Original Article

Cloning and Expression of HBV Infection Related Novel Gene C12orf49

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Objective To clone, express and purify C12orf49 recombinant protein. To prepare rabbit anti-C12orf49 protein polyclonal antibody in order to further elucidate its biological function.

Methods PCR was used to amplify the gene C12orf49 in vitro. pET-32a (+)-C12orf49, the recombinant protein prokaryotic expression vector, was transformed into *E. coli*. IPTG was used as the inductive agent to obtain C12orf49 recombinant protein, and the recombinant protein was analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. Specific polyclonal antibody was derived from rabbits that immunized by recombinant protein. ELISA and Western blot were used to test its titer and specificity, respectively. MTT cell proliferation experiment was carried out to observe effect of the protein on proliferation of HepG2 cells.

Results The C12orf49 recombinant protein was expressed in a large quantity. Data of ELISA indicated that the titer of polyclonal antibody was higher than 1:1 280 000. And the antibody also had a good specificity, confirmed by Western blot. C12orf49 recombinant protein may had a advanced effect on the proliferation of HepG2 cells.

Conclusions Using C12orf49 recombinant protein, we can obtain the polyclonal antibody with great titer and good specificity. Human novel gene C12orf49 encoded protein could promote the proliferation of HepG2 cells.

Key words: Hepatitis B virus; Novel genes; Antibodies; Recombinant proteins

Replication and release of hepatitis B virus (HBV) from hepatocytes lead to a decrease of the adaptive immune response, and an enhancement of the non-specific immune response, which allows HBV to withstand the host specific immune response and leads to chronic infection. At the same time, the non-specific immune response enhances the inflammation of liver. However, the mechanism of immune tolerance to hepatitis B virus, and molecular mechanism of inflammation of the liver damage caused by HBV is still not clear. In previous studies, we transfected HepG2 cells with hepatitis B virus large surface protein (LHB), and made a gene microarray analysis. The result showed that a number of genes expression changed, including novel gene C12orf49.¹ LHB has a dual transmembrane topology,² with direct hepatocyte toxicity and carcinogenicity, transactivation, enhanced virus replication and the up-regulation of HBV covalently closed circular DNA (HBV cccDNA) copies.³⁻⁶ Furthermore, accumulation of LHB in the

endoplasmic reticulum will result in endoplasmic reticulum stress, leading to the liver injury.⁵ Therefore, the expression of C12orf49 may be related to the infection of HBV, and may participate in the pathological processes of HBV. In this study, we cloned and expressed the uncharacteristic gene C12orf49, and prepared its polyclonal antibody in order to describe its functions.

MATERIALS AND METHODS

Materials

HepG2 cell line and pET-32a (+) vector were preserved by our laboratory. Taq enzyme, T4 DNA ligase, restriction endonucleases were purchased from Promega Company; mouse anti-His monoclonal antibody, HRP labeled goat anti mouse IgG was purchased from Santa Cruz Company; HRP substrate A, B were purchased from Beijing Hotgene Biotechnology Company; complete Freund's adjuvant, incomplete Freund's adjuvant, and protein assay kit were from Sigma Company. Male rabbits which weighted 2.5 kg were

purchased from Experimental Animal Center of Peking University Health Science Center. Animal licenses is No. SCXK (Beijing) 2001-0008. Primers were synthesized by Shanghai Sangon Technology Co. Ltd., China.

Gene cloning

Escherichia coli strains DH5 α and Rosetta were used for cloning and expression of the recombinant C12orf49 protein, respectively. The *E. coli* cells harbouring recombinant plasmids were grown aerobically at 37 °C in Luria-Bertani broth with 100 μ g/ml ampicillin. Plasmid pET-32a was used as an expression vector.

According to the coding region of the C12orf49 sequence, a pair of oligonucleotide primers (forward primer: 5'-GGTACCACCTTCAAG-CAGGAGGAGAGGGC-3', reverse primer: 5'-CTCGAGAGCGGGGAAGAGCTCGGG-3') was designed and synthesized, incorporated with *Kpn* I and *Xho* I restriction enzyme site, respectively. Then C12orf49 was amplified with PCR technology using HepG2 cDNA as the template. The reaction conditions was 95 °C for 5 minutes; 95 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 1 minute, 35 cycles total; 72 °C for 10 minutes; 4 °C placed. The amplification products was identified and purified by 1% agarose gel. The purified C12orf49 fragment was ligated into pGEM-T vector. pGEM-T-C12orf49 plasmid was digested with restriction enzymes *Kpn* I and *Xho* I, and identified by sequencing. Correct C12orf49 fragment was digested from pGEM-T-C12orf49 and ligated into the *Kpn* I - *Xho* I sites of pET32a vector, which provides six his residues at the N-terminus of the expressed protein. Recombinant vector pET32a-C12orf49 was transformed into competent *E. coli* DH5 α and identified by enzyme digestion and sequencing.

Expression of recombinant C12orf49 protein

For expression of the recombinant protein, correct recombinant vector pET32a-C12orf49 plasmid was transformed into competent *E. coli* Rosetta. *E. coli* Rosetta harbouring pET32a-C12orf49 vector were grown in LB medium supplemented with ampicillin (100 μ g/ml) at 37 °C with shaking (250 rpm) until the absorbance reached approximately 0.6~0.8. Then, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mmol/L. The cells were incubated for a further 6 hours before being harvested. Pellet of BL-21 cells harbouring C12orf49 was repeatedly frozen and thawed, and then

be suspended by gentle stirring in buffer (20 mmol/L Tris-HCl, 5 mmol/L EDTA, pH 8.0). The suspension was subjected to ultrasonication (Pulse on 5 s, pulse off 5 s, for 5 min; pulse on 20 s, pulse off 20 s, until *A* value never decreased any more).¹ After centrifugation, the supernatant was denoted as A, and the precipitate was washed by wash buffer (8 mol/L urea, 20 mmol/L Tris-HCl, 5 mmol/L EDTA, 100 mmol/L NaCl, 1% Triton X-100) and ddH₂O, supernatants were denoted as B and C. Then precipitate was soluted by solution buffer (10 mmol/L imidazole, 8 mol/L urea). Then A, B, C and D were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to verify the expression of recombinant C12orf49 protein. Supernatants D was slowly dialysis at 4 °C to remove the high concentrations of urea and make protein refolding. PEG20 000 was used to concentrate the protein, and BCA method was use to determine the protein concentration. Western blot was used to identify the recombinant protein.²

Preparation of rabbit anti-C12orf49 protein

Male rabbits were immunized with recombinant C12orf49 protein in the 1 st, 3 rd, and 4 th week. Protein was injected in subcutaneous of neck, back or groin of the rabbits, and the dose of C12orf49 protein was 1 mg (the first week, with equal volume of Freund's complete adjuvant emulsion), 0.5 mg (the second week), and 0.5 mg (the third week). Blood was collected from ear vein of rabbits at 0 week, 1 week, 3 week, and 4 week, and the serum were preserved to test the titer of antibody with ELISA. In the fifth week, on the third day after immunization, we collected all serum of the rabbits, and preserved it at -80 °C. Purification of polyclonal antibody and specificity was detected by affinity chromatography using protein A and SDS-PAGE electrophoresis, respectively.

Effects of C12orf49 recombinant protein on proliferation of HepG2 cell

HepG2 cells were collected at logarithmic phase, replated in the plate with 2 000 cells per well and incubated at 2.5% CO₂, 37 °C for 12 h. The cells were directly stimulated by C12orf49 recombinant protein at the concentration of 0, 1, 10, 20, 50 and 100 ng/ml, and cultured for a further 48 hours. And then each well was added with 10 μ L 0.5% MTT solution, supernatant was discarded after another 4 more hours, and each well was added with 150 μ L dimethyl sulfoxide (DMSO) to solute the formazan. According to the *A* value (60 repeats each) reading at 570 nm of each well, the

effect of C12orf49 on proliferation of HepG2 cell was analyzed.

Statistical methods

Mean differences was tested with One Way ANOVA analysis using SPSS13.0 statistical software. $P < 0.05$ was considered significant.

RESULTS

Specific primers were designed to amplify C12orf49 gene, and C12orf49 gene fragment was successfully amplified from the HepG2 cDNA template (Figure 1). The C12orf49 gene fragment was cloned into pET32a(+) vector. By the identification of double digestion of pET32a-C12orf49 using *Kpn* I and *Xho* I

restriction enzymes (Figure 2) and gene sequencing, we successfully constructed the prokaryotic expression plasmid of C12orf49. Cloned C12orf49 gene sequence showed 100% homology with the reference sequences. *E. coli* BL-21(DE3) cells harbouring pET32a-C12orf49 plasmid were cultured at 37 °C in the presence and absence of IPTG. Induction of the cells with IPTG (1 mmol/L) at 37 °C for 6 h was found to be optimal to achieve high-level expression of C12orf49, and it had been identified by Western blot that it was recombinant protein induced by IPTG (Figure 3). A, B, C and D supernatant were tested for the presence of recombinant proteins, and the majority of the expressed protein was detected in D supernatant with higher concentration and purity (Figure 3).

Figure 1. The amplification of C12orf49 gene in agarose gel.

Notes: The left lane is amplified C12orf49 gene, and the right lane is 2 000 bp plus DNA ladder.

Figure 2. pET32a-C12orf49 double digestion by *Kpn* I and *Xho* I.

Notes: The left lane is 2 000 bp plus DNA ladder, and those two lanes on the right are both degradation products of pET32a-C12orf49. Bands indicated by the arrow are objective fragments.

Figure 3. *E. coli* BL-21 (DE3) cells harbouring pET32a-C12orf49 plasmid were cultured at 37 °C induced by IPTG for 2 h, 4 h and 6 h (Western blot).

Notes: Above: *E. coli* BL-21 (DE3) cells harbouring pET32a-C12orf49 plasmid were cultured at 37 °C induced by IPTG for 2 h, 4 h and 6 h (Western blot). Below: *E. coli* BL-21 (DE3) cells induced by IPTG were ultrasonicated to obtain target recombinant protein. A: supernatant of degradation products B1-B3, C1-C2: cell lysate supernatant after washing by ddH₂O; D1-D2: solution of precipitation. M: protein marker.

Table 1. Test of the titer of the antibody by ELISA

	1:5 000	1:20 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000
Negative	1.20	0.25	0.16	0.21	0.13	0.25	0.14
1 st week	5.40	5.13	4.92	4.14	2.91	1.74	0.97
2 nd week	5.66	5.55	5.69	5.19	5.04	4.44	3.23
3 rd week	5.56	5.48	5.64	5.19	4.88	4.55	3.41
blank	0.07	0.18	0.06	0.07	0.07	0.06	0.07

Figure 4. Purification of anti-C12orf49 polyclonal antibody.

Notes: Lanes are all purified anti C12orf49 polyclonal antibody (polyacrylamide gel electrophoresis). Heavy chain and light chain of antibody has been detected (arrows).

Figure 5. Effects of C12orf49 recombinant protein on proliferation of HepG2 cells.

Notes: Abscissa represents the concentration of the recombinant protein (ng/ml); Ordinate represents the degree of HepG2 cell proliferation. "*" represents significant difference compared with control (0 ng/ml).

To further characterize the functions of C12orf49 gene, we prepared the rabbit anti-C12orf49 antibody. The titer of the antibody could reach 1:1 280 000 (Table1), and purified C12orf49 antibody had a good concentration and a high specificity (Figure 4).

MTT method was used to observe the effects of C12orf49 protein on the proliferation of HepG2 cell

(Figure 5). Compared with control (0 ng/ml group), 1 ng/ml group and 10 ng/ml group had a significant difference with it.

DISCUSSION

After HBV infection, the host immune system can start

a variety of responses to protect the body and clear the virus, meanwhile minimize damage to the body. HBV is a non-cytopathic virus, but the virus removal reaction and excessive inflammatory process can often lead to severe hepatocellular damage. Once inflammatory lesion in liver started, and it will not intervene, and it can progress to liver fibrosis, cirrhosis, and even liver cancer. In this study, the micro-array results showed that the C12orf49 gene expression was up-regulated in LHBs transfected HepG2 cells.³ It suggested that the gene might be involved in the development of related liver diseases with HBV infection. Bioinformatics analysis showed that the novel gene C12orf49 is located at human chromosome 12, open reading frame encoded 205 aa, with 1 - 33 aa as a signal peptide domain, and the Asn136 was N-GlcNAc. It means to be a secreted glycoprotein. Cloning, expression of recombinant protein and preparation of polyclonal antibody put a solid foundation for the further study of its function.^{4,5}

The process of HBV infects host and causes liver cells injury contains a complex mechanism. It needs the participation of innate and HBV specific adaptive immune system.^{6,7} Animal model studies also indicated that antigen-specific cells, activation antigen non-specific cells and effector molecules in liver were all involved in the process of viral hepatitis. So apoptosis, autophagy, and endoplasmic reticulum stress concepts have become the new theories of liver cell injury.⁸⁻¹¹

In this study, MTT method was used to observe the effects of C12orf49 protein on the proliferation of HepG2 cell (Figure 5). According to the present results, low level recombinant C12orf49 protein stimulation made a significant effect (MTT experiment) on the proliferation of HepG2 cells. It suggested that C12orf49 protein might have an advanced effect on HepG2 cells at a suitable concentration. The effect of

the gene on hepatic parenchymal and stromal cell and its role in the process of HBV replication was still uncertain. C12orf49 as a new gene whose function is still unknown, may be related to the multi-links after HBV infection process and its functional studies are still in progress.

REFERENCES

1. Qiao Y, Chang LS, Wei HL, Xiao F, Hao XH, Zhang RW, et al. Expression of function-unknown gene C16orf68 in different hepatic cell lines. *Wei Chang Bing Xue Yu Gan Zang Bing Xue Za Zhi (Chin)* 2011; 20:327-330.
2. Sikarwar J, Kaushik S, Sinha M, Kaur P, Sharma S, Singh TP. Cloning, expression, and purification of nucleoside diphosphate kinase from *acinetobacter baumannii*. *Enzyme Res* 2013; 2013:597028.
3. Wei HS, Huang YB, Song SJ, Dong QM, Li GL, Cheng J. The relationship between serum LHBs and HBV DNA copies in patients with HBeAg-negative chronic hepatitis B. *Zhonghua Gan Zang Bing Za Zhi (Chin)* 2006; 14:543-546.
4. Zhang HJ, Liu H, Lv LY, Chen GQ, Li ZL. Research progress on sceretome. *Fen Xi Hua Xue (Chin)* 2007; 35:912-918.
5. Hart GW, Copeland RJ. Glycomics hits the big time. *Cell* 2010; 143(5): 672-676.
6. Fiscaro P, Valdatta C, Boni C, Massari M, Mori C, Zerbini A, et al. Early kinetics of innate and adaptive immune responses during hepatitis B virus infection. *Gut* 2009; 58(7): 974-982.
7. Røskov Ø, Sioud M. Discovery of differentially expressed genes: technical considerations. *Methods Mol Biol* 2007; 360: 115-129.
8. Ni Y, Sonnabend J, Seitz S, Urban S. The pre-S2 domain of the hepatitis B virus is dispensable for infectivity but serves a spacer function for L-protein-connected virus assembly. *J Virol* 2010; 84:3879-3888.
9. Chua PK, Wang RY, Lin MH, Masuda T, Suk FM, Shih C. Reduced secretion of virions and hepatitis B virus (HBV) surface antigen of a naturally occurring HBV variant correlates with the accumulation of the small S envelope protein in the endoplasmic reticulum and Golgi apparatus. *J Virol* 2005; 79: 13483-13496.
10. Lepère-Douard C, Trotard M, Le Seyec J, Gripon P. The first transmembrane domain of the hepatitis B virus large envelop protein is crucial for infectivity. *J Virol* 2009; 83:11819-11829.
11. Wang NY, Zhang D, Zhao W, Li BA, Lin CQ. Hepatitis B virus large surface protein in serum as a candidate biomarker for evaluating hepatitis B virus infection. *Clin Biochem* 2011; 44: 1199-1204.