Influence of *Artemisia annua* extract derivatives on Proliferation, apoptosis and metastasis of osteosarcoma cells

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Abstract: Regarding the *Artemisia annua* extract derivatives called dihydroarteminin (DHA) as the object, we studied about its influence to the proliferation, apoptosis and metastasis of human osteosarcoma cells. First, we cultured in vitro the osteosarcoma cell strain and divided them into groups, then detected the cell proliferation, apoptosis and cell metastasis, etc by multiple measurement technique. Finally, we observed the influence of DHA to human osteosarcoma cells. Osteosarcoma cells were all sensitive to DHA, and the appropriate concentration range was 10–40μM. DHA could effectively restrain its protein expression, and there was a significant difference between experimental group and control group. These finding suggest that, the *Artemisia annua* extract derivatives (DHA) has a biological effect of observably restraining the proliferation and metastasis of human osteosarcoma cells and promoting the tumour cell apoptosis.

Keywords: *Artemisia annua* extract derivatives, osteosarcoma, cell proliferation, cell apoptosis.

INTRODUCTION

Osteosarcoma is a non-hematological malignancy, which is most likely seen in children with high morbidity and common in adults. It is characterized by high pulmonary metastasis rate (about 10%-20%) and recurrence rate. Nowadays, the clinical treatment of osteosarcoma faces many challenges, such as, the toxic and side effect of chemotherapeutics, drug resistance of cancer cells, cancer relapse and lung metastases, etc. Patients with osteosarcoma can rarely gain the recovery even by the systemic treatment (Yuqin *et al.*, 2011). Therefore, the side effects mentioned above can be avoided or reduced in theory by using new developed chemotherapeutics or combined application of new medicine and traditional chemotherapy.

Dihydroartemisinin (DHA) is one of the *Artemisia annua* extract derivatives with a strong and quick killing effect to the plasmodium, and it can effectively control the increasing clinical symptoms and cure the disease. Mechanism of action of artemisinin and its derivatives: it changes the structure of some functional protein in blood-stage cell cytoplasm by disturbing the normal function of mitochondria in plasmodium cytomembrane, and it can influence the ingestion of plasmodium film system to nutrition vacuolar membrane and interdict the nutrition supply. Its mode of action is: resolving the hemoglobin to produce free iron ion by the peroxide (dioxgen) bridge in its chemical construction; producing mediator of the unstable organic free radical or other electrophilic mediator by mediating, and the latter one can form covalent compound with plasmodium cytoplasm or nuclear protein, thereby starting the death program of plasmodium and play function of anti-malarial (Man, 2013). The recent research shown that, its effective function of anti-malarial relates to its well water solubility and weak side effect. The DHA not only has a function of anti-malarial, but also has an obvious effect of anti-tumor. It has been reported that, it can effectively resist the prostate cancer, ovarian cancer, skin cancer, etc (Dingsheng *et al.*, 2011).

This paper is aimed to studying the influence of DHA to proliferation, metastasis and apoptosis of human osteosarcoma cells and its possible molecular mechanism, thus to provide the significative theoretical basis to the clinic treatment of human osteosarcoma by DHA. The early results showed that, DHA could effectively restrain the proliferation and metastasis of human osteosarcoma cells, moreover, promote its apoptosis, and obviously restrain the survival vitality and clone formation of human osteosarcoma cells. *In vivo* experiment of mice showed that, DHA had a prominent inhibiting effect to osteosarcoma and the result was the same to the experimental result in vitro. In addition, Wnt/β-catenin signal transduction pathway played an important role in the influence of DHA to human osteosarcoma. The DHA could observably restrain protein regulatory factor in the Wnt/β-catenin signal transduction pathway, which can promote the occurrence and development of cancer, such as the oncogene Bcl-2. At the same time, some cancer suppressor gene can be activated, and other effective molecular mechanism may exist (Xiaohui and Xiaorong, 2014).

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MATERIALS AND METHODS

Equipments and materials
The main equipments and materials are shown in table 1.

Groups
Based on the requirement of the drug research experiment and experimental result in earlier stage, the materials were divided into five groups: Control group, solvent control group (1% DMSO), and DHA experimental groups (15µM, 25µM and 35µM).

Cell proliferation experiment
Single-layer adherent 143B cells with well growing status, exuberant proliferation (in logarithmic phase) were chosen, and we prepared the 143B cell suspension with cell density of 5×10^5 cells/ml. Add 100µl cell suspension into each hole of 96 well plate, and it would be disposed according to the scheduled experiment groups when the cells were in a good adherence and growth condition. Every group preceded 8 experimental repeated holes, and 20µl MTT (5 g/L) would be put into each hole for 4 hours before the end of the treatment. After 4 hours, absorbance value (OD value) was detected in 492 nm, and then we drew the cell survival curve. The experiment was repeated for three times.

Cell apoptosis experiment
Single-layer adherent 143B cells with well growing status, exuberant proliferation (in logarithmic phase) were chosen, and we prepared the 143B cell suspension with cell density of 5×10^4 cells/ml. Add 500µl cell suspension into each hole of 24 well plate, and it would be disposed according to the scheduled experiment groups when the cells were in a good adherence and growth condition. Hoechst 33258 solution (1 mg/ml) was put into it after treatment, and treated it with water bath in 37°C for 7 min, then cool it on ice for 4 min. The dye liquor would be abandoned after centrifugation. Resuspend PBS, and then we observed the fluorescence in ultraviolet 450 nm. Normal cells (such as blank control) and non-apoptotic cell appeared to be weak blue and karyopyknosis can be observed. The experiment was repeated for three times.

Tumour metastasis and invasion experiment
We operated based on the specification of protein extraction kit according to western blotting, and then extracted and collected the protein specimen of human osteosarcoma cells 143B lysate which was treated by DHA. BCA method was used to detect the protein concentration, and SDS-PAGE loading buffer was put into the collected protein specimen. The protein was made to be sufficient degeneration by boiling water bath for 10 min. Then SDS-PAGE electrophoresis was preceded. The experiment was repeated for three times.

Statistical treatment
Data analyses were performed using SPSS 19.0 software, and experimental data were expressed as Mean ±SD. Analysis of variance was used in comparison among groups and p<0.05 were considered to have significant difference.

RESULTS

DHA obviously restrained the proliferative activity and survival rate of human osteosarcoma cells 143B
We cultured the human osteosarcoma cell strains with different grade malignancy (MG63, U2OS, 143B and Saos2) in vitro, and all of them showed sensitive to DHA after treating by DHA for 24h and 48h (shown in fig.1). Among them, the paper showed that the 143B was the one with highest-grade malignancy, high and early occurrence rate of pulmonary metastasis; however, we also found in the study that, its sensibility to DHA was also the highest, thereby; we chose it for our later experiment. We used the MTT colorimetric method to detect the cell viability of 143B cells after treating by DHA for 24h. As shown in fig. 2, the proliferative activity of cell in experimental group obviously reduced compared with control group, and the inhibitory effect was more obvious with the increasing concentration and longer time. As shown in the fig. 1 crystal violet staining result, the living cell population in treatment group was obviously lower than it in control group, and the effect was most obvious when the concentration of DHA reached 35µM. Cytotoxicity damage had no statistical significance (P>0.1).
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DHA obviously restrained the expression of 143B cell proliferation Gap-associated protein (Western Blotting)

DHA had the function of restra ining the proliferation of human osteosarcoma cells 143B. Thereby, this paper supposed that, it might restrain the expression of certain proliferation related proteins in protein level. Therefore, we detected the proliferating cell nuclear antigen (PCNA). The expression of PCNA protein reduced after treating the 143B cells with DHA for 24h, and it showed the same testing result with the subdued cell reproductive capacity; the results might be explained that, the PCNA with reduced expression was unable to exert the function of accessory protein of DNA polymerase-δ, and the composition of DNA in 143B cells were limited, thereby their proliferation were restrained. We detected the major composition of Wnt/β-catenin signal transduction pathway, and it was the expression of target gene c-Myc and Cyclin D1 which could promote the tumour cell proliferation; we found that, their expressions also prominently reduced by treating with DHA (as shown in fig. 3 and fig. 4), and the effect was more obvious particularly when the concentration of DHA reached 40µM. Drug toxic effect had no statistical significance (P>0.1).

DHA could effectively induce the apoptosis of human osteosarcoma cells 143B

The cell apoptosis was dyed by Hoechst33258, and the experimental damage was shown above. As shown in fig. 5, the size of nucleus in control group was consistent and cellular morphology was integrity, and the fluorescent in weak blue had a distribution with homogeneous, diffuse after treating the 143B with DHA for 24h; while in the experimental group, cell nucleus obviously shrank with concentration hyperchromatic in its chromatin, and fluorescent staining was uneven distribution of crumb that hinted the cell apoptosis. Apoptotic cell counting under fluorescence microscope was shown in fig. 6, apoptotic cell percentage in DHA experimental group was much higher than that in control group, and the apoptotic cell percentage in treatment group with high concentration was likely 4 times as in control group. The conclusion was that, DHA had the function of inducing the apoptosis of human osteosarcoma cell 143B.
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Fig. 7 and Fig. 8: Detection the related protein landmark of regulating tumour cell metastasis and invasion by western blotting (H: qualitative result; I: quantitative analysis, analyzing by Image J software)

**DHA in vitro had a active suppression to metastasis and invasion of human osteosarcoma cells 143B**

DHA had a prominent inhibiting ability to the metastasis and invasion of human osteosarcoma cells 143B. Thereby, we supposed that DHA came into play function by restraining protein related to tumor metastasis and invasion. Therefore, we used western blotting to detect the important protein landmark (MMP-9 and VEGF) of regulating tumour cell metastasis and invasion. The results was shown in fig 7 and fig 8 and the protein expression of MMP-9 and VEGF all obviously reduced in DHA treatment groups whatever with low concentration, medium concentration, or high concentration. It showed a prominent effect of restraining the metastasis and invasion, and the phenomenon of result was the same as it above. The conclusion: DHA restraining the metastasis and invasion of human osteosarcoma cells might by restraining the important regulation protein of metastasis and invasion such as MMP-9 and VEGF.

**CONCLUSION**

According to experimental result at earlier stage, we chose the cell strains 143B that is sensitive to DHA and has the highest malignancy and easiest occurrence of metastasis from four human osteosarcoma cell strains in different grade malignancy. Drug concentration was between 10 40μM. It could not exert an influence to human osteosarcoma cells with too low concentration, and it showed a prominent lethal effect to cells with too high concentration (P<0.05).

The detection result of cells proliferation and clonality showed that, DHA could obviously restrain tumour cell proliferation and clone (P<0.01). That was consistent with the attenuation of PCNA, which was the marker of tumour cells proliferation. PCNA obviously expressed in normal cells in proliferation cycle and tumour cells, however, it expressed quite low in cells with stationary phrase, which was related to the synthesis of DNA and played an important role in the start-up of cell proliferation. Therefore, it was always selected to be the marker to influence cell proliferation state, particularly in the aspect of cells proliferation and tumour cells (Dongyue, 2011).

This paper found that, the expression of PCNA received significant inhibition (P<0.05) by treating the osteosarcoma cell with DHA, and it was accordance with the results of obviously reducing of cancer cell proliferative activity treated by DHA. The conclusion was that, PCNA landmark was associated with the proliferation of tumour cells; DHA restrained the cell proliferation of tumour cells might concern with the restraint of the expression of PNCA protein.

We detected migration and invasion ability change of 143B cell after treated with DHA by experimental method. The result showed that, the metastasis ability of cancer cell received enormous restraint when the human osteosarcoma cell 143B was treated by DHA and the inhibitory effect became more obvious with the long time and increasing concentration. It could be concluded combining with testing result of western blotting: DHA...
had the capacity of effectively restraining the metastasis and invasion of human osteosarcoma cell; MMP9 and VEGF protein had an auxo-action in the metastasis and invasion of cancer; DHA could obviously restrain the expression of human osteosarcoma cell to MMP9 and VEGF. It was accordance with the experimental result of metastasis and invasion: Apoptosis of human osteosarcoma cells were increasing, including, increasing of apoptotic cell (strong fluorescent cells increasing, that was, instructions of apoptotic cell quantity) and appearing of apoptotic cell form, such as, expansion, invagination, dissolution of cell nuclear membrane, shrinking of nucleus, chromatin aggregation and anachromasis, large granular material appearing in cytoplasm. The primary biochemical character of apoptosis is the concentration of its nucleus chromatin. Bcl-2 showed out significant expression limitation, thus to restrain it’s resistance to most of DNA damage factors, promote the target cell apoptosis of chemotherapeutics, and reduce its capacity of promoting DHA damage repair. It has reported that, Bcl-2 could form the homologous protein dimer with the Bcl-X1, Bcl-Xs, Bax, Bcl-2, Bad and Mc1-1, etc, which were the member of Bcl-2 family. The especial dimer possessed the on-off action in the signal path of cell death. For example, Bcl-2 could form dimer with apoptosis-promoting Bad, and it could promote the cell death when the content of Bad was in the ascendant; otherwise, it could restrain the target cell death (Xiaofeng and Gang, 2012).

DISCUSSION

As reported in the earliest study in our country, artemisinin is a kind of effective medicine to resist malaria, and then it was recognized and ranked as the antimalarial drug in frontline. The early researches of artemisinin were mainly about its efficient anti-malaria activity, and they preceded multiple chemical structural modifications and compounded multiple artemisinin derivatives in order to reduce the side effects of drugs; among them, dihydroartemisinin had the best water solubility and least toxic and side effect. In recent ten years, the researches were mainly about physiological activity of the artemisinin and its ramification, and it was found that artemisinin and its ramification not only had the function to resist tumour, treat schistosomiasis, enhance the immunity of living body, but also had a high biological activity.

Dihydroartemisinin is superior to other ramification of the same kind in water solubility, cure rate, toxic and side effect and resistance of tolerance by restoring artemisinim and modifying its No.12 carbon atom. Dihydroartemisinin has the special effects in treating the cerebral malaria and malignant malaria, which are able to resist the chloroquine or multiple drugs (Folkert et al., 2011). Therefore, it is extensively used in clinic, and the WHO ratifies it as the choice drug in treating malaria. Compared with the artemisinin, dihydroartemisinin has the advantage of good water solubility, easy to assimilate, rapid in metabolism and excretion, weak toxic and side effect, etc. With the deepening of the research, people found that the dihydroartemisinin not only has the efficient treatment of malaria, but also has the obvious regulatory function in the aspects including: anti-inflammatory, immune regulation, suppression of scar hyperplasia. It is widely concerned because of its strong pesticide effect in antineoplastic activity and low toxicity to normal tissue cells.

Long time, multi-step and multi-stage of risk factors of disease promotes the occurrence and development of tumor. Among them, its maximal changes are excessive proliferation and incontrollable apoptosis, especially the prominent biological characteristics of the cancer. Therefore, it is the entry point in tumour treatment research to intervene the tumour cell proliferation and promote tumour cell apoptosis. Another most prominent biological characteristic of cancer is metastasis and infiltrative growth, and it accomplishes through two stages: metastasis and infiltration, and there have no strict difference between them whatever in biological characteristics or in concept definition (Rulin et al., 2012). Infiltration, that is, the tumour cells fall off from the primary site and pass through the basilar membrane into surrounding mesenchyme to grow; metastasis, that is, the tumour cells pass through the basilar membrane, then approach and pass through the partial blood capillary tube wall or lymph tube wall into lumen, and they will pass through the tube wall and basilar membrane into the organization mesenchyme interval again when they arrive the suitable organization position to grow by flowing of

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**Table 1: Main equipments and materials**

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<tr>
<th>Cells incubator</th>
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<tr>
<td>Gel-imaging analytic system</td>
<td>BIO-RAD, Bio-Rad Laboratories, Inc.</td>
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<td>Chemiluminiscence imager</td>
<td>ChemiDoc XRS+, Bio-Rad Laboratories, Inc.</td>
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<td>Human osteosarcoma cells</td>
<td>American Type Culture Collection</td>
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<td>DMEM medium</td>
<td>Thermo Scientific Company</td>
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<td>DMSO</td>
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<td>Protein detection kits BCA</td>
<td>Thermo Scientific</td>
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<td>Artemisia annua extract derivatives: dihydroartemisin (DHA)</td>
<td>Sichuan Chengdu Ruifengsi Biotechnology Ltd</td>
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They form the micrometastasis by proliferating and then form the metastatic tumour by later proliferating. The multi-mechanism research shows that, tumour cells’ metastasis and infiltration might be related to decrease and easy falling of homogenous adhesion and enhancement of homogenous adhesion of tumour cell itself. In addition, compared with the normal structure cell, tumour cell has multiple enzymes, which have superstrong synthesis potency and can degrade ECM, such as MMPs. It can clear away the obstacle for tumour cell to transfer and then promote generation of booth cells to provide the nutrition to tumour cells by compounding and secreting cytokines to induce the generation of new blood vessel, such as VEGF. In addition, movability of cancer cell enhances (its athletic ability is far bigger than normal structure cell), and it can penetrates ECM and basilar membrane of vascular wall into cycle; the change of protein structure in the cytomembrane surface of tumour cell can make it escape from the recognition and destruction by immune system in the cycle, that is immune escape (Xiaoling et al., 2011). Infiltration is the premise of metastasis, and the metastasis maybe not happen, but the metastasis is certainly including the process of infiltration. Therefore, it is significant to study the rule and occurrence mechanism of tumour invasion and metastasis for prophylaxis and treatment of cancer. Most of the studies are about MMP-9 among protein factors, which are secreted by tumour cell and have degradation to the extra cellular matrix, and MMP-9 is from matrix metalloproteinase family MMPs. The function of MMP-9 is most prominent and can degrade a variety of collagen and protein composition in cellular matrix (Dong-Wan et al., 2011). It can destroy normal structure protective screen and promote cancer cell to occur infiltration, metastasis by through the basilar membrane protective screen. The formation of new vessels and its nutrition supply can provide the condition to metastasis of tumour cell. VEGF is one of the most powerful cytokines to promote the angiogenesis in studies at present, and it has the prominent biological function, including increasing the permeability of blood vessel, promoting the proliferation of endothelial cells, promoting the generation of new vessels, etc. Tumor cell can abnormally compound and secrete the protein, thus to provide sufficient nutrient for tumor cells and promote its metastasis.

Through acting on human osteosarcoma cell 143B by DHA in different concentration, the MTT colorimetric method testing result showed that, DHA could restrain the cell proliferation of 143B and the inhibitory effect became more obvious with the prolonged time and increasing concentration. The suppress proliferation effect was the most obvious compared with the control group when the concentration reached 35µM after 48h, and there had a significant difference (P<0.01), meanwhile, the clonality of 143B was obviously restrained. Cell apoptosis is an initiative genes involved in regulation, programmed cell death process and organism can maintain its own tissue cells function stability and regulate normal cell function and quantity through it. Various apoptosis regulator genes are the most important members in apoptosis regulatory protein family, including Bad and Bcl-2 and they widely express in tissue. Bcl-2 protein factor anchor in the mitochondrial outer membrane by hydrophobic amino acid in c-terminal and the Bad factor anchor intersperse in the ground substance. Bad protein after activation shifts to the mitochondria surface and combines with Bcl-2 by the influence of cell microenvironment factor and it changes the membrane potential and membrane permeability of mitochondria. Then the pro-apoptotic protein in mitochondrial membrane interval will be released, such as endonuclease G (endog), Smac/DIABLO, cytochrome C and AIF, etc. These pro-apoptotic proteins activate caspase family and then trigger caspase cascade reaction. Ultimately, they activate the apoptosis procedure, start the cell apoptosis, facilitate the formation of apoptosis vesicles, and make the cell nucleus to pyknosis, cataclastic. Finally, the cell apoptosis realizes (Gregg L, 2011). The balance between Bcl-2 and Bad in normal histocyte decide the activation or not of the apoptosis induction procedure; the apoptosis procedure excitation can be restrained when the Bcl-2 protein factor is excessive, and the procedure will start when the Bad is excessive. This study found that, DHA could restrain the expression of Bcl-2 protein, and at the same time, enhance the expression of Bad protein and caspase 3 proteins. The balance between Bcl-2 and Bad was destroyed and it trended to apoptosis. The incorporation of Bad protein and Bcl-2 protein increased, and the anti-apoptosis effect of Bcl-2 was restrained. At the same time, apoptosis executive capacity of highly expressive caspase 3, especially its activated form increased, and it could urge the increasing of 143B apoptosis. The underlying cause of death in patients with osteosarcoma is high pulmonary metastasis rate in early stage (Bo et al., 2012), and it is also the biggest bewilderment in clinic treatment. However, the high expression of MMP-9 and VEGF protein in human osteosarcoma cell 143B is unable to part from its high metastasis and infiltration, and that indicated that, MMP-9 and VEGF play an important function in metastasis of human osteosarcoma. DHA can effectively restrain the metastasis and infiltration of human osteosarcoma cell, and it may because it can restrain the degradation of extracellular matrix and abnormal formation of new vessels. MMP-9 and VEGF may become the new therapeutic target in human osteosarcoma treatment. The objective of early prevention of human osteosarcoma metastasis can be realized by restraining the expression of MMP-9 and VEGF, disturbing the degradation of extracellular matrix and formation of new vessels and restraining the metastasis and infiltration of tumour.
REFERENCES


