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Original Article

Effect of cytokine-induced apoptosis inhibitor 1 on liver cancer cell apoptosis

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Abstract: CIAPIN1 is recently recognized as a key player in a variety of prevalent cancers. In this study, we sought to explore the correlation of CIAPIN1 expression with cell apoptosis in liver cancer cell line. SI CIAPIN1 was transfected into HepG2 and Huh7 cell line to interfere the expression of CIAPIN1. Real-time polymerase chain reaction (RT-PCR) was performed to determine the expression level of CIAPIN1 at mRNA, and Western blot were performed to test the apoptosis protein level. SI CIAPIN1 successfully down-regulate the expression level of CIAPIN1 in HepG2 and Huh7 at mRNA level. Moreover, the apoptosis rate of HepG2 and Huh7 cells in SI CIAPIN1 transfected group notably increased compared with control group. Besides, the western blot results shown that the transfection of siCIAPIN1 in HepG2 and Huh7 could interfere with the expression of BCL-2 and BAX. Our study found that suppression of CIAPIN1 expression could increase cell apoptosis in liver cancer cells. All these findings suggest that CIAPIN1 may be involved in progression of liver cancer and could be a new therapeutic target for this disease.

Keywords: Liver cancer, cytokine-induced apoptosis inhibitors, apoptosis protein

Introduction

The impact of underlying patient condition on treatment and outcomes of recurrence of HCC has been investigated for many years. Until now, there are no established prognostic factors or standardized therapies for HCC recurrence. The understanding of the etiology and pathogenesis is vital for survival after HCC recurrence.

CIAPIN1 (cytokine-induced apoptosis inhibitor 1) alternate name Anamors-in, V62, is a newly confirmed molecule related to tumor apoptosis and multi-resistance, it has been proposed as an attractive target for new anticancer interventions, owing to its prognostic value for human tumors and involvement in cancer progression and tumor cell resistance to anticancer agents [1-5]. CIAPIN1 gene located in the 16th q13 by seven 8 exons and introns, relative molecular mass is 39,000, N end 60~99 amino acid sites found gener-IC methyltranferase profile structure domain, the sequence of C end partly homologous with conservative COG5636 genes and conservative DUF689 gene families. Bioinformatics analysis of CIAPIN1 indicated that it contains a common methyltransferase motif, and a Zn-ribbon-like base sequence. Thus, CIAPINI was speculated as a RNA or DNA methyltransferase. CIAPIN1 genes have a wide range of expression in both embryonic and adult tissues and organs, and in the expression of the two similar models. In different organs, however, CIAPIN1 gene expression level was different. In the heart, liver, pancreas, esophagus, skin and other organs it has higher expression, while in the stomach, colon and lung organ it was positive expressed [3, 6-9]. The expression of CIAPIN is also different in different tumor tissues. CIAPIN gene expression level is significantly lower or missing in lung cancer, kidney cancer, esophageal cancer and colon cancer than that in normal tissues [10], while, by immunohistochemical detection, CIAPIN1 genes showed a significantly higher expression in primary HCC than that in normal tissues. CIAPIN1 were proved to play key roles in many other vital cancers, such as pancreatic cancer, gastric cancer, multiple myeloma, ovarian cancer, breast cancer [5, 7, 9, 11]. Moreover, it is prov-

en to be a mediator of the RAS signaling pathway and plays a vitally important role in fetal liver (FL) hematopoiesis, the proliferation of cancer cells is limited after the knock down of CIAPIN in liver cancer [12], while, until now, there is no more exploration about CIAPIN in liver cancer.

In the present study, we sought to explore the expression level of CIAPIN1 in liver cancer cells. Meanwhile, silencing of CIAPIN1 expression was performed to further elucidate the role of CIAPIN1 in liver cancer by miRNA interference. Then western blot assay was used to investigate the correlation between the BCL-2 family proteins and CIAPIN1 expression. The finally objective of our study was to evaluate the correlation of CIAPIN1 expression with cell apoptosis in liver cancer cell lines, as well as its underlying mechanism. All of our efforts will provide theoretical basis and new insights into the treatment of liver cancer.

Materials and methods

Cell culture

HepG2 cell line was obtained from American Type Culture Collection recommendations. Huh7 cell line was acquired from the Health Science Research Resources Bank (Osaka, Japan). HepG2 and Huh7 were cultured according to recommendations in Dulbecco's modified Eagle's medium containing 10% FBS (Gibco BRL) [13].

Plasmids and siRNA transfection

The shRNA expressing plasmids specifically targeting CIAPIN1 and control siRNA (no silencing) were synthesized by GenePharma Co (Shanghai, China). A CIAPIN1 expression vector (pcDNA3.1-CIAPIN1) was constructed by sub-cloning the full-length wild-type CIAPIN1 coding sequence into pcDNA3.1 (+), and confirmed by sequencing. The empty construct pcDNA3.1 was transfected as a control.

Cell transfections were conducted using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. Stable CIAPIN1 transfectants were generated under G418 (Gibco, Paisley, UK) selection as described before [14, 15].

Apoptosis assay

The cells which transfected with siRNA CIAPIN1 for 48 h were assayed by annexin V and PI staining (BD PharMingen, San Diego, CA, USA) according to the manufacturer's instructions, the cells were analyzed with a FACS Calibur flow cytometer (Becton-Dickinson). The percentage of total apoptotic events was defined as the sum of the cells in the early stage (annexin V positive/PI negative) and late stage (annexin V positive/PI positive) of apoptosis as previously described [16].

Detection of CIAPIN1 mRNA by real-time PCR

Total mRNA was isolated from cells and islets as previously described (Rosengren et al, 2012). Complementary DNA (cDNA) was produced using reverse transcriptase (iScript™ cDNA Synthesis Kit; Bio-Rad Laboratories). The expression levels of mRNAs were measured by SYBR green-based quantitative RT-PCR (SYBR Green Master mix; Thermo Scientific, Waltham, MA, USA) [17].

Western blot analysis

Protein samples (the same concentration per lane) were separated on a 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted onto polyvinylidene difluoride membranes, blocked in PBST (0.1% triton in 19 PBS) and probed with primary antibodies overnight at 4°C. The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. The immunoreactive protein bands were developed by enhanced chemiluminescence. The immunoreactive bands were analyzed by a densitometer [18].

Statistics analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean ± SD. Statistical analyses were performed using SPSS 19.0 statistical software. The *P*-values were calculated using a one-way analysis of variance (ANOVA). A *P*-value of < 0.05 was considered to indicate a statistically significant result.

Results

Si CIAPIN1 successfully interfered mRNA levels of CIAPIN1

RT-PCR analysis displayed the expression of CIAPIN1 at mRNA level. As shown in **Figure 1A-C**), HepG2 and Huh7 were respectively transfected siCIAPIN1, the transfection of si

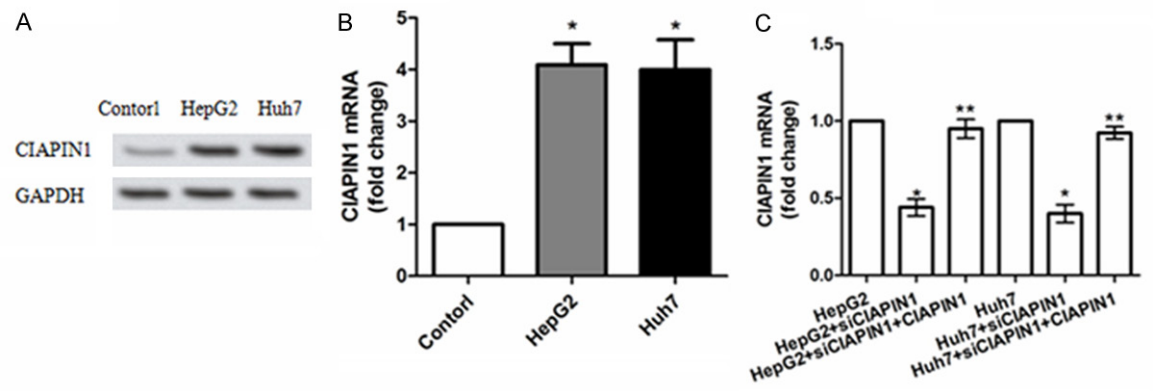

Figure 1. A. mRNA levels of si CIAPIN1 in both HepG2 and Huh7 cells, after HepG2 and Huh7 were respectively transfected siCIAPIN1. B. Statistical analysis results of CIAPIN1 mRNA levels in both HepG2 and Huh7 cells. C. Real-time RT-PCR results of CIAPIN1 mRNA from HepG2 and Huh7 cells after infection. *, $P < 0.05$; **, $P < 0.01$.

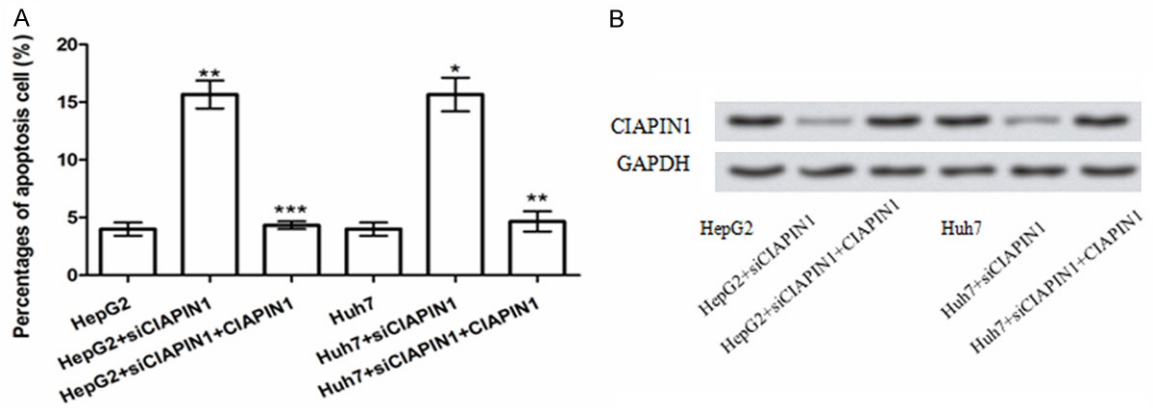

Figure 2. Cell viability of HepG2 and Huh7 transfected si CIAPIN1. A. The apoptosis rate induced by the si CIAPIN1 was examined; B. A real-time RT-PCR was performed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

CIAPIN1 successfully interfered the mRNA levels in both HepG2 and Huh7 cells.

SICIAPIN1 up-regulated cell apoptosis rate

To determine the apoptosis rate of HepG2 and Huh7 cells after 48 h of transfection. The results showed that the cell apoptosis rate of HepG2 and Huh7 transfected with siRNA CIAPIN1 significantly increased to 15% (**Figure 2A** and **2B**). This may suggest that the suppression of CIAPIN1 expression could significantly increase the apoptosis of HepG2 and Huh7 cells.

CIAPIN1 regulated apoptosis protein expression

Bax and BCL-2, are both apoptosis protein, and BCL-2 is a kind of anti- apoptosis protein [19, 20], to further confirm the mechanism of CIAPIN1 expression with cell apoptosis in liver cancer cell lines, we tested the relative density of Bax, BCL-2 after transfected siCIAPIN1. As shown in **Figure 3A** and **3B**, the CIAPIN1 could obviously regulate these apoptosis proteins, the si CIAPIN1 up-regulate the expression of Bax in both HepG2 and Huh7 cells, while down-regulate BCL-2 in both of them. This stated CIAPIN1 may affect the apoptosis of liver cells via regulating the apoptosis proteins.

Discussion

HCC, the predominant form of primary liver cancer, is a common cancer worldwide and the third leading cause of cancer death without effective treatment strategy [21]. Though many years of studies have been conducted, the mechanisms underlying HCC are poor under-

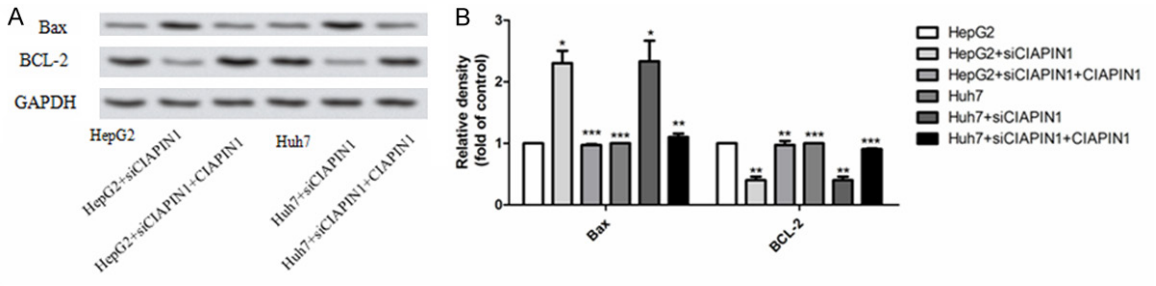

Figure 3. A. Western blot analysis of Bcl-2 family mediators of apoptosis after transfected siCIAPIN1 with a single test. B. Statistical analysis results of CIAPIN1 regulated apoptosis protein expression. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

stood. So far, no reliable molecular targets exist for early detection of HCC at surgically manageable stages [21-23].

CIAPIN1 is a recently reported anti-apoptotic molecule playing an essential role in mouse definitive hematopoiesis, is considered a downstream effecter of the receptor tyrosine kinase-Ras signaling pathway, and the knock down of CIAPIN1 by adenovirus-delivered siRNA may be a potential therapeutic strategy for the treatment of HCC [12]. Recently, there are studies confirm CIAPIN1 shows higher expression level in liver cancer than in normal control group, moreover, down-regulation of CIAPIN1 can inhibit proliferation of liver cancer cell is also well-proved. Thus, CIAPIN1 may be recognized as a key player in liver cancers. CIAPIN1 has been proved to be relevant with many tumors, however, the biophysical function of CIAPIN1 is far from elucidated. Furthermore, researches are needed to confirm the role of CIAPIN1 in the molecular pathogenesis of liver cancer.

In order to make clear the underlying mechanism of the effect of CIAPIN1 on apoptosis of liver cancer cells, we conducted several researches on the CIAPIN1 expression. First, we successfully transfected siCIAPIN1 into HepG2 and Huh7 cells to interfere the expression of CIAPIN1. The results showed that suppression of CIAPIN1 expression notably increased the apoptosis of liver cancer cells. At the same time, its effect on the promotion of apoptosis of liver cancer cells disappears when the CIAPIN1 expressed in a normal level.

CIAPIN1 is a novel ant apoptotic molecule and plays a vitally important role in malignant phenotypes of cancers. Yang Z et al, proved that CIAPIN1 siRNA inhibits proliferation, migration and promotes apoptosis of VSMCs by regulating Bcl-2 and Bax [24]. Zhang YF et al, revealed that CIAPIN1 conferred the MDR phenotype in LoVo/Adr cells through up-regulating expression of MDR-1 (P-gp) and Bcl-xL [25]. Thus, the regulation of CIAPIN1 on apoptosis of liver cancer cells may have connection with the apoptosis proteins.

So, next, we introduced western blot assay, the analysis indicated that the relative density of BCL-2 and BAX were relevant with the expression of CIAPIN1. It can therefore be hypothesized that CIAPIN1 may induce apoptosis of liver cancer cells via activation of BCL-2 family proteins.

Conclusion

Taken together, our study found that suppression of CIAPIN1 expression could increase cell apoptosis in liver cancer cells. Moreover, the further research illustrated the underlying mechanism of liver cancer apoptosis. All these findings suggest that CIAPIN1 may be involved in progression of liver cancer and could be a new therapeutic target for this disease. This study provides research foundation for further understand the role of CIAPIN1 in liver cancer.

Disclosure of conflict of interest

None.

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