

# The study on expression of CIAPIN1 interfering hepatocellular carcinoma cell proliferation and its mechanisms

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**Abstract. – OBJECTIVE:** Liver cancer is one of the common gastrointestinal cancers. This study was designed to investigate the effect of the cytokine-induced apoptosis inhibitor 1 (CIAPIN1) on hepatocellular carcinoma cell proliferation and invasion.

**MATERIALS AND METHODS:** To establish a low and high expression of CIAPIN1 in hepatoma cell lines, pGPU6/GFP/Neo and CIAPIN1 siRNA vectors were constructed. The growth curve of liver cancer cells with a low and high expression of CIAPIN1 was measured by MTT assay and colony formation in soft. The effect of overexpression and inhibition of CIAPIN1 on the expressions of cell cycle proteins Cyclin D1, CDK2, CDK4, and Cyclin E were detected by western blot.

**RESULTS:** As compared with the low expression group, the cells in CIAPIN1 high expression group showed a significant decrease in proliferation ( $p < 0.05$ ). In addition, the colony-forming ability of cells with high expression of CIAPIN1 was decreased significantly ( $p < 0.01$ ). Furthermore, the expressions of Cyclin D1 CDK2, CDK4, and Cyclin E in high expression group were significantly increased ( $p < 0.01$ ).

**CONCLUSIONS:** CIAPIN1 played an important role in the proliferation of liver cancer cells through increasing the expressions of cell cycle related proteins Cyclin D1, CDK2, CDK4, and Cyclin E.

Key Words:

Cytokine-induced apoptosis inhibitory factor 1 (CIAPIN1), Liver cancer, proliferation, Cell cycle-related proteins.

more than 80% of patients have benefited from the first-line chemotherapy, there is still a high recurrence rate and low overall survival rate in patients with advanced liver cancer<sup>4</sup>. A previous study<sup>5</sup> found that the development of liver cancer is a complicated process involving the interaction of various factors, which has a close relationship with the abnormality in the multi-genes family. This suggests that determination of the molecular targets should become the goal, which would help to understand the development and progression of liver cancer.

Cytokine-induced apoptosis inhibitory factor 1 (CIAPIN1), is a new gene identified by Digestive Disease Hospital of China's Fourth Military Medical University<sup>5</sup> through new molecular genetic screening of patients with gastric cancer in 2004. In 2004, Japanese scholars Shibayama et al<sup>6</sup> reported a new mouse-derived anti-apoptotic gene-Anamorsin, whose structure having 88% amino acid sequence homology with that of CIAPIN1, both of which are considered to be the homologous molecule among different species. The homolog Dre2 also has been found in yeast<sup>7,8</sup>. Since 2004, lots of studies on CIAPIN1 have been launched in several laboratories from different countries, from which the results suggested that CIAPIN1 played different roles under different physiological and pathological conditions.

In the development of several different human tumors, the mechanisms of CIAPIN1 are different. For example, it exerts anti-apoptosis and promoting proliferation effect in ovarian cancer and large B-cell lymphoma<sup>9</sup>, but inhibits tumor cell proliferation in renal cell carcinoma and esophageal cancer<sup>10</sup>. However, the exact role of CIAPIN1 in the development of liver cancer has

## Introduction

As one of the most common gastrointestinal malignancies<sup>1</sup>, liver cancer has a very high mortality rate<sup>2</sup>. Currently, the main treatments for liver cancer are surgery and chemotherapy<sup>3</sup>. Although

not been reported. In the present study, we aimed to investigate the distribution of CIAPIN1 in liver cancer cells, and further explore the mechanisms of CIAPIN1 in liver cancer as well as its relationship with the occurrence of liver cancer.

## Materials and Methods

### Cells

Human hepatoma cell line QGY-7703 was purchased from Sun Yat-sen University.

### Reagents

Methyl thiazolyl tetrazolium (MTT; Sigma-Aldrich, St. Louis, MO, USA); DMEM medium (Gibco, Grand Island, NY, USA); CIAPIN1 mouse anti-human monoclonal antibody (TNMED1007, Gene Technology Co., Ltd. Shanghai, China); Olympus BX51 optical microscope (Olympus, Shinjuku, Tokyo, Japan); automatic protein visualizer (Bio-Rad, Hercules, CA, USA).

### Construction of pGPU6/GFP/Neo CIAPIN1 siRNA Plasmids

5' and 3' terminals of the target sequence of the sense strand encoding small interfering RNA have been designed containing BbsI and BamHI restriction sites respectively, while there is an anti-19nt repetitive DNA sequence encoding siRNA antisense strand in the middle of the sequence as the hairpin transcript, wherein TTTTTT was served as transcription termination signals. The loop structure TTCAAGAGA was designed in shRNA template to avoid the formation of a stop signal, while the shRNA transcription termination sequence was T6 structure. The sticky terminal in 5' terminal of the sense strand of the template could be complementary after BbsI digestion; the sticky terminal in 5' GATC of antisense strand could be complementary after BamHI digestion. Core design includes CIAPIN1 siRNA, CIAPIN1 siRNA1, CIAPIN1 siRNA2, Con-siRNA and negative control, with GAPDH as the positive control.

### Transfection of CIAPIN1 siRNA

After trypsin digestion, QGY-7703 cells were cultured in high glucose medium containing 10% fetal bovine serum (excluding double-antibody) in 24-well plates until reaching 80% of the density and then washed twice with serum high glucose Dulbecco's Modified Eagle Medium (DMEM). The liposome/DNA complexes were prepared as follows: antisense plasmid pGPU6/GFP Neo CIA-

PIN1 siRNA plasmid, negative control of pGPU6/GFP/Neo against nuclear and positive control of pGPU6/GFP/Neo GAPDH were added into 50  $\mu$ L Opti-MEM, respectively. Then, 1.5  $\mu$ L lipofectamine<sup>TM</sup>2000 liposomes were added under gently pipetting, respectively and maintained at room temperature for 5 minutes. DNA and liposomes are thoroughly mixed at room temperature for 20 minutes; then, the mixture were added into the QGY-7703 cell culture sugar-free DMEM for 5 hours. Transfected cells were named as the CIAPIN1 siRNA, CIAPIN1 siRNA1, CIAPIN1 siRNA2 experimental groups, negative control and positive control of Con-siRNA GAPDH.

### The effect of CIAPIN1 on QGY-7703 Hepatocellular Carcinoma Cell Proliferation in vitro by MTT Assay

QGY-7703 human hepatoma cells in the logarithmic growth phase were digested and resuspended to be a single cell suspension.  $5 \times 10^3$  cells/96 well plate were seeded. Cells were cultured at 37°C and 5% carbon dioxide incubator overnight. After medium replaces, 100  $\mu$ L medium with different concentrations of metformin was added into each well. After another 72 hours cultured, 20  $\mu$ L 5 mg/ml MTT was added to each well. 4 hours later, 150  $\mu$ L dimethyl sulfoxide (DMSO) solution was added into plates, and the plates were shaken for 10 minutes under dark conditions.

### Clonogenic Assay

QGY-7703 human hepatoma cells in the logarithmic growth phase were digested and resuspended to be a single cell suspension.  $1 \times 10^3$  cells were seeded in 6-well plates and cultured at 37°C and 5% carbon dioxide incubator overnight. After medium had replaced in every time, 100  $\mu$ L medium with different concentrations of metformin was added into each well. After two weeks of culture, medium was removed and, then, the cells were fixed with 4% DMSO for 15 minutes, stained with 0.1% crystal purple for 5 minutes. Pictures were taken by a digital camera and archived.

### Western Blot

Human hepatoma cell QGY-7703 was lysed with radioimmunoprecipitation assay (RIPA) buffer, and the particles were discarded after centrifugation. The concentration of extracted protein was measured by the bicinchoninic acid (BCA) assay. The sample was mixed with 5 x loading buffer, and boiled for 5 minutes.

After separated by 10% polyacrylamide gel electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes; then, they were blocked with 5% milk at room temperature for 1 hour. Membranes were incubated with antibody at 4°C overnight. After 3 times rinsing, membranes were incubated with secondary antibody at room temperature. After addition of enhanced chemiluminescence (ECL) reagent for 5 minutes, the membranes were developed with gel image analyzer.

### Statistical Analysis

Statistical analysis was conducted by IBM SPSS 19.0 software (SPSS Inc., Armonk, NY USA), and data expresses as mean  $\pm$  standard deviation (SD). The groups were compared using the *t*-test, and count data were compared using the chi-square test.  $p < 0.05$  was considered statistically significant.

## Results

### CIAPIN1 siRNA Plasmid was Constructed Successfully

The mRNA sequence of CIAPIN1 gene was obtained from [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov) database. According to the design principle, three pairs interference sequences were designed as follows: CIAPIN1 siRNA, CIAPIN1 siRNA1,

CIAPIN1 siRNA2, and CIAPIN1 siRNA2) and a negative control -Con-siRNA and a positive control, namely GAPDH fragment sequences. The pGPU6/GFP/Neo plasmid map was shown in Figure 1.

### The Efficiency and Effect of CIAPIN1 siRNA Transfection into QGY-7703 Cells

Image acquisition is completed in an inverted fluorescence microscope 24 hours after green fluorescent labeled with 7703 cells. After the cells had been transfected with a eukaryotic expression vector pGPU6/GFP/Neo CIAPIN1 siRNA plasmid, and green fluorescence was observed under a fluorescence microscope, it indicated that the expression of CIAPIN1 was found in cells, and was positively correlated with the expression of GFP. QGY-7703 cells were used as the control group without GFP expression, and the number of cell apoptosis is minimal. As shown in Figure 2, the system CIAPIN1 siRNA and Con-siRNA transfection efficiency were reached to 80%.

### Cell Transformation Proliferation Assay to Detect the Effect of siRNA for CIAPIN1 on Hepatoma Cell Proliferation

MTT results showed that compared with cells transfected with empty vector control and parental cells, transfection of antisense expression vector CIAPIN1 siRNA decreased cell prolifera-

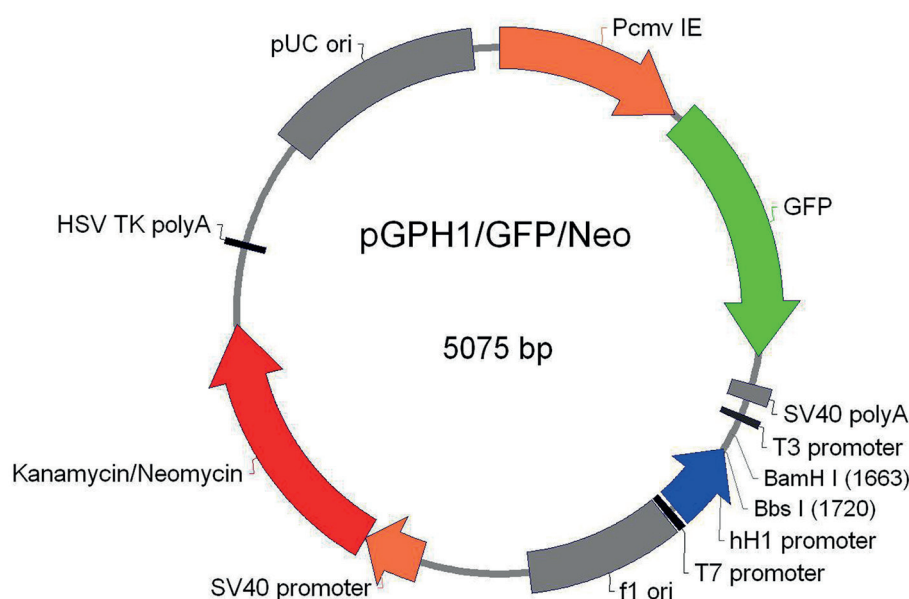
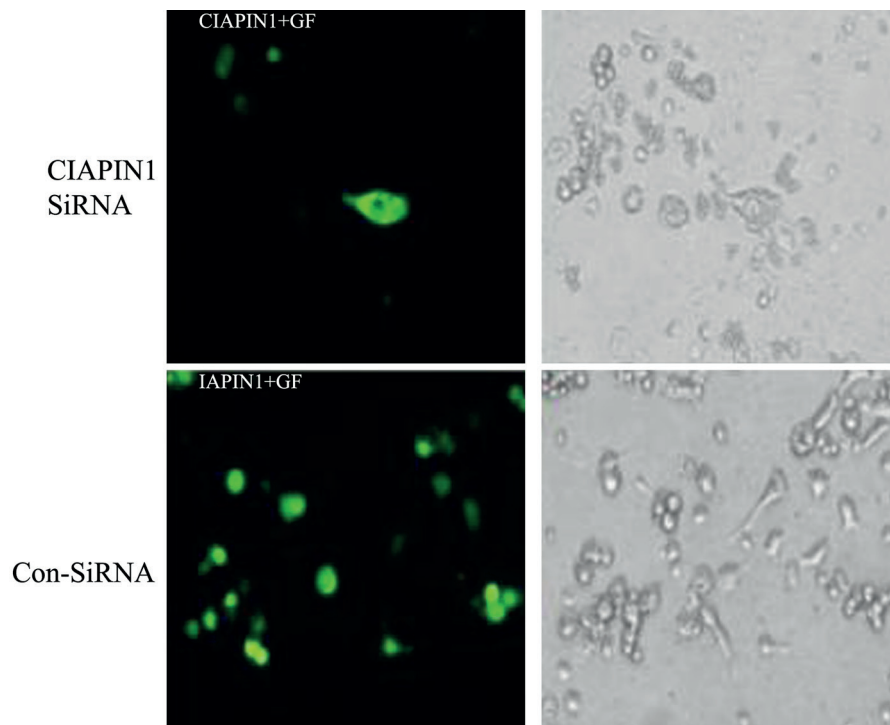
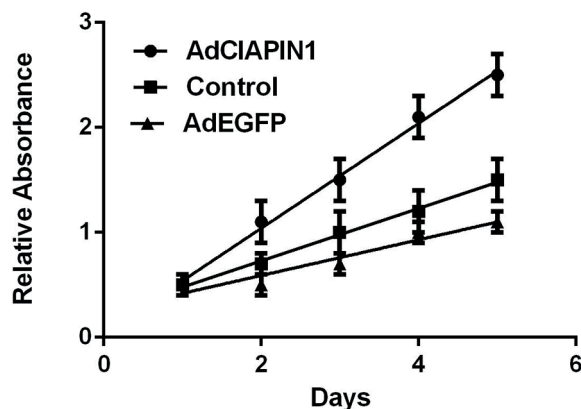


Figure 1. Plasmid pGPU6/GFP/Neo map.

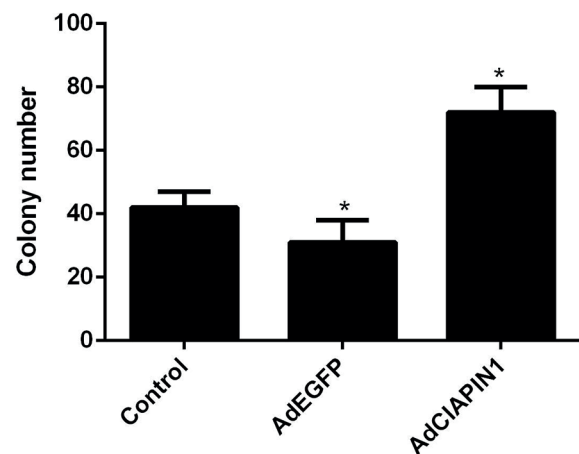


**Figure 2.** Morphological effects of CIAPIN1 siRNA in QGY-7703 cells (48 hour). The top layer is CIAPIN1 siRNA QGY-7703 for 48 hours after transfection, showed obvious apoptotic cell debris, early apoptotic cell rounding, cell attachment is poor; the underlying negative control plasmid QGY-7703 infection effect, compared with the state of cell growth well, less the number of apoptotic cells



**Figure 3.** The proliferation curve of liver cancer cells was measured by MTT assay. The cell growth rate of QGY-7703 cells infected with adenoviral CIAPIN1 was significantly higher than the control ( $p < 0.05$ ).

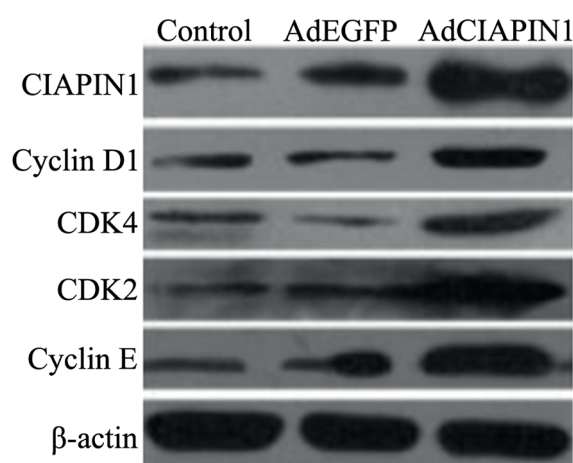
tion *in vitro*. However, infection with the recombinant adenovirus could significantly increase the growth rate of AdCIAPIN1 comparing with the AdEGFP group and control (Figure 3). The results indicated that CIAPIN1 can promote the proliferation of liver cancer cells.



**Figure 4.** The effect of CIAPIN1 on hepatoma cell anchorage-independent growth. After QGY-7703 cells were infected with adenovirus, the expression of AdCIAPIN1 increased and the ability to form colony in soft agar experiment was significantly higher than that of control, which showed a significant difference ( $*p < 0.05$ ).

#### CIAPIN1 Hepatoma cell Anchorage-Independent Growth of Influence

To further understand the effect of molecular CIAPIN1 on the anchorage-dependent growth



**Figure 5.** The expression of CIAPIN1 and cell cycle-related proteins in hepatoma cells.

of QGY-7703 liver cancer cell, soft agar colony formation assay was performed to determine the growth rate. Figure 4 showed that compared with control cells and cells infected with empty virus of AdEGFP, the number of AdCIAPIN1 tumor cells increased significantly. Further, CIAPIN1 could promote the anchorage-independent growth ability of liver cancer cells *in vitro*, as well as the growth ability of ovarian cancer cells.

#### **CIAPIN1 Promote the Proliferation of Ovarian Cancer Cells Through up-Regulation of Cyclin Proteins**

In order to analyze the mechanisms of CIAPIN1 in promoting cell cycle progression of liver cancer cells, we examined the expressions of cell cycle-related proteins cyclinD1, CDK4, CDK2, cyclin E. Western blot results showed that after the expression of CIAPIN1 was increased, the expression of cell cycle-related proteins cyclinD1, CDK4, CDK2, and cyclin E was also increased

(Figure 5; Table I). On the contrary, after down-regulation of the expression of CIAPIN1, the expression levels of cell cycle-related proteins were decreased.

### **Discussion**

Due to the onset of liver cancer are not easy to find out, and the clinical pathological diagnosis is too late, gastrointestinal cancer has the highest mortality rate<sup>11,12</sup>. To date, the standard treatment for liver cancer is mainly combined the ideal cytoreductive surgery with the platinum and paclitaxel-based chemotherapy<sup>7,13</sup>. After years of exploration by clinicians, the liver tissue could be removed under the microscope by means of advanced microsurgical technique<sup>14</sup>. However, the 5-year survival rate of liver cancer patients after surgery is still at around 15%<sup>15</sup>. After several chemotherapies, some patients might be sensitive to the drugs, while others could not stand the powerful killing effect of chemotherapy drugs. Tumor cells together with non-tumor cells are killed at the same time without targeted therapy. The characteristics of poor prognosis in patients with liver cancer lead to research on the development mechanism of liver cancer as a new hot spot. To improve the survival rate of patients with liver cancer, targeted therapy provides a new hope<sup>16</sup>.

The results of the present study suggested that CIAPIN1 can promote liver cancer cell growth and proliferation with the main mechanism being through up-regulating the expressions of cell cycle related proteins cyclinD1, CDK4, cyclinE, and CDK2. As a new anti-apoptotic molecule, CIAPIN1 is different from traditional apoptosis regulatory molecule Bcl-2 family and caspase family members. It is a key molecule in the Ras signaling. The expression of CIAPIN1 was entirely dependent on growth factors such as IL3, stem cell factor and the stimu-

**Table I.** The IOD value of CIAPIN1, Cyclin D1, CDK4, CDK2, Cyclin E ( $\bar{X} \pm \text{SD}$ ).

|           | Control (no. 3) | AdEGFP (no. 3) | AdCIAPIN (no. 3)            |
|-----------|-----------------|----------------|-----------------------------|
| CIAPIN1   | 7741.20±65.34   | 7997.14±45.67  | 17803.64±30.50 <sup>#</sup> |
| Cyclin D1 | 7322.34±73.31   | 7721.30±51.20  | 8261.10±27.23 <sup>#</sup>  |
| CDK4      | 8045.00±64.55   | 8323.13±41.51  | 9662.33±55.78 <sup>*</sup>  |
| CDK2      | 7445.13±34.55   | 8455.00±55.45  | 9456.34±54.45 <sup>*</sup>  |
| Cyclin E  | 7445.13±34.55   | 8545.11±45.45  | 9455.45±45.11 <sup>*</sup>  |

<sup>#</sup> $p < 0.01$ ; <sup>\*</sup> $p < 0.05$

lation of TPO. However, based on a biological point of view, its specific function primarily depends on the interaction effect with protein molecules and molecules involved in signaling pathways in cells. This experiment showed that CIAPIN1 promoted liver cancer cell proliferation. CyclinD1/CDK4 and cyclinE/CDK2 are regulatory proteins in cell cycle G1/S phase and play an important role in the process of dividing and replicate in normal cells and cancer cells<sup>17</sup>. Up-regulation of cyclinD1 could promote cell proliferation and cell replication in G1 phase, and be good to the mitosis in S phase, while down-regulation of cyclinD1 would inhibit the cell proliferation, and cell cycle would be arrested in G1 phase, leading to reduction of the ability to replicate. This experiment confirmed that up-regulation of CIAPIN1 could improve the expression of cyclin-related molecules cyclinD1, CDK2, CDK4, and cyclinE. CyclinD is a key molecule in cell division<sup>18</sup>. CIAPIN1 could negatively regulate p38MAPK<sup>19</sup>, while p38MAPK could negatively regulate cyclinD1 to promote tumor cell proliferation. Furthermore, cyclinD1 is the activated protein for NF- $\kappa$ B transcription factor, which also indicates that cyclinD1 could promote liver cancer cell proliferation. Therefore, it suggested that CIAPIN1 could play a major role in promoting the proliferation of liver cancer cells through affecting the transcription levels of the genes.

## Conclusions

CIAPIN1 plays a role in promoting the proliferation of liver cancer cells in the development of liver cancer *in vitro* experiment which might be through the regulation of the expression of cell cycle-related proteins Cyclin D1, CDK4, CDK2, and Cyclin E. Furthermore, there was no expression or weak expression of CIAPIN1 in normal liver tissue, but with high expression in liver cancer cells, indicating that there are a high sensitivity and specificity of CIAPIN1 in the diagnosis. As a result, CIAPIN1 might be used as a new therapeutic target in liver cancer diagnosis.

## Acknowledgments

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## Conflict of interest

The authors declare no conflicts of interest.

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