

• VIRAL HEPATITIS •

# Interaction between hepatitis C virus core protein and translin protein- a possible molecular mechanism for hepatocellular carcinoma and lymphoma caused by hepatitis C virus

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## Abstract

**AIM:** To investigate the interaction between hepatitis C virus core protein and translin protein and its role in the pathogenesis of hepatocellular carcinoma and lymphoma.

**METHODS:** With the components of the yeast two hybrid system 3, "bait" plasmids of HCV core the gene was constructed. After proving that hepatitis C virus core protein could be firmly expressed in AH109 yeast strains, yeast two-hybrid screening was performed by mating AH109 with Y187 that transformed with liver cDNA library plasmids - pACT2 and then plated on quadruple dropout (QDO) medium and then assayed for  $\alpha$ -gal activity. Sequencing analysis of the genes of library plasmids in yeast colonies that could grow on QDO with  $\alpha$ -gal activity was performed. The interaction between HCV core protein and the protein we obtained from positive colony was further confirmed by repeating yeast two - hybrid analysis and coimmunoprecipitation *in vitro*.

**RESULTS:** A gene from a positive colony was the gene of translin, a recombination hotspot binding protein. The interaction between HCV core protein and translin protein could be proved not only in yeast, but also *in vitro*.

**CONCLUSION:** The core protein of HCV can interact with translin protein. This can partly explain the molecular mechanism for hepatocellular carcinoma and lymphoma caused by HCV.

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## INTRODUCTION

The core protein of hepatitis C virus (HCV) is the structural protein of the virus<sup>[1-4]</sup>. However, some evidences suggested

that this protein has a pleiotropic nature. In addition to having a packaging function, the core protein has been shown to act *in trans* on the viral and cellular promoters and it is also capable of transformation of rat embryonic fibroblasts through cooperation with the *ras* oncogene. Previous studies showed that the core protein could interact with several proteins such as lymphotoxin- $\beta$  Receptor, heterogeneous nuclear ribonucleoprotein K, RNA helicase<sup>[5-8]</sup>. In order to understand the pathogenesis of HCV infection we examined the possibility that the HCV core protein interacts with cellular proteins.

## MATERIALS AND METHODS

### Material

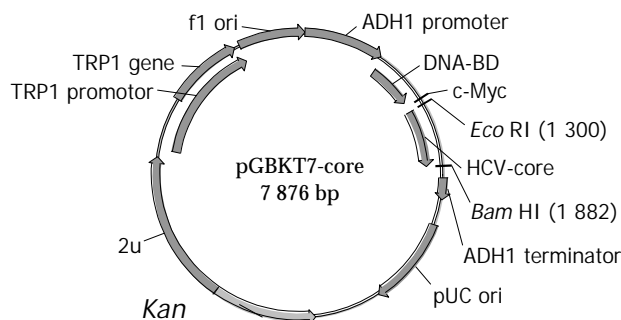
**Bacterial, yeast strains and Plasmids** All yeast strains and plasmids for yeast two-hybrid experiments were obtained from Clontech (Palo Alto, Calif., USA) as components of the MATCHMAKER Two Hybrid System 3. Yeast strain AH109 (MAT $\alpha$ , *trp1-901*, *leu2-3,112*, *ura3-52*, *his3-200*, *gal4*  $\Delta$ , *gal80*  $\Delta$ , *LYS2::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-HIS3*, *GAL2<sub>UAS</sub>-GAL2<sub>TATA</sub>-ADE2 URA3::MEL1<sub>TATA</sub>-lacZ MEL1*) containing pGBKT7-53, coding for DNA-BD/mouse p53 fusing protein and AH109 used for cloning of bait plasmids, yeast strain Y187 (MAT $\alpha$  *ura3-52*, *his3-200*, *Ade2-101*, *trp1-901*, *leu2-3, 112*, *gal4*  $\Delta$ , *gal80*  $\Delta$ , *met-*, *URA3::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-lacZ MEL1*) containing pTD1-1, in which pACT2 coding for AD/SV40 large T antigen fusing protein and Y187 used for cloning of library plasmids. Pretransformed cDNA liver cell library Y187. Bacterial strain DH5 $\alpha$  used for cloning of every shuttle plasmid. Yeast-*Escherichia coli* shuttle plasmids pGBKT7 DNA-BD cloning plasmid, pGADT7 AD cloning plasmid, pGBKT7-53 control plasmid, pGADT7, pGBKT7-Lam control plasmid, pCL1 plasmid from Clontech L.T.D Company(K1612-1). pGEM T vector from Promega Company, USA.

**Chemical agents and cultural media** Taq DNA polymerase purchased from MBI Company, T4 DNA ligase, EcoRI and BamHI restriction endonuclease from Takara. c-Myc monoclonal antibody secreted by 1-9E10.2 hybridoma (ATCC), goat anti-mouse IgG conjugated with horseradish peroxidase from Zhongshan Company, China. Lithium Acetate, semi-sulfate adenine, Acrylamide and N, N' -Bis-acrylamide from Sigma, TEMED from Boehringer Mannheim. Tryptone and yeast extracts from OXOID. X- $\alpha$ -Gal and Cultural media: YPDA, SD/-Trp SD/-Leu, SD/-Trp/-Leu, SD/-Trp/-Leu/-His, SD/-Trp/-Leu/-His/-Ade from Clontech L.T.D Company. protein-G agarose from Roche. RT-PCR kit and TNT<sup>®</sup>Coupled Reticulocyte Lysate Systems from Promega. [<sup>35</sup>S]-methionine (.1 000 Ci/mmol; 10 mCi/ml) from Isotope company of china. Amplify Fluorographic Reagent (#NAMP100) from Amersham Life Sciences. Others from Sigma company.

### Methods

**Construction of "bait" plasmid and expression of HCV core protein** Plasmid pGBKT7-core (Figure 1) containing full-

length HCV core gene was constructed by insertion of HCV core gene in-frame into EcoRI/BamHI site, which could direct expression of DNA binding domain, *c-myc* and core fusion protein. After the plasmid was transformed into yeast strain AH109 by using Lithium Acetate method<sup>[9]</sup>. Western blotting was performed to confirm the expression of the fusion protein by using *c-myc* monoclonal antibody. Transformed AH109 was cultured on quadropole dropout media to exclude the auto-activity.

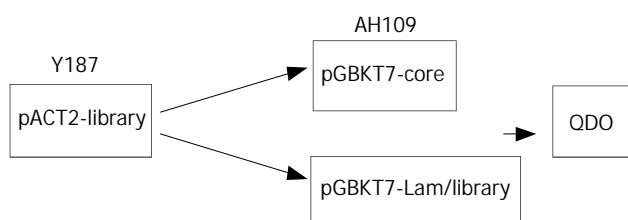


**Figure 1** "bait" plasmid pGBKT7-core.

**Two-hybrid library screening using yeast mating** One large (2-3-mm), fresh (<2 months old) colony of AH109[bait] was inoculated into 50 ml of SD/-Trp and incubated at 30 °C overnight (16-24 hr) with shaking at 250-270 rpm. Then the cells were spun down by centrifuging the entire 50-ml culture at 1 000×g for 5 min and supernatant Decanted the cell pellet was resuspended in the residual liquid by vortexing. The entire AH109[bait] culture and the 1-ml library were combined and cultured in a 2-L sterile flask and Add 45 ml of 2X YPDA/Kan was added and swirl gently. After 20 h mating, the cells were spun down and resuspended then spreaded on -50 large (150-mm) plates, containing 200 ml of SD/-Ade/-His/-Leu/-Trp (QDO). After 6-18 days grew, the yeast colonies were transferred onto the plates containing X-a-Gal to check for expression of the MEL1 reporter gene(blue colonies).

**Plasmid isolation from yeast and transforming *E.coli* with yeast plasmid** Yeast plasmid was isolated with Lyticase method (provided by Clontech), and transformed into *E. coli* by using electroporation<sup>[10]</sup>, transformants were plated on ampicilin LB selection media, then, isolating plasmids from *E.coli* and sequencing analysis.

**To confirm the true interaction in yeast** To confirm the true protein-protein interaction and exclude false positives, the plasmids of positive colonies were transformed into yeast strain Y187, next mating experiments were carried out by mating with yeast strain AH109 containing pGBKT7-core or pGBKT7-Lam. After mating ,the diploids yeast were plated on QDO covered with X-α-gal (Figure 2).



**Figure 2** To confirm the interaction.

**Bioinformatic analysis** After sequencing the positive colonies, the sequences blasted with GenBank to analogize the function of the genes.

**RT-PCR** In order to clone the full-length gene, RT-PCRs were conducted by using PCR primers the designing based on the information of GenBank. The genes amplified by RT-PCR were ligated into yeast plasmid pGADT7.

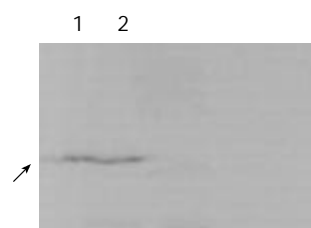
**In vitro translation** Mixture of TNT®reticulocyte 25 µl, TNT®reactio buffer 2 µl, T7 TNT®RNA polymerase 1µl, amino acids mixture (minus methionine, 1 mM) 1µl, [<sup>35</sup>S]methionine 2 µl, Rnasin®nuclease inhibitor (40u/µl) 1µl, DNA template (pGBKT7-core or pGADT7-library gene) (0.5 µg/µl) 2 µl, ddH<sub>2</sub>O 50 µl, 30 °C incubated 90 minutes.

**Coimmunoprecipitation** The following reactants were combined in a 1.5-ml microcentrifuge tube on ice: Five µl *in vitro* translated bait protein, 5 µl *in vitro* translated library protein. The control only added 10 µl pGBKT7-core plasmid. The mixtures were incubated at 30 °C for 1 hr. Then, the following reagents added into the reaction tubes: 470 ml coimmunoprecipitation buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 5 µg/ml aprotinin, 0.5 mM PMSF, 0.1 % Tween 20), 10 µl Protein-G Agarose Beads, 10 µl c-Myc Monoclonal Antibody. Incubated at 4 for 2 hr with continuous rocking. The tubes were centrifuged at 14 000 rpm for 1-2 min. The supernatants were removed. 0.5 ml TBST added to the tubes. Rinse steps were repeated three times. 15 ml SDS-loading buffer were added. The samples was heated at 80 °C for 5 min. The tubes were placed on ice. Briefly centrifuged, and 10 µl loaded onto an SDS-PAGE minigel to begin the electrophoretic separation. After electrophoresis, the gel was transferred to a tray containing Gel Fixation Solution, and placed on a rotary shaker for 10 min at room temperature. Rinsed the gel with H<sub>2</sub>O, then Amplify Fluorographic Reagent was added shaken for 20 min at room, then dried at 80 °C under constant vacuum. The gel was exposed to a X-ray film overnight at room temperature. The film was developed by using standard techniques.

## RESULTS

### Expression of the "bait" fusion protein

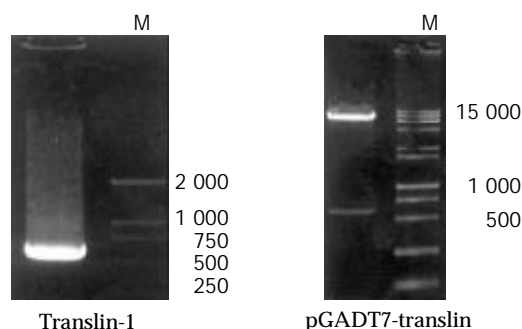
Yeast strain AH109 transformed with pGBKT7-core could stably express the fusion protein at high level (Figure 3) and could only grow on SD/-Trp medium and could not grow on QDO medium. Thus, the transformed yeast could be used for yeast hybrid analysis.



**Figure 3** Western blotting showed the expression of HCV core protein in yeast (arrow indicated). lane 1 is HCV core protein and lane 2 is negative control.

**RT-PCR experiments** The yeast two hybrid analysis showed 30 blue colonies grew on QDO plates containing X-α-Gal. After confirming the true interaction in yeast, we isolated the plasmids from the blue colonies containing only pGBKT7-core and one library plasmid other than other plasmids. Sequencing the gene and blasted with the data from GenBank, a gene is translin. To further prove the interaction between HCV core protein and translin protein (Translin), a pair of primer were designed based on the gene of translin (Forward: 5' -GAA TTC ATG TCT GTG AGC GAG ATC TTC GTG G -3', down: 5' -GGA TCC CTA TTT TTC AAC ACA AGC

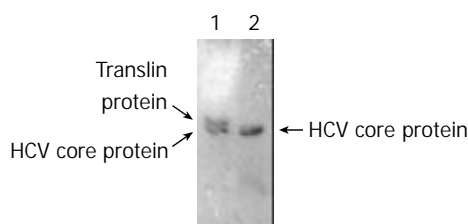
TGC TGC C-3'), up and down primers containing EcoRI and BamHI restriction endonuclease site, respectively. Total RNAs were prepared from HepG2, A 687bp fragment was amplified by using RT-PCR (Figure 4A). After cut by EcoRI/BamHI, the fragment was in-frame ligated into pGADT7 EcoRI/BamHI site (Figure 4B).



**Figure 4** a 687bp fragment-translin, amplified by RT-PCR. (A) pGADT7-translin cut by EcoRI/BamHI (B).

### *In vitro* coimmunoprecipitation

HCV core protein containing 192 aa,  $M_r=20\ 968$ , is smaller than Translin containing 228aa,  $M_r=26\ 182$ . Lane 1 showed two protein could interact with each other, lane 2 was only HCV core protein.(Figure 5).



**Figure 5** lane 1 HCV core protein and translin protein, lane 2 HCV core protein.

## DISCUSSION

Hepatitis C virus infects an estimated 170 million persons worldwide and thus represents a viral pandemic. Progression to chronic disease occurs in the majority of HCV-infected persons<sup>[11-17]</sup>, and some patients can develop to hepatocellular carcinoma<sup>[18]</sup> and lymphoma. Infection with the virus has become the main indication for liver transplantation. Although research advances have been impeded by the inability to grow HCV easily in culture, there have been some insights into pathogenesis of the infection and improvements in treatment options. The core protein of HCV is a multifunctional protein involved in several processes; it is phosphorylated and has both cytoplasmic and nuclear localization and thus it may play multiple roles in the viral life cycle<sup>[3,4]</sup>. Several studies also suggested that it has regulatory roles for viral and cellular genes<sup>[19,20]</sup> and possesses transformation activity. More recent studies revealed that the core protein can interact with tumor necrosis factor receptor-1, lymphotoxin  $\beta$ -R and viral envelope protein 1 (E1) and also forms a complex with apolipoprotein AII of the lipid droplet<sup>[21]</sup>. A recent report showed that the HCV core protein can suppress the cisplatin- and c-Myc-mediated apoptotic effect, supporting its role in the establishment of persistent HCV infection<sup>[22]</sup>. But there have reverse results that the core protein also has the ability to enhance cell death triggered by LT- $\beta$ R ligand or anti-Fas monoclonal antibody. Some research considered that the core protein of HCV can induce steatosis and hepatocellular carcinoma in transgenic mice<sup>[23,24]</sup>.

Thus, the molecular mechanism by which the HCV core protein and HCV induce hepatocellular carcinoma is not clear. Recent epidemical research indicated that a significant increase in the prevalence of HCV infection in a group of B-cell non-Hodgkin's lymphoma(NHL)<sup>[25-29]</sup>.

In this study, yeast two-hybrid system was used to clone oncogenic gene. Yeast two-hybrid system 3 based on the system originally designed by Fields and Song<sup>[30]</sup> is developed by Bendixen<sup>[31]</sup>, which is commercially available from Clontech Company L.t.d. In this system, the promoters controlling *HIS3*, *ADE2*, and *MEL1* expression in AH109 have significantly fewer false positives and the simple mating protocol significantly reduces the labor and time involved in performing a two-hybrid library screening and improves the chances of finding rare protein-protein interactions and leads to more reproducible results.

The "bait" plasmid pGBKT7-core was transformed into yeast strain, After mating with liver cDNA library yeast strain Y187, the diploid yeast cells were plated on QDO media containing X- $\alpha$ -gal, 30 true positives were obtained. Sequencing analysis of isolated library plasmids, we find one of the genes is translin<sup>[32]</sup> - a recombination hotspot binding protein. In order to further conform the interaction between the expressed protein and HCV core protein, we performed the experiment of coimmunoprecipitation of both proteins. A strong interaction between the HCV core protein and Translin protein *in vitro* was observed.

A number of studies have shown that chromosomal translocations either result in the activation of proto-oncogenes by joining them to immunoglobulin (Ig) or T-cell receptor genes or lead to the creation of tumor-specific fusion proteins. In man, such translocations consistently occur at particular sites in the genome. Translin protein, which specifically binds to the consensus sequences ATGCAG and GCCC (A/T) (G/C) (G/C) (A/T) found at the breakpoint junctions in many cases of chromosomal translocations, is a unique DNA binding protein<sup>[33]</sup>. The nuclear translocation of translin protein only happened in the time when the cells were treated with mutagen. translin protein may be a typical DNA end binding protein, which is in contrast with one of the other DNA binding proteins, the Ku antigen, that initially binds to DNA ends and then moves to internal positions within the DNA molecule<sup>[34]</sup>. Previous report showed that translin protein was not found in liver tumors. But in this study, we found the gene expressed in liver tumor cell HepG2 and in liver cDNA library. And the interaction between translin protein and HCV core protein not only existed in yeast, but also *in vitro*. The results suggested that translin protein may play a role in hepatocellular carcinoma. But there has had no report giving the evidence that the patients infected with HCV other than HBV have the chromosomal translocation, whether the hepatocellular carcinomas caused by infection of HCV have chromosomal translocation worthy of further studying.

The effects of translin protein on normal lymphocytes need the induction by some factors such as mutagens or biological factors (HCV infection). In lymphoproliferative disorder patients infected with HCV, some reports showed chromosomal translocation happened in B-cell<sup>[35,36]</sup>. Therefore, our report indicated a molecular mechanism that the interaction between HCV core protein and translin protein may trigger the B-cell progressing into lymphoma in patients infected with HCV. How the interaction between the HCV core protein and translin protein causes chromosomal translocation or rather, causes lymphoma, more experiments are necessary to elucidate it.

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