Preparation of human single chain Fv antibody against hepatitis C virus E2 protein and its identification in immunohistochemistry

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Abstract
AIM: To screen human single chain Fv antibody (scFv) against hepatitis C virus E2 antigen and identify its application in immunohistochemistry.

METHODS: The phage antibody library was panned by HCV E2 antigen, which was coated in microtiter plate. After five rounds of biopanning, 56 phage clones were identified specific to HCV E2 antigen. The selected scFv clones were digested by SfiI/NotI and DNA was sequenced. Then it was subcloned into the vector pCANTAB5E for expression as E-tagged soluble scFv. The liver tissue sections from normal person and patients with chronic hepatitis B and chronic hepatitis C were immunostained with HCV E2 scFv antibody.

RESULTS: The data of scFv-E2 DNA digestion and DNA sequencing showed that the scFv gene is composed of 750 bp. ELISA and immunohistochemistry demonstrated that the human single chain Fv antibody against hepatitis C E2 antigen has a specific binding character with hepatitis virus E2 antigen and paraffin-embedded tissue, but did not react with liver tissues from healthy persons or patients with chronic hepatitis B.

CONCLUSION: We have successfully screened and identified HCV E2 scFv and the scFv could be used in the immunostaining of liver tissue sections from patients with chronic hepatitis C.

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INTRODUCTION
Hepatitis C virus (HCV) has been identified as the major etiological agent of post-transfusion non-A non-B hepatitis, responsible for most cases of non-A non-B hepatitis. Hepatitis C is a disease of clinical importance because of its high infection rate in blood donors and its persistence as chronic infections which may lead to cirrhosis and hepatocellular carcinoma in the long term. The variability of the HCV genome has difficulties in serological detection and vaccine design. Recent advance in phage technology offers a means of cloning human anti-HCV antibodies of a defined specificity that may have potential therapeutic use.

MATERIALS AND METHODS
Materials
Humanized scFv antibody phage library in which the variable region coding gene of VL and VH were amplified by polymerase chain reaction (PCR) with degenerate primers and connected with a glycin linker ([Gly4Ser]3) was widely used in the screen and identification of humanized scFv to various antigens. The recombinant HCV E2 protein was purchased from Virostat Co, USA. Phage M13K07 was purchased from Pharmacia Co., Sweden. Other reagents used in this experiment are all domestic products of analytical grade.

Biopanning
The phage library was amplified in 37 °C. The host E. coli TG1 was infected with phage M13K07 and incubated at 37 °C for 12 hours, the phage in the supernatant was harvested and concentrated by PEG. Culture plate (Nunc) was coated with recombinant HCV E2 protein at the concentration of 80 mg/L. The coating buffer was 0.05 mol/L NaHCO3, pH 9.6. The plate was blocked with BSA at the concentration of 20 g/L for 2 hours and the concentrated phages were added to the well of the plate, incubated at the room temperature for 90 min. The plates were washed 20 times with PBST and PBS buffer respectively. The bound phage was eluted by the 0.1M of triethylamine, and neutralized with 1M Tris buffer (pH 7.4). Recovered phages were used to infect the host E. coli TG1 at the log phase growth and HCV E2 protein-binding phages were amplified. The procedure of absorption-elution-amplification was repeated 5 times.

Identification of phage clones
After 5 rounds of biopanning, 56 phage clones were selected randomly. The clones grew in 400 μl 2×TY-AMP-Glu at 37 °C overnight. The culture was transformed to another Eppendorf tube when its A600 nm reached 0.5. The culture was continued at 30 °C overnight after adding helper phage. ELISA for determining the supernatants was repeated at least two times. The cross-reaction of the supernatants to the BSA antigen was conducted. According to the ELISA results to the HCV E2 and BSA, one clone with high reaction to HCV E2 and low reaction to BSA was selected.
Sequencing analysis
The plasmid DNA was prepared using Wizard plus miniprep DNA Purification System (Promega Co., USA) and sequenced using ABI automated DNA sequencing machine.

Expression of human HCV E2-scFv in E. coli
The selected HCV E2 scFv clone was subcloned as SfiI/NotI fragments into the vector pCANTAB5E for expression as E-tagged soluble scFv. DNA digestion and electrophoresis confirmed the recombinant vector pCANTAB5E-E2-scFv. Competent E. coli XL1-Blue was transformed with pCANTAB5E-E2-scFv and transformed XL1-Blue was induced by IPTG for 20 h. The E. coli was harvested by centrifugation at 10 000 rpm. The culture supernatant was rendered for ELISA test according to the standard procedure. In ELISA detection, Nunc plate was coated with 1µg/well of recombinant HCV E2 antigen and blocked with 2 % bovine serum albumin (BSA) at 37 ºC for 2 h. The supernatants from induced and non-induced transformed E. coli were added and incubated at 37 ºC for 2 h. The plate was washed with PBS buffer, and 100 µl of HRP/anti-E Tag 1:4000 ratio diluted in PBS buffer containing 1 % BSA was added, and incubated at 37 ºC for 1 h. The substrate solution was added and A450nm value was measured.

Immunohistochemical identification of scFv in liver tissue
Paraffin-embedded liver tissue slices were from patients with positive anti-HCV antibodies and HCV-RNA. After deactivating endogenous peroxidase, these slices were submersed in the methanol solution with 0.5 % H2O2 in the room temperature. Fifty min later, they were washed with PBS buffer for 3 times,5 % BSA was added and slices were stored overnight at 4 ºC. Self-made scFv primary anti-HCV E2 single-chain antibodies were diluted at 1:100 ratio and added on to the slice. They were kept in the 37 ºC incubator for 1 h, then 4 ºC refrigerator overnight. HRP-sheep anti-M13 antibodies (diluted to 1:200) solutions were dropped on to the tissue sections, incubated at 37 ºC for 40 min. After ten times with PBS buffer, DAB solutions (9 mg DAB, 13.5 ml Tris.cl, 1.5 ml CoCl2, 15 µl 30 % H2O2) were dropped on to the tissue sections at room temperature. After ten minutes, the slices were washed with PBS buffer for 3 times again, and 1 % heamatin solution was used to stain the cell nucleus. Gradient ethanol was utilized to dehydrate and dimethylbenzene to clear the sections, then neutral resin to envelope them. The resultant slices were observed under microscope. The set was as follows: 1) PBS buffer instead of anti- HCV E2 scFv; 2) HBsAg, HBCag double-positive liver tissue sections; and 3) Normal liver tissue sections.

RESULTS
 Screening and identification phage clones
Using HCV E2 protein as immobilized antigen, the humanized scFv phage library was biopanned. After 5 rounds of biopanning, 56 phage clones were selected randomly. ELISA and cross-reaction of these clones to BSA confirmed their specificity to HCV E2. Among the 56 phage clones, 16 showed good reactivity to the recombinant HCV E2 protein with high A value in the ELISA. In the cross-reaction screen, 6 among the 16 showed low cross-reaction with BSA. The combined results indicated that 1 of the 6 showed the highest reaction to HCV E2 protein and lowest reaction to BSA. One clone has been utilized for further DNA digestion and sequence analysis. The DNA sequence digestion was made by SfiI/NotI in Figure 1. Its nucleic acid sequence and deduced amino acid sequence about HCV-E2-scFv fragment are shown in Figure 2.

![Figure 2](https://example.com/figure2.png)

**Figure 2** Nucleic acid and deduced amino acid sequences of scFv for HCV E2 protein GenBank accession number for this sequence is AF317001

![Absorbances of HCV-E2-scFv binding to E2 antigen by ELISA.](https://example.com/figure3.png)

**Figure 3** Absorbances of HCV-E2-scFv binding to E2 antigen by ELISA. (a), supernant from induced XL1-blue transformed with pCANTAB5E-E2-scFv; (b), posive control; (c), supernant from non-induced XL1-blue transformed with pCANTAB5E-E2-scFv, d. negative control

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Expression of human HCV-E2-scFv in E. coli.
The expressed HCV-E2-scFv antibody from E. coli XL1-blue transformed by pCANTAB5E and induced by IPTG was confirmed by ELISA as shown in Figure 3. The recombinant HCV E2 antigen was taken as the positive control. The protein from induced and non-induced E. coli XL1-blue transformed by expression vector was positive. But the protein derived from the E. coli non-transformed by pCANTAB5E- HCV E2- scFv was negative. These results indicated that the soluble form of human HCV E2- scFv antibody has been successfully expressed in this procedure.

Immunostaining of HCV E2 antigen of liver tissue sections
The different sections from liver tissues of healthy persons and patients with chronic hepatitis B or C were immunostained. The positive immunostaining was seen only in the liver tissue section of patients with chronic hepatitis C, but not in the liver tissue of normal person and patients with chronic hepatitis B as seen in (Figure 4A). The HCV E2 antigen was mainly located in the cytoplasm of the hepatocytes infected by HCV virus (Figure 4B).

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