Identification of Human Hepatocyte Proliferation Related Gene C2orf69

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Objective To construct the prokaryotic expression vector pET-32a(+)-C2orf69 and induce the expression of recombinant proteins in vitro. Then the possible effects of recombinant protein on cell proliferation was observed and rabbit-anti-C2orf69 protein polyclonal antibodies was obtained.

Methods Gene fragment of C2orf69 was amplified by PCR and then prokaryotic expression plasmid pET-32a(+)-C2orf69 was constructed. Recombinant protein C2orf69 expression was identified by SDS-PAGE and Western blot. The white-ear rabbits were immunized with purified recombinant protein C2orf69, and the potency and specificity of polyclonal antibody were evaluated by enzyme-linked immunosorbent assay (ELISA) and Western blot. Also, different liver cells were incubated with recombinant protein C2orf69 in vitro.

Results C2orf69 gene fragment was successfully amplified, results of gene sequencing were consistent with the sequence in GenBank. Recombinant protein of C2orf69 was successfully induced and expressed. The polyclonal antibody titer was up to 1 : 1 280 000 through enzyme-linked immunosorbent assay. Results of cell proliferation showed that the recombinant protein could inhibit the proliferation of different liver cells.

Conclusions The recombinant protein C2orf69 could inhibit the proliferation of different liver cells, and we speculated that it may be a widely roled inhibitor of hepatocyte proliferation. Our experiment showed that the proliferation inhibition of cells may be realized by G1 phase extending and S phase shortening.

Key words: C2orf69; Polyclonal antibody; Hepatocyte proliferation

HBV infection is one of the world's most important health problems. According to the World Health Organization reports, there are about 20 million persons infected with HBV all over the world.¹ Most HBV infection in adults lead to self-limited acute hepatitis B, which can be completely removed. However, there are more than 350 million HBV infections, which will eventually progress to chronic hepatitis B or HBV carriers. The vast majority of patients with chronic hepatitis B have a high risk of hepatocellular carcinoma.² According to the latest data of international cancer research organizations,

the incidence of liver cancer in men ranked the fifth of cancer (523 000 cases per year, accounting for 7.9% of all cancer cases), and in women ranked the seventh (226 000 cases per year, accounting for 6.5% of all cancer cases). Hepatocellular carcinoma has a high mortality rate, which was associated with geographic distribution. Liver cancer occurs frequently in developing countries, the rate is about 85%. And hepatocellular carcinoma (HCC) is the most common liver cancer.³

There is a strong correlation between HBV infection and the progress of hepatocellular carcinoma. However, the mechanism of that HBV infection mediated HCC progress has not yet been elucidated.⁴ Most patients with chronic hepatitis B will eventually progress to severe liver diseases, and there is a very high risk of developing into hepatocellular carcinoma.⁵ So the elucidation of the pathogenesis of hepatitis B is particularly important.

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By means of gene chip technology as well as techniques of differential proteins expression, comparing with the normal group, we found that genes in tissues and cells infected with the HBV changed significantly, including the human gene C2orf69, the function of which was still unknown. We speculated that C2orf69 gene expression may be associated with the damage of liver cells infected with HBV and the mechanism of hepatocellular carcinoma.⁶ In this study, gene C2orf69 was cloned and expressed in vitro, and recombinant protein of C2orf69 was purified for polyclonal antibody preparation. By clarifying the gene expression distribution characteristics of different liver cell lines and its effect on liver cell proliferation, it will provide a basis for elucidation of the mechanism of liver cell injury and the occurrence of hepatocellular carcinoma, as well as new research direction for future clinical applications.

MATERIALS AND METHODS

Cell lines and reagents

The human hepatoblastoma cells HepG2, normal liver cells L02, lymphoma cells Jurket 77 and hepatic stellate cells LX-2, pET-32a(+) vector were kept in our laboratory; *E. coli* DH5 α competent cells and BL21 competent cells were purchased from TransGen Biotech; Taq enzyme, dNTP, MgCl₂, pGEM-T-Vector, T4 DNA ligase, *Xho* I and *Kpn* I restriction endonuclease, IPTG (isopropyl- β -D-thiogalactopyranoside), N, N'-methylene-bis-acrylamide were purchased from Promega Corporation (USA); RT-PCR reverse transcription kit was purchased from Fermentas; Freund's complete and incomplete adjuvant, 7-aminoactinomycin D (7-AAD), dimethyl sulfoxide (DMSO) and protein assay kit (BCA Protein Assay Kit) were purchased from Sigma (USA); his-probe mouse monoclonal IgG1, cyclin antibody (cyclin A, cyclin B1, cyclin D1 and cyclin E) and GAR-HRP were purchased from Santa Cruz Biotechnology; GAM (goat anti-mouse)-HRP (horseradish peroxidase) was purchased from Bio-Rad; Ampicillin, MTT were purchased from Amresco Company (USA); CCK-8 reagent kit was purchased from the Institute of Dojindo Laboratories.

Plasmid construction

C2orf69 gene fragment was successfully amplified by polymerase chain reaction (PCR) from HepG2 cDNA with the forward primer 5'-GGATCCTCGTCCTGC

TCTCAGGCCAG-3' and the reverse primer 5'-CTCGAGAAATACTTCATGAACCCTGA-3', and was cloned into pET-32a(+) expression vector. All the constructs were confirmed by sequencing.

Protein expression and preparation of polyclonal antibody

Successfully constructed plasmid was transformed into *E. coli* BL21 and the recombinant protein expression was induced at 30°C for 2 h with 1 mmol/L IPTG. The C2orf69 purified recombinant protein was used to immunize the big ears rabbits to obtain polyclonal antibody. The potency and specificity of polyclonal antibody were evaluated by enzyme-linked immunosorbent assay (ELISA) and Western blot.

Enzyme-linked immunosorbent assay (ELISA)

Briefly, this assay used polyclonal antibody against C2orf69 for the capture of C2orf69 protein to detect the potency and specificity of polyclonal antibody. Anti-rabbit IgG-HRP was used as the second antibody, the absorbance was measured at 450 nm/630 nm.

Western blot analysis

The protein of sample was separated by SDS-PAGE using 10% polyacryl-amide gels and transferred into, which was incubated with milk for 2 hours to close non-specific binding sites. Then the PVDF membrane was incubated with rabbit polyclonal antibody to C2orf69 (from 1 : 5 000 to 1 : 1 280 000, fold dilution), anti-C2orf69 (1 : 500) for 4°C overnight, and then they were immunodetected by incubation with HRP-linked secondary antibody (at an appropriate ratio) and detected using an ECL detection kit.

Cell culture

Human hepatoblastoma cells (HepG2), normal liver cell (L02), human hepatic stellate cells (LX-2) were grown in DMEM, supplemented with 10% fetal bovine serum at normal experimental conditions. Cell proliferation was detected with the kits of CCK-8 and MTT, and the possible molecular mechanisms were explored by the detection of cell cycle. The culture medium of cell proliferation assay was supplemented with less than 2% fetal bovine serum. Lymphoma cells Jurket 77 was maintained in RPMI 1640 medium, the proportion of fetal bovine serum was the same as the other cells.

MTT assay

Various cells (HepG2, L02, Jurket 77 and LX-2) were

divided into 96-well plates (4 000 wells) for 24 h before C2orf69 recombinant protein treatment. Then, cells were incubated with purified C2orf69 recombinant protein at various concentrations (1 ng/ml, 10 ng/ml, 20 ng/ml, 30 ng/ml, 40 ng/ml, 50 ng/ml, 80 ng/ml and 100 ng/ml) for 24 h. The medium was absorbed, and fresh medium was added with 20 μ l MTT (5 mg/ml) and incubated for 4 h at 37°C. The solution was absorbed, and formazan product was dissolved by adding 150 μ l DMSO to each well and incubated for 10 min at room temperature. It was measured at 490 nm/630 nm with a microplate reader.

Analysis of the cell cycle

HepG2 cells were plated into 6-well plates at 1×10^5 cells/well, and were incubated with different concentrations of purified C2orf69 recombinant protein for 48 hours, respectively. The cells were harvested for the analysis of cell cycle. Cells were washed with cold PBS for three times and stained with 7-aminoactinomycin D. DNA content was analyzed by flow cytometry.

Statistical analysis

Statistical analysis was performed with a one-way analysis of variance (ANOVA) and student's *t* test. *P* value of less than 0.05 was considered statistically significant. GraphPad Prism were used for calculations.

RESULTS

Plasmid construct

C2orf69 gene fragments was cloned from HepG2 cells, and the gene was successfully inserted into prokaryotic expression plasmid, its sequence was varified by sequencing (Figure 1).

Protein expression and preparation of polyclonal antibody

This prokaryotic expression plasmid was introduced into competent cells of *E. coli* BL21, 1 mmol/L IPTG was used to induce the expression of recombinant proteins of the gene, and the recombinant protein of C2orf69 was identified by SDS-PAGE and Western

Figure 1. C2orf69 prokaryotic expression plasmid.

Notes: A. C2orf69 PCR products. M. DL2 000 bp marker; 1-2. the target gene fragment; B. double-enzyme digestion of pGEM-T-C2orf69 plasmid using *Bam*H I and *Xho* I. M. DL500 bp ladder marker; 1-2. double-enzyme digestion of the pGEM-T-C2orf69 plasmid by the same enzymes. C. double digestion products of pET32a (+)-C2orf69 prokaryotic expression plasmid. M. DL15 000 bp marker, 1-2. double digestion products of pET32a (+)-C2orf69; 3. pET32a (+)-C2orf69 original plasmid.

Figure 2. The identification of recombinant proteins of C2orf69 by SDS-PAGE and Western blot.

Notes: A. The SDS-PAGE of recombinant proteins of C2orf69. M. protein marker, 1.1 mmol/L IPTG induced at 30 °C for 2 h; 2. 30 °C non-induced for 2 h under the same condition; 3.5.7. under the same conditions induced for 4 h, 6 h, 8 h; 4.6.8. under the same conditions non-induced for 4 h, 6 h, 8 h. B. Western blot of C2orf69 recombinant protein. 1.1 mmol/L IPTG induced at 30°C for 2 h; 2. 30°C non-induced for 2 h under the same condition; 3.5.7. under the same conditions induced for 4 h, 6 h, 8 h; 4.6.8. under the same conditions non-induced for 4 h, 6 h, 8 h.

blot (Figures 2A, 2B).

The purified recombinant C2orf69 protein was used to immune big ear white rabbits, anti-C2orf69 protein polyclonal antibody was obtained (Figures 3A, 3B). Its polyclonal antibody titer was detected by ELISA and the specificity of the antibody was detected by Western blot (Figures 3C, 3D and Table 1).

The distribution of C2orf69 in different cell lines

With the anti-C2orf69 polyclonal antibody, we found C2orf69 expressed differently in various cell lines (HepG2, L02, LX-2 and Jurket 77) by Western blot analysis (Figure 4).

The impact of different concentrations of recombinant proteins on proliferation in different cell lines in vitro

Before the cell proliferation assay, all the cells were required to reach cell cycle synchronization. At the present experiment, it was realized by serum starvation method (using less than 2% serum cell

culture medium).

The C2orf69 recombinant protein was obtained by the experimentally induction and purification. With serum-free medium (DMEM), recombinant protein was respectively diluted into different concentrations (1 ng/ml, 10 ng/ml, 20 ng/ml, 30 ng/ml, 40 ng/ml, 50 ng/ml, 80 ng/ml and 100 ng/ml), and liver tumor cell lines (HepG2), liver cell line (L02), hepatic stellate cells (LX-2) and lymphoma cell lines (Jurket 77) were incubated respectively with the protein at 37°C for 24 h and then added with 20 μ l MTT, and then incubated at 37°C for 4 h. After termination of the reaction, the datas were measured at 490 nm/570 nm. HepG2 cells were set 120 duplicate wells; L02 cells were set 150 duplicate wells; Jurket 77 cells were set 108 duplicate wells; the LX-2 cells were set 60 duplicate wells.

With the concentration of the C2orf69 recombinant protein increased, the overall trend was that C2orf69 inhibited proliferation of HepG2 cells, at the concentration of 10-100 ng/ml, the inhibition was most significant. Compared with the control

Figure 3. The identification of anti-C2orf69 protein polyclonal antibody.

Notes: A. Recombinant protein preparation. M. Marker, Lane 1. total protein, Lanes 2-5. contaminant proteins, Lanes 6,7. Expressed protein. B. Purified C2orf69 recombinant protein. C. Purification of rabbit anti-human polyclonal antibody (the arrow above: heavy chain; the other: light chain). D. Identification of recombinant protein using Western blot. Lanes 1-6. specific bands.

Figure 4. The distribution characteristics of C2orf69 in each cell line.

Notes: Lane 1, 2, 3, 4 are GAPDH and C2orf69 in liver tumor cell line (HepG2), lymphoma cell line (Jurket 77), liver cell line (L02) and hepatic stellate cells (LX-2), respectively.

group, the inhibition effects at 10-100 ng/ml were statistically significant ($P < 0.05$) (Figure 5A).

As for the impact of the C2orf69 recombinant protein on the proliferation of L02 cells, with the concentration increased, the overall trend was to inhibit the proliferation of L02 cells, but compared with the control group, the inhibition effects of

1-100 ng/ml concentration range was not statistically significant ($P > 0.05$) (Figure 5B). Thus the impact of C2orf69 recombinant protein on L02 cell proliferation needs further verification.

As for the impact of the C2orf69 recombinant protein on the cell proliferation of Jurket 77, at low concentration, it inhibited Jurket 77 cell proliferation,

Figure 5. The impact of different concentrations of recombinant proteins on different cell lines in vitro.

Notes: A. The impact of C2orf69 recombinant protein on proliferation of the HepG2 cells in vitro. B. The impact of C2orf69 recombinant protein on proliferation of the L02 cells in vitro. C. The impact of C2orf69 recombinant protein on proliferation of the Jurket 77 cells in vitro. D. The impact of C2orf69 recombinant protein on proliferation of the LX-2 cells in vitro.

Figure 6. The inhibition effect of C2orf69 recombinant protein on the proliferation of HepG2 cells.

Notes: P was control group, the concentration of recombinant protein of C2orf69 was respectively 1 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml and 100 ng/ml, this test was repeated 48 times to take the average.

Figure 7. The impact of different concentrations of the C2orf69 recombinant protein on HepG2 cell cycle.

Notes: A was control group, B was treated with a recombinant protein concentration of 1 ng/ml, C was treated with a recombinant protein concentration of 10 ng/ml, D was treated with a recombinant protein concentration of 20 ng/ml, E was treated with a recombinant protein concentration of 50 ng/ml, F was treated with a recombinant protein concentration of 100 ng/ml.

Table 1. The titer of purified C2orf69 polyclonal antibody

	Antibody titers					
	1 : 5 000	1 : 20 000	1 : 80 000	1 : 320 000	1 : 640 000	1 : 1 280 000
Control	1.4560	0.5591	0.2467	0.2126	0.1843	0.3793
Duplicate	1.6662	0.4877	0.2339	0.1832	0.1998	0.2491
First immunization	4.5515	3.7857	4.0656	2.8572	1.7906	1.2200
Duplicate	4.4821	3.5247	3.9697	2.7980	1.7369	1.1667
Second immunization	4.8945	4.1334	4.4823	4.2247	3.9259	2.9740
Duplicate	4.7229	4.0290	4.6225	4.3640	3.8514	3.2333
Third immunization	4.9319	4.4121	4.6564	4.3330	4.1089	3.6815
Duplicate	5.2559	4.5328	4.7475	4.2300	4.3067	3.5087

Table 2. The data of cell cycle

	The concentrations of the recombinant protein (ng/ml) (Mean $\pm$ SD)					
	P	1	10	20	50	100
G1	61.98 $\pm$ 7.607	65.75 $\pm$ 7.447	66.62 $\pm$ 6.834	66.78 $\pm$ 6.356	66.38 $\pm$ 7.539	63.55 $\pm$ 10.12
S	31.47 $\pm$ 7.395	25.99 $\pm$ 6.051	24.94 $\pm$ 4.943	24.88 $\pm$ 4.539	24.73 $\pm$ 5.282	26.13 $\pm$ 5.642
G2	6.564 $\pm$ 7.905	8.265 $\pm$ 5.964	8.441 $\pm$ 5.047	8.333 $\pm$ 4.436	8.93 $\pm$ 6.262	10.31 $\pm$ 9.263

Notes: P was control group.

with the concentration increased, it gradually promoted cell proliferation. Compared with the control group, the effect of 1-40 ng/ml was statistically significant ($P < 0.05$), while the effect of 50-100 ng/ml was not statistically significant ($P > 0.05$) (Figure 5C).

As for the impact of the C2orf69 recombinant protein on LX-2 cell proliferation, with the concentration increased, the overall trend was that it inhibited LX-2 cell proliferation. An effective concentration was 1-100 ng/ml, compared with the control group, it was statistically significant respectively ($P < 0.05$) (Figure 5D).

The impact of C2orf69 recombinant proteins with different concentrations on the cell cycle of HepG2 cells

The impact of C2orf69 recombinant proteins with different concentrations on the cell cycle of HepG2 cells was analyzed by flow cytometry, the experiment was repeated 48 times and it was found that the C2orf69 recombinant protein can inhibit the proliferation of HepG2 cells in a certain concentration range (Figure 6, Table 2).

With the increase of concentration of the recombinant protein, S period shortened and G1 phase prolonged. When the concentration of the recombinant protein was 100 ng/ml, the effect of C2orf69 recombinant protein on the proliferation of HepG2 cells was little.

Various parts of the cell cycle were analyzed by flow cytometry software ModFit (Figure 7).

DISCUSSION

The new gene C2orf69 with unknown function is human chromosome 2 open reading frame 69, the total length of the gene encoding protein is 385 amino acids, 24 amino acids in the N-terminal are its signal peptides and it is the secreted protein of N-linked glycosylation. Gene Cards results displayed at the RNA level the distribution of the gene in the liver, kidney, heart, lymph nodes and other organs. We found that the gene C2orf69 was expressed in different cell lines (HepG2, L02, Jurket 77 and LX-2) by the constuction of prokaryotic expression plasmid, induction of recombinant protein expression and the preparation of polyclonal antibody.

There is a strong correlation between HBV infection and hepatocellular carcinoma. However, the mechanisms of HCC has not yet been elucidated. HBV, as a non-invasive hepadnavirus, doesn't directly damage the liver cells after infecting organisms, but their antigen-antibody expression system and the specific immune response induced liver cell injury.⁷ The pathogenesis of hepatitis B immune damage is the immune response mainly caused by clearing HBV virus, in which CTL (cytotoxic T cell) played a key role. HBV-specific CTL recognition of infected liver

cells is considered to be the central link of causing the damage of liver cell and removing viruses.⁸

One of the treatments for liver cancer is liver transplantation. There is a high correlation between surgical resection and recurrence of liver cancer, it may be the results of some occult metastases or regional effects kept in residual liver after liver resection.⁹ Therefore, due to the poor prognosis of patients with hepatocellular carcinoma, it is an urgent need for new treatment strategies. The specific inhibitor targeted treatment for cancer is a developing strategy, and has shown a promising anti-tumor effect.¹⁰

It was found that the recombinant protein within a certain range of concentration could inhibit the proliferation of liver hepatocellular carcinoma cells (HepG2), hepatic stellate cells (LX-2) and lymphoma cell lines (Jurket 77) by cell proliferation assay (MTT and CCK-8) and cell cycle. At the same time compared to the control group, it showed a significant statistical difference ($P < 0.05$). Thus we speculated that C2orf69 may be a broad cell proliferation inhibitory factor. The results of HepG2 cell proliferation was verified by cell cycle, it showed that the S shortened and G1 phase prolonged, this result was consistent with previous results of cell proliferation. However, whether it could really become a specific inhibitor for the treatment of tumors also remains to be seen, but the role of the inhibition of cell proliferation of C2orf69 provided a new direction in its subsequent functional studies.

The cell cycle was regulated through the respective specific cyclins of the respective time, including cyclins, CDK-dependent kinases (CDKs) and CDK-inhibitory factors (CKIs). They are the main factors involved in the regulation of cell cycle.¹¹ Cyclin D was first isolated by Motokura et al, including cyclin D1, D2 and D3 three subtypes.¹² In recent years, it have been reported that the abnormal activation of Wnt/beta-catenin signaling pathway could promote the occurrence of the progress of liver cancer.¹³ Cyclin D is the target molecules in the Wnt/beta-catenin signaling pathway, after wnt protein binding its corresponding ligand, cytoplasmic beta-catenin protein increased and transferred into the nucleus, combined with the transcription complex and promoted the transcription of target genes. The study found that Wnt/beta-catenin signaling pathway is very important for the growth, the abnormal activation of the signaling pathway is associated with cancer occurrence and development,¹⁴ and the inhibition of the signaling pathway could also

inhibit cell proliferation.

This experiment showed that the proliferation inhibition of the protein may be realized by extending G1 phase and shortening the S phase. It is worth mentioning that cyclin D1 is the major protein of the G1 regulation, and it is crucial for the regulation of cell cycle.¹⁵

On the other hand, in addition to inhibit cell proliferation of liver parenchymal cells, the recombinant proteins also inhibited the proliferation of liver mesenchymal cells. The development of HCC was associated with chronic liver inflammation, fibrosis, and concurrent irreversible cirrhosis. Most important, liver fibrosis has been considered to be a contributing factor in the development process of hepatocellular carcinoma of cirrhosis patients.¹⁶ Hepatic stellate cells are main cell types that play a decisive role in the excessive production and deposition of extracellular matrix of the progression of liver fibrosis, this process also regulated by different inflammatory factors.^{17,18} In addition, the activated HSCs might contribute to the progress of HCC by mutual interference in cancer microenvironment.¹⁹⁻²¹

The inhibition of C2orf69 recombinant protein on the proliferation of liver mesenchymal cells (hepatic stellate cells LX-2), on the one hand, as previously mentioned, could impede the progress of HCC by cancer microenvironment intervention; on the other hand, inhibition the proliferation of hepatic stellate cells (HSC) can make liver fibrosis and even cirrhosis reversible. Thus, the research had suggested that inhibition of hepatic stellate cell activation and proliferation might be a treatment strategy for cirrhosis and liver fibrosis which eventually lead to hepatocellular carcinoma.²²

Through this study, we found that C2orf69 may be a broad cell proliferation inhibition factor, but for specific mechanisms of the inhibition of cell proliferation and many other issues still need more in-depth experiments to verify, the study of relevant mechanism is in progress.

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