RESEARCH COMMUNICATION

Efficient Killing Effect of Osteosarcoma Cells by Cinobufacini and Cisplatin in Combination

Tao Huang[1]*, Wei-Hua Gong[2], Xiu-Cheng Li[1], Chun-Ping Zou[1], Guang-Jian Jiang[2], Xu-Hui Li[2], Hao Qian[1]

Abstract

Purpose: To study the killing effects on osteosarcoma cells of cinobufacini and cisplatin in combination and the related mechanisms so as to explore the chemotherapeutic method with integrated traditional Chinese and Western medicines. Methods: Cinobufacini and cisplatin were applied to OS732 cells singly or jointly and survival rates were measured by MTT assay. Changes in cellular shape were observed with inverted phase contrast and fluorescence microscopy and apoptosis rates were analyzed with flow cytometry (FCM). Immunocytochemistry were used to examine the Fas expression of OS732 cells. Results: The combination of cinobufacini and cisplatin had the effect of up-regulating Fas expression and inducing apoptosis. The survival rate of combined application of 100 μg/ml cinobufacini and 1 μg/ml cisplatin on OS-732 cells was significantly lower than with either of the agents alone ($p<0.01$). Changes in cellular shape and apoptotic rates also indicated the apoptosis-inducing effects of combined application were much enhanced. Conclusion: The combination of cinobufacini and cisplatin demonstrated strong killing effects on OS-732 cells which might be related to up-regulation of Fas expression.

Keywords: Osteosarcoma - cinobufacini - cisplatin - combined treatment - apoptosis

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Introduction

Osteosarcoma is the most common primary tumor of bone, and early pulmonary metastasis is liable to occur with bad prognosis (Bhurgri et al., 2009; Dotan et al., 2010; Broadhead et al., 2011; Limmahakhun et al., 2011). Although the chemotherapeutic agents like cisplatin has obvious killing effect on osteosarcoma cells, the resistance and toxic side effects after long-term application remain the main obstacle for clinical doctors. Cinobufacini, an aqueous extract from the skins of Bufo bufo gargarizans Cantor, is a well-known traditional Chinese medicine widely used in clinical cancer therapy in China and some Asian pacific countries (Cui et al., 2010; Wang et al., 2011; Chen et al., 2012). Currently, it has been proved clinically that cinobufacini may enhance the anti-tumor effect as well as reduce the doses of chemotherapeutic agents. However, there has been few reports about the application of cinobufacini in osteosarcoma chemotherapy (Li et al., 2006; Qi et al., 2008; Wang et al., 2009), therefore, we used cinobufacini and cisplatin respectively or jointly on OS732 cells so as to explore whether cinobufacini could induce cellular apoptosis and we also examined whether the effect was related with up-regulating the expression of Fas on osteosarcoma cells so as to analyze its anti-tumor mechanism and provide theoretic evidence for the integrated traditional Chinese and western medicine treatment of osteosarcoma.

Materials and Methods

The osteosarcoma OS732 cell line was bought from Beijing Jishuitan hospital. RPMI-1640 powder were from the Gibco company. Trypsin, MTT were from Huamei biological company. Cinobufacini injection was provided by Anhui Jinchan Biochemical Co., Ltd. and cisplatin by Qilu pharmaceutical factory. Multi-clone antibody of Fas was bought from Fuzhou Maixin biological company.

Cell culture and research methods

The OS732 cell line was placed in the 1640 culture solution with 10% fetal bovine serum and cultured in incubator at 37°C in a humidified 5% CO_2 atmosphere. The cells that entered the logarithmic growth period were selected for experiment.

We selected the concentrations of 1 μg/ml, 10 μg/ml, 100 μg/ml and 1000 μg/ml for the cinobufacini group, the concentrations of 1 μg/ml, 5 μg/ml, 10 μg/ml and 100 μg/ml for the cisplatin group, and 100 μg/ml cinobufacini with 1 ug/ml cisplatin for the combined group, meanwhile setting an additional PBS blank control group. The experiment times were as follows : 24 h, 48 h, 72 h, after the treatment with different drugs and all trials were repeated three times.

*[1]Department of Orthopedics, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning, China, [2]Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, USA *For correspondence: huangtao@mail.cmu.edu.cn*

Measurement of the survival rates of tumor cells with MTT method

5×10^5/ml of cells were seeded in the 96-well plate with 200 ul each well, and added to the culture medium containing agents of different concentrations or control PBS with 100 ul each well, each concentration for parallel 4 wells after adherence. After culture for 24 h, newly made-up 20 μl MTT was added, and continued to incubate at 37 °C for 4 h, then the supernatant was discarded and dissolved in 150 μl DMSO. The absorptance was measured at 540 nm wavelength after mixed. Survival rate of tumor cells (%) = experimental group A value/control group A value × 100%.

Observation of the morphology of apoptotic cells

The morphology, number and adherence of tumor cells were directly observed with inverted phase contrast microscope. A cover slide was placed in the 6-well plate, and after the apoptosis of cells, they were fixed for 10min, stained with 0.5 ml Hoechst33258 staining solution for 5 min, and then camera-imaged with fluorescence microscope on the object slide covered by cover slide and dropped with anti-fading solution.

Measurement of the proportion of apoptotic cells with flow cytometer(FCM)

The cells were added with 70% cold ethanol to fix for 48 h. After centrifugation with supernatant discarded, they were added with 10 g/l RNase200 μl, water bathed at 37 °C for 30 min, washed out RNase, and stained in darkness with 100 ulPI staining solution at 4 °C for 1 h. The fluorescence strength was measured with FACScan flow cytometer. The wavelength of activated light was 488 nm, and the apoptotic rates were measured with CellQuest analysis software.

Immunocytochemistry to detect Fas expression of OS732 cells

2×10^5/ml of digestive cells were placed in a 6-well culture plate with pre-treated cover slide in each well. cultured for 24 h and then supernatant were discarded, added with medicine, continue to culture for 24 hours ,meanwhile set blank control group,The cover slide were removed, fixed with acetone at 4 °C for 10 min and stained with SP method according to manual. The brown yellow cytoplasm indicated positive, and the expression intensity of Fas was inversely determined by the average gray value obtained with image analysis system, which means the more is the average gray value, the less is the Fas level.

Statistic method

Experimental data were given as means±standarad deviations (SDs), compared between different groups by t-test with WindowsSPSS13.0 software.

Results

Changes of survival rates of tumor cells

When the concentration of cinobufacini was 1 μg/ml, 10 ug/ml, 100 μg/ml and 1000 μg/ml, the cell growth was inhibited in a dose-dependent manner. There were significant differences between different groups (P<0.05) (Figure 1A). Similarly, when the concentration of cisplatin was 1 μg/ml, 5 μg/ml, 10 μg/ml and 100 μg/ml, the cell growth was also suppressed between different groups (P<0.05) (Figure 1B). With the combination of 100 μg/ml cinobufacini and 5 μg/ml cisplatin, the survival rate was significantly lower (P<0.01) (Figure 1C), compared with the respective use of 100 μg/ml cinobufacini or 1 μg/ml cisplatin, showing that the combined use of cinobufacini and cisplatin may have stronger inhibition effect than the single agent. More importantly, we found that cell growth was inhibited in a time-denpendent manner since the survival rate of OS732 cells were the lowest at 72 h and the highest at 24 h.

Morphological changes of apoptosis of OS-732 cells

Under the inverted phase contrast microscope, The

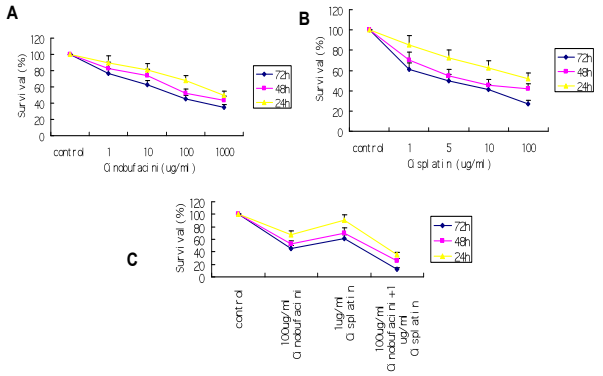

Figure 1. The Inhibition Effect on OS732 after 24h, 48h, 72h Measured by MTT. (A) The survival rate of OS732 with different concentration of cinobufacini. (B) The survival rate of OS732 with different concentration of cisplatin. (C) The survival rate of OS732 with combination of 100 μg/ml cinobufacini and 1 μg /ml cisplatin

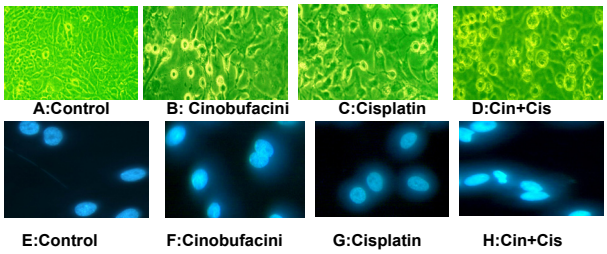

Figure 2. Morphological Changes and Fluorescent Staining of OS732 Cells after 72h with Different Drugs. (A) Morphological appearance of OS732 under inverted phase contrast microscope ×400. (B) Morphological changes of OS732 treated with 100 μg /ml cinobufacini under inverted phase contrast microscope ×400. (C) Morphological changes of OS732 treated with 1 μg /ml cisplatin under inverted phase contrast microscope ×400. (D) Morphological changes of OS732 treated with combination of 100μg /ml cinobufacini and1μg/ml cisplatin under inverted phase contrast microscope × 400. (E) Fluorescent staining of OS732 cells under the fluorescence microscope ×400. (F) Fluorescent staining of OS732 cells treated with 100 μg/ml cinobufacini under the fluorescence microscope × 400 (G) Fluorescent staining of OS732 cells treated with 1μg /ml cisplatin under the fluorescence microscope × 400. (H) Fluorescent staining of OS732 cells treated with 100 μg/ml cinobufacini and 1 μg/ml cisplatin under the fluorescence microscope × 400

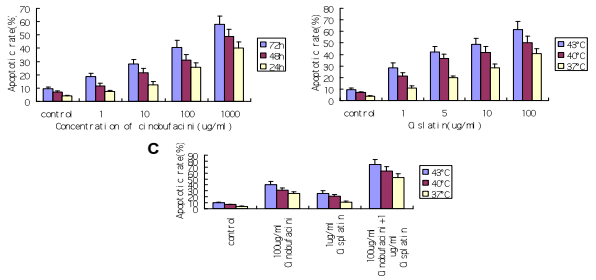

Figure 3. Apoptotic Effect of OS732 Cells after 24h, 48h, 72h Treated with Different Drugs. (A) Apoptotic rate of OS732 treated with different concentration of cinobufacini. (B) Apoptotic rate of OS732 treated with different concentration of cisplatin. (C) Apoptotic rate of OS732 treated with combination of 100 μg/ml cinobufacini and 1 μg/ml cisplatin

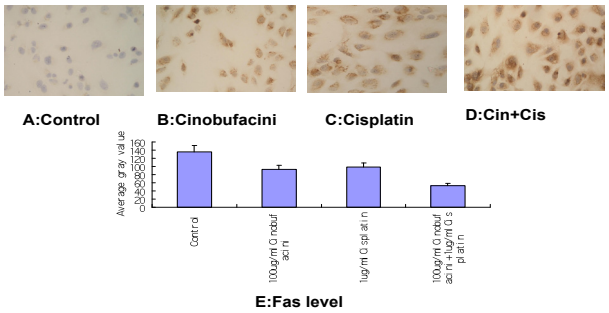

Figure 4. Fas Expression of OS732 Cells after 72h with Different Drugs. (A) Fas expression of OS732 by immunocytochemistry × 400. (B) Fas expression of OS732 treated with 100 μg/ml cinobufacini by immunocytochemistry ×400. (C) Fas expression of OS732 reated with 1 μg/ml cisplatin immunocytochemistry × 400. (D) Fas expression of OS732 treated with combination of 100 μg/ml cinobufacini l and 1 μg/ml cisplatin by immunocytochemistry × 400. (E) Quantitative analysis of Fas level by comparing the average gray value of different groups with Meta Morph automatic image analyzer

normal OS-732 cells were attached to the dish, the cells were rhombus-like and angular (Figure 2A). with individual application of cinobufacini (100 μg/ml) or cisplatin (1μg/ml), only part of the cells became small and round (Figure 2B, 2C), However, with the combined application, chromatin and cytoplasm were condensed, and many cells became nonadherent and suspended in the culture medium (Figure 2D). Under fluorescence microscope, OS cells were lightly-stained (Figure 2E), whereas with individual application of cinobufacini (100 μg /ml) or cisplatin (1 μg/ml) , only only a few cells showed condensed and flared fluorescence (Figure 2F, 2G). With the combination of cinobufacini (100 μg/ml) and cisplatin (1 μg/ml), obvious condensed and flared fluorescence was observed, revealing the presence of many apoptotic cells (Figure 2H).

Comparison of apoptotic rates of OS-732 cells

After the treatment of the cells for 24h, 48h and 72h, with various concentration of cinobufacini and cisplatin, the apoptotic rate increased in a dosage-dependent manner. There were significant differences between the different groups (P<0.05) (Figure 3A,3B). The apoptotic rate of the combination of cinobufacini (100 μg/ml) and cisplatin (1 μg/ml) is significantly higher than the individual application of cinobufacini (100 μg/ml) or cisplatin (1 μg/ml (P<0.01) (Figure 3C).

Fas expression of OS732 by Immunocytochemistry

We observed only a small amount of brown particles in the cytoplasm of OS732 cells in the control group (Figure 4A). Deeper staining cytoplasm of OS732 cells with 100 μg/ml cinobufacini (Figure 4B) or 1 μg/ml cisplatin (Figure 4C); With the combination of 100 μg/ml cinobufacini and 1 μg /ml cisplatin, Deepest staining in cytoplasm, deformed, huge osteosarcoma cells is clearly visible, all Vision are covered with dye range (Figure 4D). we further measured the fas level quantitatively with Meta Morph automatic image analyzer by comparing average gray value which is Inversely proportional to the Fas expression (Figure 4E).

Discussion

Cinobufacini is a Chinese medicine prepared from the toad skin which has been extensively used in clinics to treat a number of diseases, such as malignant tumors (Chen et al., 2003; Dong et al., 2007; Qi et al., 2010a; Ma et al., 2012), chronic hepatitis B and systemic and local infection (Cui et al., 2010). However, little is known about its killing effect on osteosarcoma. Experimental pharmacodynamics and clinical study both indicate that cinobufacini has obvious killing effect on many kinds of tumor cells, and the mechanism may be related with the apoptosis-inducing effect on tumor cells (Qi et al., 2008; Qi et al., 2010b; Wang et al., 2010). Our research found that cinobufacini may inhibit the proliferation of OS-732 cells in a dose-dependent manner which was consistent with the above research results. Our results indicated, after 72 h of the treatment, when the concentration of cinobufotalin increased from 1 ug/ml to 1000 ug/ml, the survival rate only reduced from 76.28% to 34.55%, which revealed that Cinobufacini could inhibit the osteosarcoma cells, but osteosarcoma cell is not very sensitive to cinobufacini (Figure1A). We also found when the concentration of cisplatin increased from 1 ug/ml to 100 ug/ml, the survival rate reduced from 61.36% to 26.73 whcih indicated that osteosarcoma cells were more sensitive to cisplatin and the anti-tumor effect of cisplatin was stronger than cinobufacini (Figure1B). All these results were consistent with the main view in the clinical treatment that cisplatin is the first-line anticancer drugs for regular chemotherapy and the cinobufacini is the adjuvant second-line anticancer drugs which could be conducive to improve patient's immunity function so as to prevent the recurrence of tumor after the regular chemotherapy is over. According to our results, we deduce, cinobufacini is suitable for the treatment of osteosarcoma. To our surprise, after 72 h of combination of 100 ug/ml cinobufacini and 1 ug/ml of cisplatin on OS732 cell line, the survival rate dropped sharply to 12.65%. Obviously, the synergistic-killing effect is much stronger than the individual use of 100 ug/ml cinobufacini or 1 ug/ml cisplatin (P<0.01, Figure1C). This suggested us that cinobufacini could enhance killing effect of osteosarcoma cells when combined with cisplatin, therefore, a greater anti-tumor effect would be triggered if cinabufotalin were

used in the chemotherapy. Since there were a great number reports about side effects on digestive, nervous, blood and other body systems due to application of cisplatin during the regular chemotherapy of osteosarcoma patients (Konstantakou et al., 2009; LaPensee et al., 2010; Maroto et al., 2011; Rotte et al., 2010; Sprowl et al., 2012), we suppose ,combination of cinobufacini and cisplatin will avoid all these side effects of chemotherapy, and by this way, cinobufacini will promote from the second-line to the first-line anti-tumor drugs and will play a more important role in osteosarcoma therapy. In addition, our research showed the killing effect by the application of cinobufotalin and cisplatin were fulfilled by inducing apoptosis of osteosarcoma cells which were revealed from the cell morphology (Figure2A-2D), cell apoptosis (Figure2E -2H) and FCM analysis (Figure3A-3B).

Fas is a kind of trans-membrane protein, belonging to the super-family of TNF receptors (Moulian and Berrih-Aknin, 1998; O'Callaghan et al., 2008; Inoue et al., 2009; Matsuda et al., 2009; Akimzhanov et al., 2010; Liedtke and Trautwein, 2012), and can be expressed extensively on many normal and tumor tissues. When it is ligated with Fas antibody or ligand, the apoptotic signal may be transfered into cells, and the apoptosis of cells triggered by Fas pathway may be induced. Many anti-tumor agents induce the apoptosis of tumor cells by up-regulating the expression of Fas (Okamoto et al., 2006; Wei et al., 2006; Kim et al., 2007; Dai et al., 2009). Our research indicated 72 hours after the combination of 100 ug/ml cinobufotalin and 1ug/ml cisplatin, the apoptosis rate is as high as 74.63 percent, far higher than individual application of 100 ug/ml cinobufotalin or 1 ug/ml cisplatin (Figure3C), meanwhile, our results revealed that Fas expression of OS732 cell line improved significantly in combination group compared with respective administration (Figure4A-4E). All these results indicated that such a synergistic effect is probably achieved through triggering Fas Pathway. Currently, Many chemotherapy drugs, including cisplatin, were reported to induce apoptosis by up-regulating Fas expression (Shiu et al., 2007). As far as our research is concerned, the combined application of cinobufacini and cisplatin significantly increased the Fas expression of OS732 cells, therefore, we deduce that Fas-FADD pathway plays a critical role in achieving this synergistic effect. After all, the exact mechanism is yet up to deep research and verification.

In conclusion, cinobufacini seems to have prospective view concerning about the treatment of osteosarcoma. Although the individual application of cinobufacini on chemotherapy may still have some limitation, cinobufacini is suitable for the osteosarcoma therapy as a kind of sensitivity-enhancing agents of chemotherapy by combination with cisplatin. Our research indicated cinobufacini could not only increase the anti-tumor effect of cisplatin by overcoming the resistance to ciplatin but also supress the side effects of chemotherapy by decreasing the dose of cispaltin. Moreover, our results also showed, to some extent, individual application of cinobufacini could also inhibit the osteosarcoma cells which suggested us it could be used respectively in preoperative and postoperative or unconventional chemotherapeutic course, in order to enhance the immunity of patients as an adjuvant treatment. Therefore we believe cinobufacini will be a prospective anti-tumor method worthwhile to spread in the field of clinical treatment on osteosarcoma .

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