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Original Article

Overexpression of cytokine induced apoptosis inhibitor 1 inhibits cell proliferation and induces apoptosis in human MG-63 osteosarcoma cells

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Abstract: Osteosarcoma (OS) is the high-grade malignant tumor with low cure rates. We aimed to investigate the roles of cytokine induced apoptosis inhibitor 1 (CIAPIN1) in cell proliferation and apoptosis in human MG-63 OS cells. The human MG-63 OS cell line as an *in vitro* model was transfected with plasmid PCDNA3.1-CIAPIN1 and siRNA expression vector specifically targeting CIAPIN1 (siCIAPIN1) with Lipofectamine 2000 reagent. MTT assay and flow cytometer using annexin V and propidium iodide (PI) staining were then used to explore the proliferation and apoptosis of human MG-63 OS cells *in vitro*, respectively. The expression levels of CIAPIN1 mRNA and protein in different transfected cells were respectively determined with measured using qRT-PCR analysis and western bolt. Besides, the phosphorylation level of c-Jun N-terminal kinase (JNK) was also measured with western bolt. The expression level of CIAPIN1 was significantly increased in PCDNA3.1-CIAPIN1 transfected cells compared with blank group, and the elevated CIAPIN1 expression was markedly decreased after cells were transfected with both PCDNA3.1-CIAPIN1 and siCIAPIN1 simultaneously. Moreover, overexpression of CIAPIN1 significantly decreased cell viability and induced cell apoptosis in human OS MG63 cells, whereas down-regulation of CIAPIN1 by siCIAPIN-1counteracted these effects. Besides, the elevated phosphorylation level of JNK after PCDNA3.1-CIAPIN1 transfection markedly decreased after cells were transfected with both PCDNA3.1-CIAPIN1 and siCIAPIN1. Our findings indicate that CIAPIN1 may inhibit cell proliferation and induce cell apoptosis in OS development via affecting the phosphorylation level of JNK. CIAPIN1 may be used as a potent therapeutic target in OS.

Keywords: Osteosarcoma, cytokine induced apoptosis inhibitor 1, proliferation, apoptosis

Introduction

Osteosarcoma (OS) is the most common bone cancer and remains a leading cause of cancer death in children and adolescents [1, 2]. Unfortunately, substantial advances in treatment or survival has not been made in three decades [3]. Therefore, identification of key molecular mechanism underlying the OS development is still a hotspot in the research field of this disease.

Cytokine induced apoptosis inhibitor 1 (CIAPIN1, initially named anamorsin) is a newly identified anti-apoptotic molecule [4] that may play a vital role in malignant phenotypes of a variety of cancers. For instance, CIAPIN1 has been considered as a potential tumor suppressor that can inhibit the growth and proliferation of multiple myeloma [5]. In addition, overexpression of CIAPIN1 can also inhibit the proliferation of pancreatic cancer cells and consequently result in good prognosis in pancreatic cancer [6]. Down-regulation of CIAPIN1 is also reported to reverse multidrug resistance in human breast cancer cells through inhibiting MDR1 gene [7]. Besides, nuclear accumulation of CIAPIN1 is also associated with poor clinical outcome in epithelial ovarian cancer [8]. CIAPIN1 is demonstrated to be a therapeutic target in a variety of cancers [9]. However, the important roles of CIAPIN1 in OS development have not yet been fully investigated.

Considering the key roles in malignant phenotypes of some cancers, we used overexpressed and knocked down the CIAPIN1 expression in the human MG-63 OS cell line. MTT assay and

flow cytometer using annexin V and propidium iodide (PI) staining were then used to determine whether overexpression or down-regulation of CIAPIN affected the proliferation and apoptosis of human MG-63 OS cells *in vitro*, respectively. Besides, the phosphorylation level of c-Jun N-terminal kinase (JNK) was also measured to explore the potential regulatory mechanism of CIAPIN. The objective of our study was to determine the potential roles of CIAPIN in the proliferation and apoptosis of human MG-63 OS cells, as well as to elucidate the possible mechanisms underlying OS development.

Materials and methods

Cell culture

The human MG-63 OS cell line was obtained from Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. MG-63 OS cells was then grown in Eagle's minimum essential medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Welgene Ltd.) at 37°C with 5% CO_2 in a humidified incubator.

Plasmids and transfection

The full-length wild-type CIAPIN1 coding sequence was inserted into pcDNA3.1(+) to construct a CIAPIN1 expression vector (pcDNA3.1-CIAPIN1), which was confirmed by sequencing. In addition, the control siRNA (no silencing) and siRNA expression vector specifically targeting CIAPIN1 (siCIAPIN1) and were synthesized by GenePharma Co (Shanghai, China). Plasmids pcDNA3.1-CIAPIN1 and siCIAPIN1 were respectively transfected into MG 63 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The blank vector (pcDNA3.1) and control siRNA were transfected as a control.

MTT assay

Cell viability was determined using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MG63 cells (5×10^4 cells/mL) in logarithmic growth phase were cultured in a 96-well plate with 200 μL per well. After incubation for 24 h, 48, 72 h and 96 h, 20 μL of fresh medium with 0.5 mg/mL MTT 4 h was added to each well and continued to incubate for 4 h. After termination, 200 μL Dimethyl Sulfoxide (DMSO) was added to each well. The values for each well using 492 nm optical density (OD) were then measured with a microplate reader (BioTek, USA). The experiment was repeated for three times.

Flow cytometry for cell apoptosis analysis

After MG63 cells were transfected with CIAPIN1 for 48 h, the apoptosis analysis was assayed by annexin V and propidium iodide (PI) staining (BD PharMingen, San Diego, CA, USA) following the manufacturer's instructions. Briefly, MG63 cells with different transfected treatment were suspended in Binding Buffer and stained with 5 μL annexin V-FITC and 5 μL PI for 15 min in the dark. The cells were then analyzed with a FACS Calibur flow cytometer (Becton-Dickinson). The percentage of apoptosis cells was defined as the sum of the apoptosis cells in the early stage (annexin V positive/PI negative) and late stage (annexin V positive/PI positive).

qRT-PCR analysis

Total mRNA was extracted from cells using Trizol reagent (Invitrogen, Burlington, ON, Canada) following the manufacturer's recommended protocol. Complementary DNA (cDNA) was reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA). The expression levels of CIAPIN1 mRNA were measured by SYBR green-based qRT-PCR. SYBR Green Master mix was purchased from Thermo Scientific (Waltham, MA, USA). The primers for CIAPIN1 amplification were: forward, 5'-CGGAATTCATGGCAGATTTTGGGATCTC-3'; reverse, 5'-GGTCGACCTAGGCATCAAGATTGCTATC-3'. β-actin was used as the loading control. The expression level of CIAPIN1 mRNA was calculated using the comparative threshold (Ct) cycle ($2^{-\Delta\Delta Ct}$) method.

Western blot analysis

Total protein extracts were then obtained from cells by radioimmunoprecipitation assay (RIPA) buffer on ice. The protein concentration was then measured using bicinchorinic acid assay (BCA). Afterwards, the same concentration of protein samples was loaded on per lane, separated on a 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to polyvinylidene difluoride membranes. After being bl-

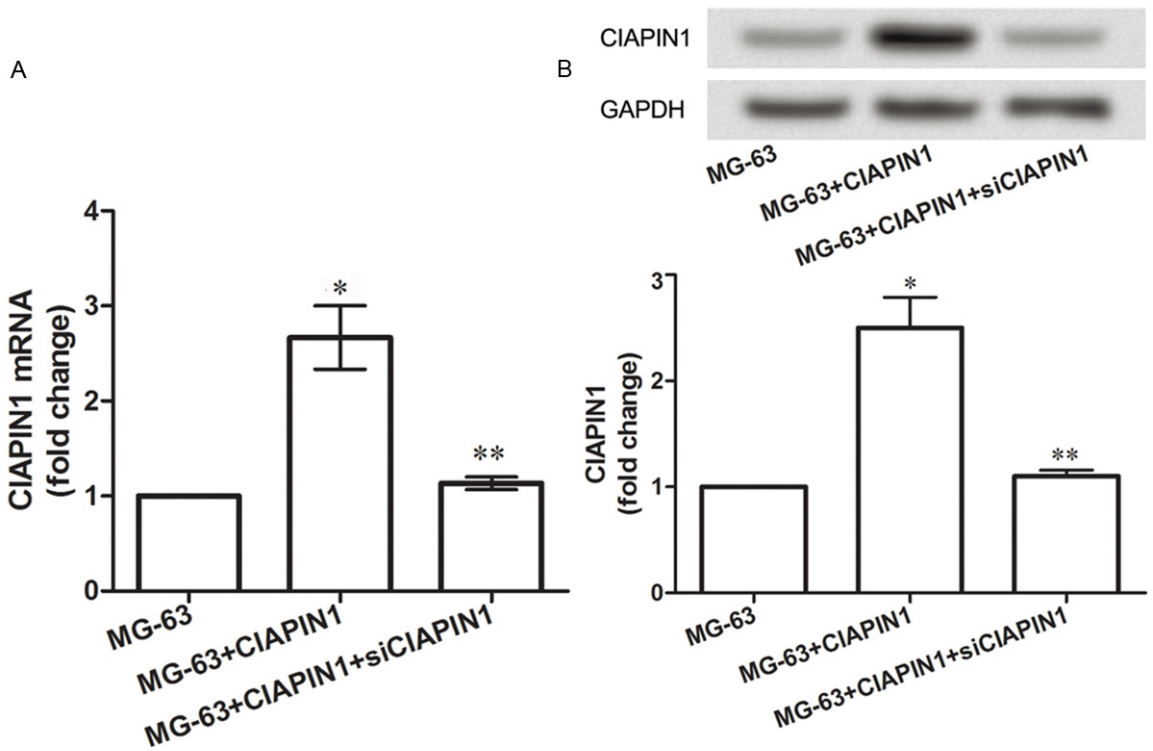

Figure 1. The expression of CIAPIN1 at mRNA and protein levels was respectively determined by qRT-PCR (A) and western blot analysis (B). Error bars indicate means ± SD. *Indicates significant difference compared with blank group ($P < 0.05$) and **indicates significant difference compared with PCDNA3.1-CIAPIN1 transfected group.

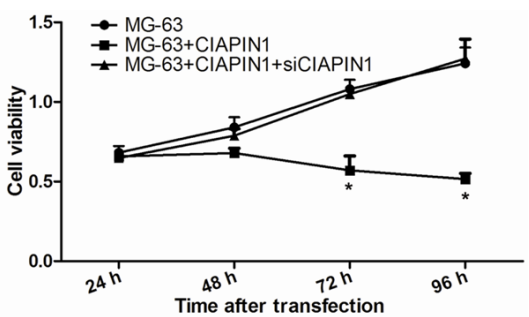

Figure 2. MTT assay displayed the cell viability of different transfected cells. Error bars indicate means ± SD. *Indicates significant difference compared with blank group ($P < 0.05$).

ocked in PBST, the membranes were probed with primary antibodies against CIAPIN1 as prepared by Hao et al. [10], JNK and p-JNK (Santa Cruz, USA) overnight at 4°C. The membranes were subsequently washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence was performed to visualize the immunoreactive protein bands. GADPH was as an internal standard to normalize loading protein. The immunoreactive bands were analyzed by NIH Imaging software and a densitometer.

Statistical analysis

All data obtained from multiple experiments were presented as the mean ± SD. Statistical analyses were then performed using a one-way analysis of variance (ANOVA) in SPSS 19.0 statistical software. A *p*-value of < 0.05 was considered statistically significant.

Results

CIAPIN1 was successfully overexpressed and knocked down

The expression of CIAPIN1 at mRNA and protein levels was respectively determined by qRT-PCR and western blot analysis. As shown in **Figure 1**, the expression level of CIAPIN1 was significantly increased in PCDNA3.1-CIAPIN1 transfected cells compared with blank group ($P < 0.05$), indicating that CIAPIN1 was successfully overexpressed in PCDNA3.1-CIAPIN1 transfected group. Moreover, compared with PCDNA3.1-CIAPIN1 transfected group, the elevated CIA-

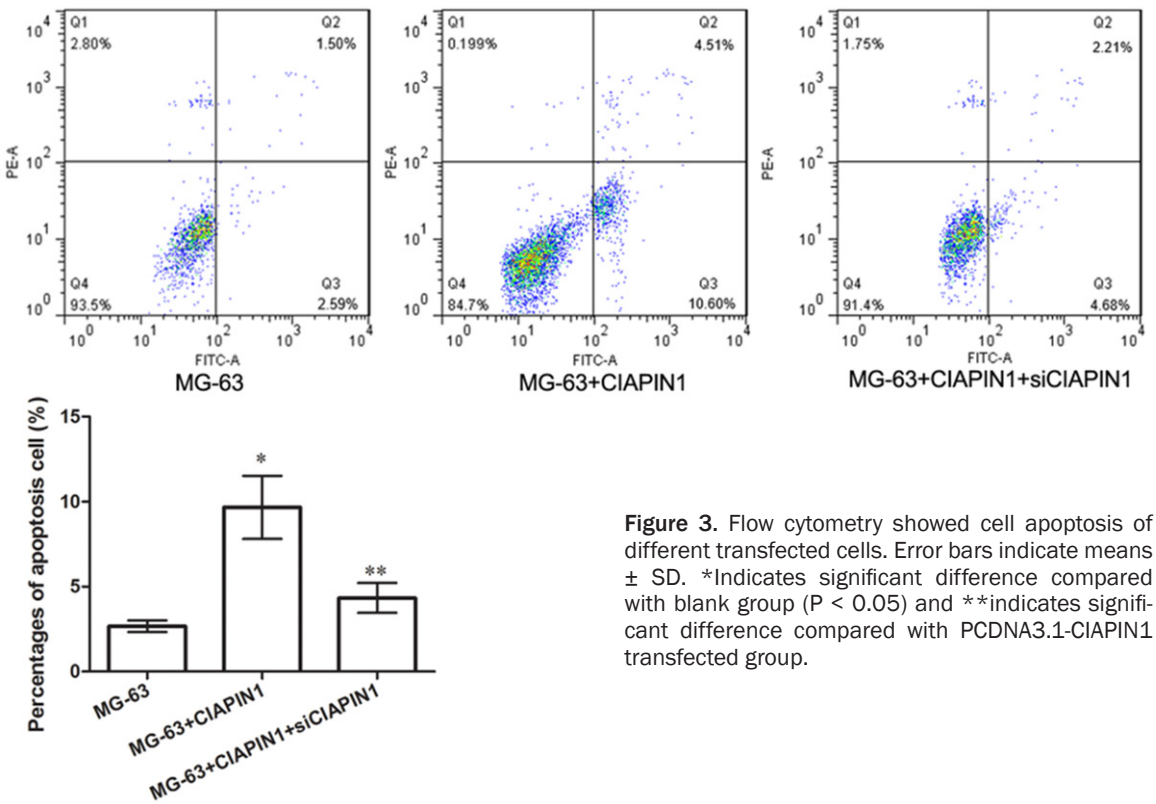

Figure 3. Flow cytometry showed cell apoptosis of different transfected cells. Error bars indicate means ± SD. *Indicates significant difference compared with blank group ($P < 0.05$) and **indicates significant difference compared with PCDNA3.1-CIAPIN1 transfected group.

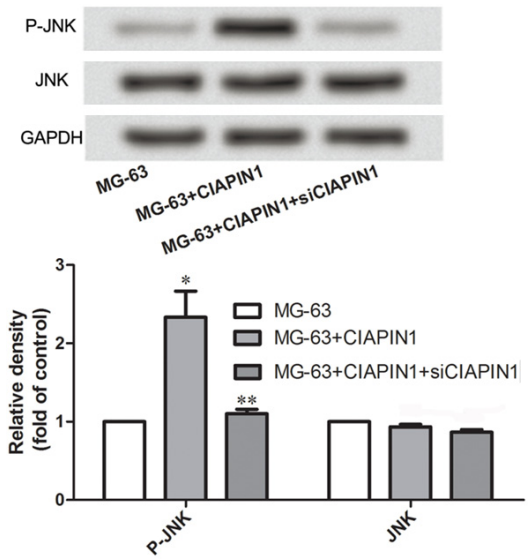

Figure 4. Western blot analysis showed the phosphorylation level of JNK in different transfected cells. Error bars indicate means ± SD. *Indicates significant difference compared with blank group ($P < 0.05$) and **indicates significant difference compared with PCDNA3.1-CIAPIN1 transfected group.

PIN1 expression was markedly decreased after cells were transfected with both PCDNA3.1-CIAPIN1 and siCIAPIN1 simultaneously ($P < 0.05$), indicating that CIAPIN1 expression was successfully knocked down by siCIAPIN1.

Overexpression of CIAPIN1 decreased cell viability in human MG-63 OS cells

MTT assay displayed the cell viability of different transfected cells. As shown in **Figure 2**, cell viability of PCDNA3.1-CIAPIN1 transfected cells significantly decreased compared with that of PCDNA3.1-CIAPIN1 and siCIAPIN1 transfected cells ($P < 0.05$), indicating that overexpression of CIAPIN1 significantly decreased cell viability.

Overexpression of CIAPIN1 induced cell apoptosis in human MG63 OS cells

Flow cytometry was used for cell apoptosis analysis. As shown in **Figure 3**, apoptosis percentage was significantly increased after PCDNA3.1-CIAPIN1 transfection and significantly decreased after cells were transfected with both PCDNA3.1-CIAPIN1 and siCIAPIN1 ($P < 0.05$).

The phosphorylation level of JNK was increased after CIAPIN1 overexpression

To explore the potential regulatory mechanism of CIAPIN1 expression in human MG63 OS

cells, the phosphorylation level of JNK was measured. We found that JNK expression had no changed after CIAPIN1 overexpression, while the phosphorylation level of JNK significantly increased ($P < 0.05$, **Figure 4**), implying that CIAPIN1 may play role in OS development via affecting the phosphorylation level of JNK.

Discussion

To data, treatment strategies for OS mainly target the primary tumor rather than metastases, leading to a limited efficacy in the treatment of this disease [11]. CIAPIN1 is recently recognized as a therapeutic target in various cancers. However, the role of CIAPIN1 in the molecular pathogenesis of OS has not been elucidated fully. In the present study, CIAPIN1 was successfully overexpressed and knocked down in human OS MG63 cells. We then found that overexpression of CIAPIN1 significantly decreased cell viability and induced cell apoptosis in human OS MG63 cells, whereas down-regulation of CIAPIN1 counteracted these effects. Besides, the elevated phosphorylation level of JNK after PCDNA3.1-CIAPIN1 transfection markedly decreased after cells were transfected with both PCDNA3.1-CIAPIN1 and siCIAPIN1. All these findings merit further discussion.

CIAPIN1 has been found to be implicated in the process of cell proliferation in a variety of cancers. The expression of CIAPIN1 is reported to be negatively related to cell proliferation in human colorectal cancer [12]. He et al. also demonstrated that up-regulating CIAPIN1 exhibited significant cell growth inhibition via inhibition of cell cycle-related proteins, such as cyclinD1, cyclinE, cdk2 and cdk4 [13]. Down-regulation of CIAPIN1 promoting cell proliferation and cell cycle progression, thus leading to gastric carcinogenesis [14]. Similar with previous findings, our results showed overexpression of CIAPIN1 significantly decreased cell viability, whereas down-regulation of CIAPIN1 counteracted these effects. It is thus intriguing to speculate that CIAPIN1 may play a key inhibitory role in OS development via inhibiting cell proliferation.

As another aspect of the present analysis, our results verified that overexpression of CIAPIN1 significantly induced cell apoptosis, whereas down-regulation of CIAPIN1 counteracted these effects. Cell apoptosis is a key mechanism involved in tumor development. CIAPIN1 is found to mediate multidrug resistance in leukemia cells through regulating Bcl-2 and Bax [15]. The BCL-2 protein family determines and controls the commitment of cells to apoptosis [16]. Bax, a Bcl-2 family member, is also proved to participate in cell apoptosis through activation by p53 [17, 18]. Bcl-2 inhibitor and Bax activator are promising approaches for cancer therapy [19, 20], implying that co-targeting CIAPIN1 with Bcl2 and/or Bax may have application prospects in cancer therapy. Moreover, down-regulation of CIAPIN1 triggered more apoptosis of K562 chronic myeloid leukemia cells with or without Imatinib treatment [21]. Besides, it has been reported that CIAPIN1 is a downstream mediator of the RAS signaling pathway [22]. Yu et al. confirmed that Ras/Raf/MEK/ERK pathway was associated with OS lung metastasis and suggested that targeting this pathway may have a potential use in the effective treatment of OS [23]. In view of the key role of CIAPIN1 in cell apoptosis, we speculate that CIAPIN1 may induce cell apoptosis to inhibit OS development.

Besides, the phosphorylation level of JNK was measured to explore the potential regulatory mechanism of CIAPIN1 in OS development. A novel berbamine derivative BBMD3 is found to increase phosphorylation of JNK, thus to induce apoptosis of chemotherapy-resistant human OS cells [24]. Yao et al. suggested that the roles of nephroblastoma overexpressed (NOV) gene in inhibiting proliferation and promoting apoptosis in OS cell lines were played through p38/MAPK and JNK/MAPK pathways [25]. Li et al. reported that celastrol induced apoptosis and autophagy in human OS cells via the ROS/JNK signaling pathway [26]. In our study, we found that JNK expression had no changed after CIAPIN1 overexpression, while the phosphorylation level of JNK significantly increased. Although the relationships of CIAPIN1 and the phosphorylation level of JNK have not been fully investigated, it can therefore be hypothesized that CIAPIN1 may play role in OS development via affecting the phosphorylation level of JNK.

Taken together, our findings indicate that CIAPIN1 may inhibit cell proliferation and induce cell apoptosis in OS development via affecting the phosphorylation level of JNK.

CIAPIN1 may be used as a potent therapeutic target in OS. However, only one cell line (human MG 63 OS cell line) was used to verify the role of CIAPIN1 in cell viability and apoptosis *in vitro*. Additionally, experimental validation *in vivo* was not performed in our study. Further studies are still needed to verify our findings and speculations.

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Disclosure of conflict of interest

None.

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