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Original Article

Down-regulation of KIAP promotes cyclophosphamide-induced apoptosis of hepatoma HEPG2 cells

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Abstract: Cyclophosphamide is a commonly used drug in clinical chemotherapy. However, the molecular mechanism of how cyclophosphamide inhibits and kills liver cancer cells is unclear. Induction of apoptosis is used to treat cancer. KIAP is an apoptosis inhibitory protein. Whether cyclophosphamide inhibits and kills liver cancer cells by regulating KIAP level is not clear. This study intends to investigate how cyclophosphamide regulates the growth and apoptosis of hepatoma HEPG2 cells. Cyclophosphamide was used to treat HEPG2 cells 48 h at a concentration of 2 μmol/L, MTT assay and flow cytometry were used to detect the proliferation and apoptosis of HEPG2 cells. Western blot was used to detect KIAP levels. After silence of KIAP by siRNA, cyclophosphamide (2 μmol/L) was used to treated HEPG2 cells, and cell apoptosis was detected. Cyclophosphamide (2 μmol/L) inhibited growth of HEPG2 cells and induced apoptosis of HEPG2 cells. Cyclophosphamide (2 μmol/L) also caused down-regulation of KIAP levels in HEPG2 cells. Silence of KIAP by siRNA significantly enhanced cyclophosphamide-induced apoptosis of HEPG2 cells. Down-regulation of KIAP enhanced cyclophosphamide-induced apoptosis of hepatoma HEPG2 cells.

Keywords: Cyclophosphamide, KIAP, hepatoma HEPG2 cells, apoptosis

Introduction

Hepatocellular carcinoma (HCC) is one of the human digestive system cancers [1, 2]. The molecular mechanism of occurrence and development of liver cancer has yet to be elucidated.

HCC threaten the lives of patients [3, 4]. Most of liver cancer patients were treatment with surgery [5, 6]. Hepatic artery chemoembolization was used for patients with unresectable tumors [7, 8]. The vast majority of patients had no surgical indications and depended on chemotherapy treatment [9, 10]. Cyclophosphamide is one of the commonly used chemotherapy drugs which play a key role in the treatment of cancers including colorectal cancer, leukemia, myeloma, breast cancer, and prostate cancer. Cyclophosphamide can alleviate the suffering of cancer patients, improve the patient's five-year survival rate, reduce mortality, and improve prognosis. Further studies have shown that anti-tumor effect of cyclophosphamide is mainly through the induction of apoptosis. However, how cyclophosphamide regulates the expression of apoptotic proteins and induces apoptosis remain to be elucidated [11, 12].

KIAP is a protein kinase which plays a role in apoptosis [13], programmed necrosis [14], autophagy [15], and NF-κB signal transduction [16]. Studies have shown that knockdown of KIAP by siRNA enhanced cyclophosphamide-induced apoptosis of hepatocellular carcinoma HEPG2 cells [17]. However, the molecular mechanism of cyclophosphamide induction of apoptosis of cancer cells is unclear. This study designed to investigate the regulation of cyclophosphamide on the growth and death of hepatoma HEPG2 cells.

Materials and methods

Reagents and consumables

MTT reagents were purchased from Sigma. Apoptosis detection reagents including specific dye for mitochondrial membrane potential, tetra-

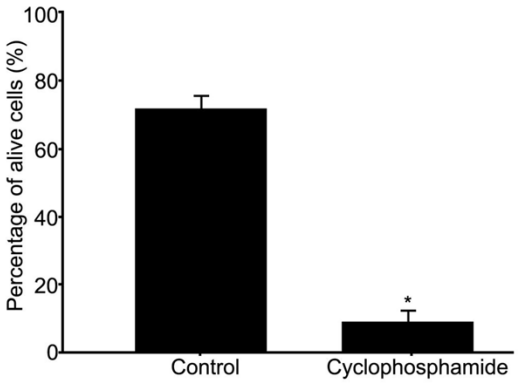


Figure 1. Cyclophosphamide (2 μmol/L, 24 h) inhibits the growth of HEPG2 cells. *Compared to control, $P < 0.05$.

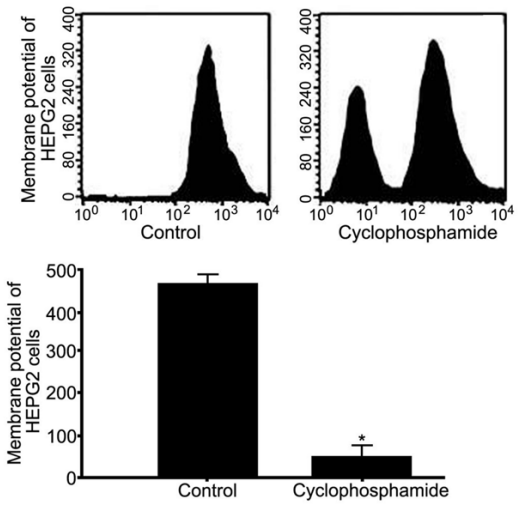


Figure 2. Cyclophosphamide treatment (2 μmol/L, 24 h) reduced membrane potential of HEPG2 cells. *Compared to control, $P < 0.05$.

methylrhodamineethylester, were purchased from Biyuntian Institute of Biotechnology.

Rabbit anti-human KIAP antibody was purchased from Sigma. siRNA of KIAP was synthesized by Shanghai Jima biotechnology company. Sequences of siRNA of KIAP and control siRNA were as follows: 5'GATTGGTTCGATGATCGT3', and 5'AATCATCATGAATAGGGT3'.

Liposomal transfection reagent Escort™ was purchased from Sigma. Other reagents were purchased from Beijing Dingguo Biotech.

Cell culture

HEPG2 cells were purchased from ATCC and cultured according to manufacturer's instruction. 2 μmol/L cyclophosphamide or DMSO was added to culture medium in cyclophosphamide group or control group in this study.

Transfection

Cells were seeded. The siRNA and liposome were mixed and transfected cells for subsequent experiments [7].

Flow cytometry

Membrane potential dye TMRE and phosphatidylserine specific dye FITC-Annexin-V were used to stain cells and detect the apoptosis of cells in each group according to previous publication [18, 19]. Briefly, cells were harvested and mixed with Annexin-V-FITC and measured at wavelength of 625 nm. For detection of mitochondrial membrane potential, the cells were resuspended in PBS, mixed with 10 μmol/L of TMRE, and incubated in dark at room temperature for 20 min and measured by flow cytometry.

MTT test

MTT test was used to measure survival and growth of cells in each group [20]. Briefly, cells were harvested and mixed with MTT. Absorbance was measured and recorded.

Detection of caspase-3 activity

Cells were collected, and activity of caspase-3 was measured by ELISA [21]. Briefly, cells were lysed on ice for 10 min, the lysate was loaded into a 96-well plate, mixed with chromogenic substrate, incubated for 5 min, and absorbance was recorded.

Western blot

HEPG2 cells were lysed, resolved by SDS-PAGE, and transferred to PVDF membranes. Protein levels of KIAP and Actin were measured [11].

TUNEL assay

The cells were treated by 1 μl TdT, 1 μl biotin labelled dUTP, and 98 μl equilibrium liquid. Next, the cells were mixed with 100 μl TUNEL reaction liquid and incubated in wet box at 37°C for 1 h avoid of light. Then the reaction was stopped at 2 × SCC for 15 min and the plate was washed by PBS for three times. The cells were further incubated in 0.3% H_2O_2 for 15 min and treated by 100 μl streptavidin

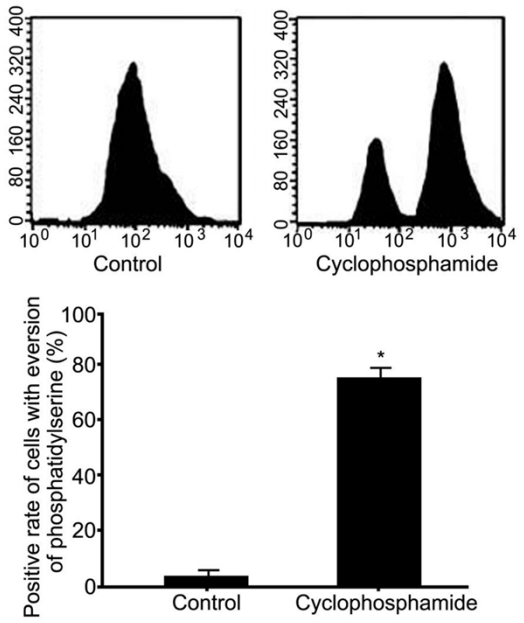


Figure 3. Cyclophosphamide (2 μmol/L, 24 h) induced eversion of phosphatidylserine in hepatoma HEPG2 cells. *Compared to control, P < 0.05.

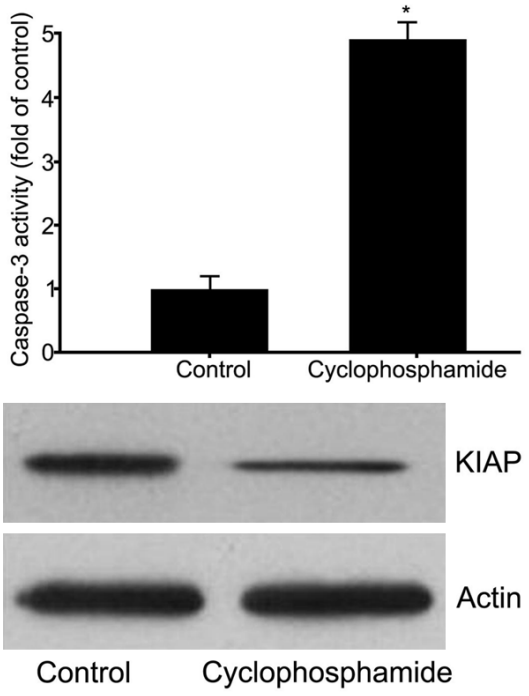


Figure 4. Cyclophosphamide treatment (2 μmol/L, 24 h) induced caspase-3 activity in hepatoma HEPG2 cells. *Compared to control, P < 0.05.

labelled HRP (1:500) for 30 min. At last, the cells were added with 100 μl DAB mixture containing 50 μl DAB, 50 μl DAB substrate buffer solution, 50 μl H_2O 220 ×, and 950 μl ddH_2O for 10 min and observed under the microscope.

Statistical analysis

SPSS 18.0 was used for statistical analysis. All data are expressed as mean ± standard deviation. One-way ANOVA was used to do comparisons between groups. P < 0.05 was considered statistically significant.

Results

Cyclophosphamide inhibits the growth of HEPG2 cells

Effect of cyclophosphamide on the survival and growth of HEPG2 cell was measured. As shown in **Figure 1**, cyclophosphamide treatment significantly inhibited the growth of HEPG2 cells, suggesting that cyclophosphamide affected the growth of hepatocellular carcinoma HEPG2 cells.

Cyclophosphamide treatment reduced membrane potential of HEPG2 cells

Mitochondrial membrane potential of HEPG2 cells treated with cyclophosphamide was measured. Results (**Figure 2**) showed that cyclophosphamide treatment significantly lowered mitochondrial membrane potential of HEPG2 cells, suggesting that cyclophosphamide may cause apoptosis of hepatoma HEPG2 cells.

Cyclophosphamide induced apoptosis of hepatoma HEPG2 cells

As shown in **Figure 3**, cyclophosphamide treatment significantly induced the eversion of phosphatidylserine in hepatoma HEPG2 cells, suggesting that cyclophosphamide treatment caused apoptosis of hepatoma HEPG2 cells.

Cyclophosphamide treatment induced caspase-3 activity in hepatoma HEPG2 cells

As shown in **Figure 4**, cyclophosphamide treatment caused caspase-3 activation in hepatoma HEPG2 cells, suggesting that cyclophosphamide induced apoptosis of hepatoma HEPG2 cells.

Cyclophosphamide treatment down-regulated KIAP expression in hepatoma HEPG2 cells

Expression levels of KIAP in hepatoma HEPG2 cells treated with cyclophosphamide were mea-

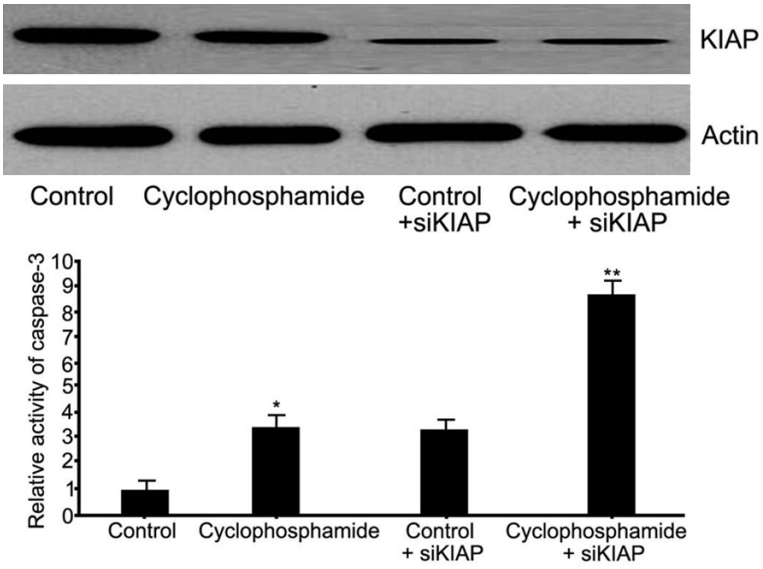


Figure 5. Cyclophosphamide treatment (2 μmol/L, 24 h) down-regulated KIAP expression in hepatoma HEPG2 cells.

sured by Western blot analysis. Results (**Figure 5**) showed that cyclophosphamide treatment significantly inhibited the expression level of KIAP in hepatoma HEPG2 cells.

Down-regulation of KIAP promoted cyclophosphamide-induced apoptosis of HEPG2 cells

As shown in **Figures 6** and **7**, transfection of siRNA reduced expression levels of KIAP. Down-regulation of KIAP enhanced cyclophosphamide-induced activation of caspase-3. These results suggested that down-regulation of KIAP enhanced cyclophosphamide-induced apoptosis of hepatoma HEPG2 cells.

Discussion

Cyclophosphamide is a clinically important anticancer drug [22]. Studies have shown that use of siRNA knockdown KIAP by siRNA enhanced cyclophosphamide-induced apoptosis of h HEPG2 cells [6]. In this study, the effect of cyclophosphamide on growth and apoptosis of hepatoma HEPG2 cells were investigated.

Our results showed that cyclophosphamide treatment inhibited the growth of HEPG2 cells, which was consistent with previous studies. Flow cytometry results showed that cyclophosphamide treatment caused decrease of mitochondrial membrane potential and apoptosis of hepatoma HEPG2 cells, suggesting that cyclophosphamide treatment resulted in the apoptosis of hepatoma HEPG2 cells, which was consistent with other studies [23, 24].

However, a variety of studies have shown that under the same concentrations of cyclophosphamide (2 μmol/L), hepatoma HEPG2 cells are more sensitive to cyclophosphamide than oral cancer cells, and hepatoma HEPG2 cells showed higher apoptosis level that oral cancer cells. This might be caused by different sensitivity of cells [15-17].

There are two main pathways of apoptosis, one is extrinsic apoptotic signaling pathways mediated by receptors [25, 26], the other is the intrinsic mitochondria-mediated signaling pathway [27, 28]. The signaling pathway of cyclophosphamide-induced apoptosis of HEPG2 cells was also discussed.

Apoptosis is achieved through different caspases. Extrinsic apoptotic pathway caused the activation of caspase-8, while the intrinsic mitochondrial pathway mainly causes the activation of caspase-3/7. Our results showed that cyclophosphamide induced the activation of caspase-3 other than the activation of caspase-8, suggesting that cyclophosphamide-induced apoptosis of HEPG2 cells was mediated by intrinsic mitochondrial pathway, which was consistent with previous studies [18, 20].

KIAP inhibits apoptosis through inhibition of NF-κB and caspase. Silencing of KIAP by siRNA enhanced cyclophosphamide-induced apoptosis of hepatoma HEPG2 cells [3]. The results of this study showed that cyclophosphamide-induced apoptosis of hepatoma HEPG2 cells was significantly increased by silence of KIAP using siRNA, suggesting that down-regulation of KIAP increased the sensitivity of hepatoma HEPG2 cells to cyclophosphamide.

In the future study, clinical liver cancer specimens of different stages will be collected to measure the apoptosis and KIAP level. Different

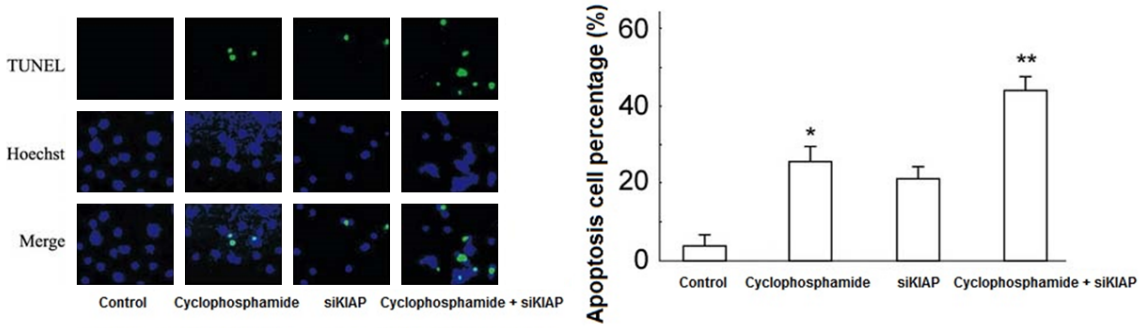
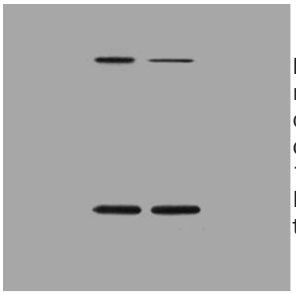


Figure 6. Down-regulation of KIAP promoted cyclophosphamide-induced apoptosis of HEPG2 cells. *Compared to control, P < 0.05, **Compared to control, P < 0.01.



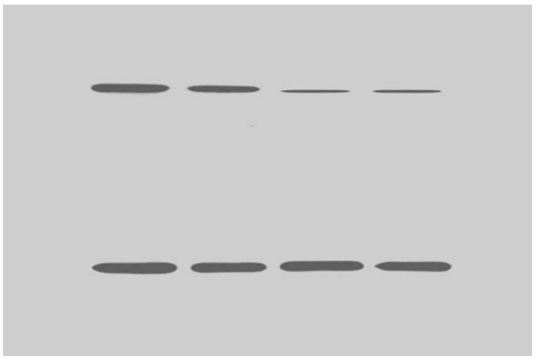


Figure 7. Cyclophosphamide induced HEPG2 cell apoptosis via inducing KIAP activation. *Compared to control, P < 0.05, **Compared to control, P < 0.01.

cyclophosphamide derivatives will be used to clarify the target and mechanism of cyclophosphamide. KIAP knockout mice should also be used to verify the results of this study. An animal model of liver cancer should be established to elaborate the relationship between KIAP and cyclophosphamide in the treatment of liver cancer.

In short, down-regulation of KIAP enhanced cyclophosphamide-induced apoptosis of hepatoma HEPG2 cells.

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Disclosure of conflict of interest

None.

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