYFDY
Hu5b3.v3(humanized)	NYWIN	DIYP--SDSFTNYNQNFKD	SSIYYGKDYVL------DY
R3-19(human)	DYAMS	FIRSKAYGGTTEYAASVKG	GMATIKGARF-------DY
R4-85(human)	DYAMS	FIRSKAYGGTTEYAASVKG	IAAADDEAYYYGM---DV
		★ # # ★ ####	★#

	LCDR1	LCDR2	LCDR3
AP33(mouse)	RASESVDG-YGNSFLH	LASNLNS	QQNNVDP--WT
3/11(rat)	RSSQSLLESDGNTYLN	SVSNLES	MQTTHA----PT
HCV1(human)	RASQ------SVSSYLA	DASNRAT	QQRSNW----IT
Hc33.1(human)	SGGSFN----IGNNYVS	DNDNRPS	GTWDSSLNVVV
Hu5b3.v3(humanized)	RASESVDN-YGISFMN	SASNHAS	HQSKEAP--YA
R3-19(human)	TGTSSDIG--GYNFVS	DVNSRPS	SSYRSDST-VV
R4-85(human)	RASQ------NINNWLA	KASALES	QQSYSSPM-YT
	# #	# # #	#

Fig. 2

Amino acid sequence alignment of the CDRs of antibodies recognizing the HCV E2 $_{412\text{-}423}$ epitope

★Indicates amino acid sites that were the same in the CDR sequences of all seven antibodies. #Indicates amino acid sites that were the same for ≥5 sequences of the seven antibodies.

Bioinformatics analysis of positive phages

The scFv sequences were highly repetitive among the 101 positive phage clones analyzed. The majority showed variations in the J segments of VL. Among these, two scFv clones with maximum S/N values (R3-19 and R4-85, with S/N values of 20.4 and 39.2, respectively) were selected for further analysis. R3-19 and R4-85 share the same germline V gene, Homsap IGHV3-49*05 F (GenBank Acc. No. AM940221). The light chain of R3-19 was identified as belonging to the lambda family, whereas that of R4-85 belonged to the kappa family (Table 2).

The CDRs of these two scFvs were compared with those of 5 previously reported antibodies with broad neutralizing activities that specifically bind to the HCV E2 $_{412\text{-}423}$ epitope. As shown in Fig. 2, sequence alignment indicated significant differences in the CDRs of the antibodies obtained from different sources. The region with the highest number of differences was in HCDR3, for which the number of residues ranged from 3 to 18. We also found 16 individual amino acid sites that were conserved to different degrees. Specifically, I51, Y59 and D101 were completely conserved in all aligned antibodies and were identical to another human mAb, 95-2 (Broering *et al.*, 2009). The results suggested a potential key role for these 16 amino acid sites in HCV E2 $_{412\text{-}423}$ epitope binding.

Conclusion

We have successfully isolated two anti-HCV E2 $_{412-423}$ low-immunogenicity epitope antibodies, R3-19 and R4-85, by using optimal library construction and bio-panning strategies. The two scFv antibodies exhibited high and specific binding to a synthetic HCV E2 $_{412-423}$ peptide, which represents a highly conserved linear epitope across multiple HCV gene subtypes and plays a direct role in virus entry by mediating CD81 binding. Sequence analysis showed that the R3-19 and R4-85 scFv antibodies are significantly different from several previously reported broadly neutralizing anti-HCV E2 $_{412-423}$ epitope antibodies, although all antibodies shared certain conserved amino acid sites. These results indicate that scFvs R3-19 and R4-85 are novel anti-HCV E2 $_{412-423}$ epitope antibodies. These antibodies may be broadly neutralizing across multiple HCV genotypes, particularly the major epidemic subtypes of HCV in China, and may prove useful for blocking HCV infection.

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Supplementary information is available in the online version of the paper.

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Supplementary information

Identification of two novel anti-HCV E2 $_{412\text{-}423}$ epitope antibodies by screening a Chinese-specific phage library

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Table S1. Primers for construction of the phage display human scFv library

Primer	Sequence
Specific primers for cDNA synthesis of human antibody variable regions	
Human heavy chain constant region primers	
HuIgG1-4CH1AS	5'- GTC CAC CTT GGT GTT GCT GGG CTT -3'
HuIgMAS	5'-TGG AAG AGG CAC GTT CTT TTC TTT-3'
Human kappa constant region primer	
HuGkAS	5'-AGA CTC TCC CCT GTT GAA GCT CTT-3'
Human lambda constant region primer	
HuCkAS	5'-TGA AGA TTC TGT AGG GGC CAC TGT CTT-3'
Primers for PCR amplification of human antibody variable regions	
HuVHlaSS	5'-*TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG* **CAG GTG CAG CTG GTG CAG TCT GG**-3'
HuVH2aSS	5'-*TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG* **CAG GTC AAC TTA AGG GAG TCT GG**-3'
HuVH3aSS	5'-*TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG* **GAG GTG CAG CTG GTG GAG TCT GG**-3'
HuVH4aSS	5'-*TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG* **CAG GTG CAG CTG CAG GAG TCG GG**-3'
HuVH5aSS	5'-*TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG* **GAG GTG CAG CTG TTG CAG TCT GC**-3'
HuVH6aSS	5'-*TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG* **CAG GTA CAG CTG CAG CAG TCA GG**-3'
HuJH1-2AS	5'-TTA CTC GAG TGC GGC CGC AAG CTT TTA **TGA GGA GAC GGT GAC CAG GGT GCC**-3'
HuJH3AS	5'-TTA CTC GAG TGC GGC CGC AAG CTT TTA **TGA AGA GAC GGT GAC CAT TGT CCC**-3'
HuJH4-5AS	5'-TTA CTC GAG TGC GGC CGC AAG CTT TTA **TGA GGA GAC GGT GAC CAG GGT TCC**-3'
HuJH6AS	5'-TTA CTC GAG TGC GGC CGC AAG CTT TTA **TGA GGA GAC GGT GAC CGT GGT CCC**-3'
HuVklaSS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **GAC ATC CAG ATG ACC CAG TCT CC**-3'
HuVk2aSS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **GAT GTT GTG ATG ACT CAG TCT CC**-3'
HuVk3aSS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **GAA ATT GTG TTG ACG CAG TCT CC**-3'
HuVk4aSS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **GAC ATC GTG ATG ACC CAG TCT CC**-3'
HuVk5aSS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **GAA ACG ACA CTC ACG CAG TCT CC**-3'
HuVk6aSS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **GAA ATT GTG CTG ACT CAG TCT CC**-3'
HuVλlSS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **CAG TCT GTG TTG ACG CAG CCG CC**-3'
HuVλ2SS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **CAG TCT GCC CTG ACT CAG CCT GC**-3'
HuVλ3aSS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **TCC TAT GTG CTG ACT CAG CCA CC**-3'
HuVλ3bSS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **TCT TCT GAG CTG ACT CAG GAC CC**-3'
HuVλ4SS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **CAC GTT ATA CTG ACT CAA CCG CC**-3'

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Table S1. Continued

Primer	Sequence
HuVλ5SS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **CAG GCT GTG CTC ACT CAG CCG TC**-3'
HuVλ6SS	5'-ATG CTC AGT GGG GAT CCG AAT TCT **AAT TTT ATG CTG ACT CAG CCC CA**-3'
HuJklAS	5'-*AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC* **ACG TTT GAT TTC CAC CTT GGT CCC**-3'
HuJk2AS	5'-*AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC* **ACG TTT GAT CTC CAG CTT GGT CCC**-3'
HuJk3AS	5'-*AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC* **ACG TTT GAT ATC CAC TTT GGT CCC**-3'
HuJk4AS	5'-*AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC* **ACG TTT GAT CTC CAC CTT GGT CCC**-3'
HuJk5AS	5'-*AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC* **ACG TTT AAT CTC CAG TCG TGT CCC**-3'
Hu JλlAS	5'-*AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC* **ACC TAG GAC GGT GAC CTT GGT CCC**-3'
Hu Jλ2-3AS	5'-*AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC* **ACC TAG GAC GGT CAG CTT GGT CCC**-3'
Hu Jλ4-5AS	5'-*AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC* **ACC TAA AAC GGT GAG CTG GGT CCC**-3'
scFvSS	5'-ATG CTC AGT GGG GAT CCG AAT TCT
scFvAS	5'-TTA CTC GAG TGC GGC CGC AAG CTT TTA TGA

*Bold font indicates sequence complementary with the V-gene segments. Recognition sites for restriction enzymes (*Eco*RI / *Hin*dIII), and linker sequence are italicized.

Table S2. Sequence analysis of the scFv clones randomly selected from the primary library

Clone	VH				VL			
	Family	Germline	Identity	Junction	Family	Germline	Identity	Junction
1	VH1	Homsap IGHV1-69*01 F, or Homsap IGHV1-69D*01 F	100.00% (288/288 nt)	CAREGGSGSPS-DYW	VK3	Homsap IGKV3-20*01 F	84.40% (238/282 nt)	CQQYGRSPRTF
2	VH2	Homsap IGHV2-70*01 F, or Homsap IGHV2-70*11 F	98.28% (286/291 nt)	CARELISGSYGDA-FDIW	VK2	Homsap IGKV2-30*02 [F]	96.60% (284/294 nt)	CMQGTHWPWTF
4	VH3	Homsap IGHV3-49*03 F	97.28% (286/294 nt)	CARTKPAPGGW-GYFDYW	VK4	Homsap IGKV4-1*01 F	100.00% (297/297 nt)	CQQYYSTPPTF
5	VH5	Homsap IGHV5-51*01 F	97.92% (282/288 nt)	CARRRADRYGSGS-FWYFDLW	VK1	Homsap IGKV1-39*01 F, or Homsap IGKV1D-39*01 F	99.28% (277/279 nt)	CQQSYSTPFTF
6	VH1	Homsap IGHV1-69*09 F	95.83% (276/288 nt)	CARGGPVTTVVT-PGAFDVW	VK3	Homsap IGKV3-15*01 F	96.77% (270/279 nt)	CQQFNNWPPEFTF
7	VH2	Homsap IGHV2-70*01 F, or Homsap IGHV2-70*11 F	96.22% (280/291 nt)	CARFDYTSIYYG-GFDYW	VL3	Homsap IGLV3-19*01 F	88.89% (248/279 nt)	CHCRDSTGDHLVF
8	VH6	Homsap IGHV6-1*01 F	97.31% (289/297 nt)	CARLPFTAT-PGTSSW	VK3	Homsap IGKV3-20*01 F	94.27% (263/279 nt)	CQQYGRSPITF
9	VH5	Homsap IGHV5-51*01 F	91.67% (264/288 nt)	CARPYYDILTGYYS-LYFDYW	VK4	Homsap IGKV4-1*01 F	93.94% (279/297 nt)	CQQYFTTPITF
10	VH1	Homsap IGHV1-69*01 F, or Homsap IGHV1-69D*01 F	93.75% (270/288 nt)	CARIGTLEVGAMD-FW	VL3	Homsap IGLV3-19*01 F	76.70% (214/279 nt)	CDSRDGSGRFWVF
11	VH5	Homsap IGHV5-51*01 F	97.22% (280/288 nt)	CVRRGENIYANDN-WFDPW	VK1	Homsap IGKV1-33*01 F, or Homsap IGKV1D-33*01 F	100.00% (279/279 nt)	CQQYDNLPLTF
12	VH1	Homsap IGHV1-18*04 F	99.31% (286/288 nt)	CAREYGDYFDYW	VL3	Homsap IGLV3-21*01 F	99.64% (278/279 nt)	CQVWDSSSDHVVF

*Sequence analysis was performed using IMGT/V-QUEST (http://www.imgt.org/IMGT_vquest/vquest).