Honokiol induces autophagy and apoptosis of osteosarcoma through PI3K/Akt/mTOR signaling pathway

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Abstract. Honokiol is the main active constituent of *Magnolia officinalis*. With effective and long-term pharmacological functions of being antibacterial, anti-oxidative, anti-inflammatory, antitumor, anti-spasmic, anti-anxiety and anti-viral, Honokiol is clinically used in the treatment of acute enteritis and chronic gastritis. The aim of the present study was to observe the possible anti-effects of honokiol on autophagy and apoptosis of osteosarcoma, and to investigate the role of the PI3K/Akt/mTOR signaling pathway in its anticancer effects. MTT assay was used to evaluate cell proliferation and Annexin V-fluorescein isothiocyanate/propidium iodide staining flow cytometry was used to analyze the apoptotic rate. The authors identified that honokiol could inhibit cell proliferation and induce the apoptotic rate of osteosarcoma cells. The expression level of Bcl-2-like protein 4, caspase-3 and p53 protein expression were induced and cyclin D1 protein expression was suppressed in osteosarcoma cells by honokiol. Autophagy-associated LC3II protein expression level was promoted, and PI3K, p-Akt and p-mTOR protein expression level was suppressed in osteosarcoma cells by honokiol. The present study demonstrated, to the best of the authors' knowledge, for the first time that honokiol induces autophagy and apoptosis of osteosarcoma cells through the PI3K/Akt/mTOR signaling pathway.

Introduction

As the most common primary malignant bone tumor occurring in children and adolescents, osteosarcoma is characterized by early lung metastasis and high recurrence rate (1). Surgery, combined with neoadjuvant chemotherapy and other comprehensive treatment approaches, can significantly improve the disease, but some local osteosarcomas still recur; at present, there have been no effective approaches for the treatment of recurrent and metastatic osteosarcoma (2). Although neoadjuvant chemotherapy combined with surgery improves the long-term survival rate of patients with osteosarcoma, short-term survival rate of patients has not been further improved in recent years (3).

Autophagy is an ancient biological phenomenon in the evolution of creatures, and extensively occurs in plants and low-ranking organisms as an important ways to maintain the stability of cellular homeostasis; autophagy is also one of the most important means by which mammals can eliminate tumor cells. Autophagy is closely related to the incidence, development, prognosis and treatment of tumor cells (4). When the body grows tumor cells, autophagy will be activated to remove damaged organelles and degrade harmful substances in cells such as peroxides, so as to prevent normal cells from developing into tumor cells (5). Some scholars observed autophagosomes wrapped parts of the nucleolus, suggesting that autophagy is involved in the metabolism of genetic materials in cells and in the elimination of mutant chromosomes (6). Regulating the expression of autophagy combined with chemotherapy drugs has synergistic or antagonistic effects in the growth of tumor cells. Compared with chemotherapy alone, the regulation autophagy changes the apoptosis rate of tumor cells (7). Inhibiting the expression of autophagy during radiotherapy or chemotherapy may cause tumor cells to be unable to eliminate damaged organelles, thereby accelerating the death of tumor cells, so as to enhance the efficacy of treatment (8).

Akt (also known as protein kinase B) is a regulator located at the downstream of the PI3K pathway. Mammalian target of rapamycin (mTOR), a kind of serine/threonine protein kinase, is a member of phosphatidylinositol 3-kinase family (9). mTOR exists in multi-protein complexes, such as mTORC1 and mTORC2. The PI3K-Akt-mTOR signaling pathway serves important roles in cells, regulating a variety of cellular behaviors such as growth, survival, proliferation, apoptosis, angiogenesis and autophagy of cells (10). Many diseases, including cancer, autoimmune diseases and neuropathy, are caused by disorders of the PI3K-Akt-mTOR signaling pathway (10). The PI3K-Akt-mTOR signaling pathway is

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related to several important mechanisms of cell growth, so a better understanding of the PI3K-Akt-mTOR signaling pathway contributes to the development of cancer drugs, and mTOR inhibitors have been previously developed and applied as novel antitumor targeting drugs (11).

As one of the two major active constituents of *Magnolia officinalis*, honokiol can effectively fight against bacteria, oxidation, inflammation and tumors, inhibit central nervous and muscular relaxation, kill pathogenic microorganisms and lower cholesterol levels, and is generally used in the treatment of acute enteritis, bacterial or amoebic dysentery and chronic gastritis (12). A large number of studies have demonstrated that honokiol inhibits the proliferation and apoptosis of cancer cells and prevents from angiogenesis *in vivo*, indicating that it has good therapeutic effect on different tumors (13). Honokiol has a better curative effect if combined with other anticancer drugs. A study suggested that Honokiol downregulates the phosphorylation of Akt and upregulates the expression of PTEN to realize the negative regulation of PI3K/Akt/mTOR pathway, so as to inhibit breast cancer (14). Honokiol promoted cycle arrest and apoptosis of breast cancer cells through the downregulation of the c-Src/EGFR signaling pathway. Furthermore, the authors investigated whether honokiol induces autophagy and apoptosis of osteosarcoma, and analyzed the possible mechanisms underlying these anticancer effects.

Materials and methods

Cell culture. The human OS cell line, MG-63, was purchased from the Wuhan Cell Bank of Sciences China (Wuhan, China) and maintained in Dulbecco's modified Eagle's medium, which contains 10% heat-inactivated FBS (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in 5% CO_2. Honokiol was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and its constitutional formula is presented in Fig. 1.

MTT assay. MG-63 cells were seeded in 96-well plates at a density of $1x10^4$ cells/well overnight and then treated with various concentrations of honokiol (0, 5, 10 and 20 μg/ml) for 24, 48 and 72 h. A total of 20 μl MTT solution (5 g/l; Thermo Fisher Scientific, Inc.) was added to each well and incubated for 4 h. DMSO (Thermo Fisher Scientific, Inc.) was added to cells and dissolved for 20 min. The absorbance value was read using an automatic multiwell spectrophotometer (PowerWave HT; Bio-Tek Instruments, Inc., Winooski, VT, USA) at 570 nm.

Apoptosis. MG-63 cells were seeded in 6-well plates at a density of $1x10^6$ cells/well overnight and then treated with various concentrations of honokiol (0, 5, 10 and 20 μg/ml) for 48 h. MG-63 cells were washed three times with PBS and stained using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's protocol. Apoptotic rate was detected using flow cytometry (FACSCanto™) and analyzed by CellQuest™ software (version 3.2) (both from BD Biosciences).

Caspase-3 and western blotting analysis. MG-63 cells were seeded in six-well plates at a density of $1x10^6$ cells/well overnight and then treated with various concentrations of Honokiol (0, 5, 10 and 20 μg/ml) for 48 h. MG-63 cells were resuspended in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) at 4˚C for 30 min and the lysate was centrifuged at 120,000 x g for 10 min at 4˚C. Protein contents were detected using the Bicinchoninic Acid protein assay kit (Thermo Fisher Scientific, Inc.). A total of 5-10 μg protein was incubated with Ac-DEVD-pNA for 1-1.5 h at 37˚C. The absorbance value was read using an automatic multiwell spectrophotometer (PowerWave HT; Bio-Tek Instruments, Inc.) at 405 nm.

Next, equal amounts of total protein (50 μg) were separated on 6-12% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific, Inc.). The membranes were blocked with fat-free milk solution (5%, w/v) for 12 h and then incubated with rabbit anti- B-cell lymphoma-2 (Bcl-2, 1:500, cat no. sc-783) and anti- Bcl-2-like protein 4 (Bax, 1:500, cat no. sc-6236), p53 (cat no. sc-6243), cyclin D1 (1:500, cat no. sc-717), LC3II (1:500, cat no. sc-292354), PI3K (1:500, cat no. sc-7174), p-Akt (1:550, cat no. sc-16646-R), p-mTOR (1:500, cat no. sc-101738) and GAPDH (1:500, cat no. sc-25778) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) primary antibodies at 4˚C for 12 h. After washing 3 times with TBS and 0.1% Tween-20, membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:5,000, cat no. sc-2004; Santa Cruz Biotechnology, Inc.) at 37˚C for 1 h and were visualized using Western Blotting Chemiluminescence Reagent (BD Biosciences). Blots blank was quantified using BandScan software (version 5.0; Glyko Inc., Novato, CA, USA).

Statistical analysis. Data are presented as the mean ± standard deviation. Student's t-tests were performed for the comparison of results between different groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Honokiol inhibited cell proliferation of osteosarcoma cells. As presented in Fig. 2, an MTT assay demonstrated that various concentrations of honokiol inhibited cell proliferation of osteosarcoma cells (MG-63) in a dose- and time-dependent manner. Treatment with osteosarcoma cell (5, 10 and 20 μg/ml honokiol for 72 h), or 10 and 20 μg/ml honokiol for 48 h or 20 μg/ml honokiol for 24 h significantly inhibited cell proliferation of MG-63 cells, compared with the control group (0 μg/ml group).

Honokiol induced apoptotic rate of osteosarcoma cell. Therefore, the authors detected the apoptotic rate of osteosarcoma cells (MG-63) by honokiol for 48 h. As demonstrated in Fig. 3, Annexin V-FITC/PI indicated that 10 and 20 μg/ml honokiol significantly induced apoptotic rate of MG-63 cells, compared with the control group (0 μg/ml group).

Honokiol induced Bax, p53 and cyclin D1 protein expression and caspase-3 activity of osteosarcoma cells. To investigate apoptosis mechanism of Honokiol on osteosarcoma cell, the authors firstly detected Bax, p53 and cyclin D1 protein

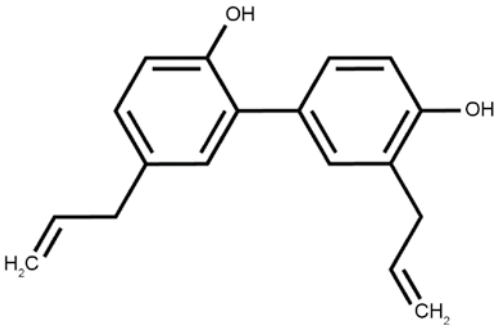

Figure 1. Constitutional formula of honokiol.

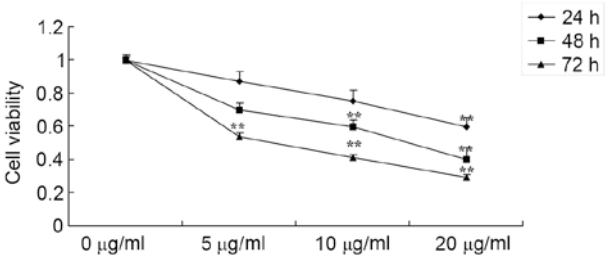

Figure 2. Honokiol inhibited cell proliferation of osteosarcoma cells. Honokiol inhibited cell proliferation of osteosarcoma cells for 24 h and 48 h. 0 μg/ml, 0 μg/ml honokiol; 5 μg/ml, 5 μg/ml honokiol; 10 μg/ml, 10 μg/ml honokiol; 20 μg/ml, 20 μg/ml honokiol. **P<0.01 vs. 0 μg/ml honokiol group.

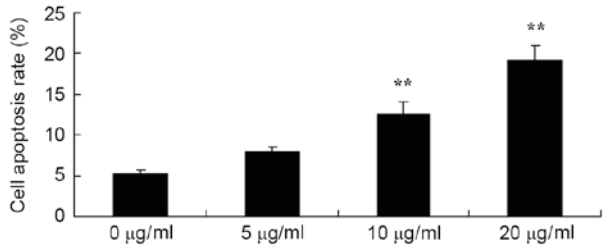

Figure 3. Honokiol induced apoptotic rate of osteosarcoma cells. 0 μg/ml, 0 μg/ml honokiol; 5 μg/ml, 5 μg/ml honokiol; 10 μg/ml, 10 μg/ml honokiol; 20 μg/ml, 20 μg/ml honokiol. **P<0.01 vs. 0 μg/ml honokiol group.

expression and caspase-3 activity in osteosarcoma cell (MG-63) by honokiol for 48 h. In Fig. 4, 10 and 20 μg/ml honokiol significantly induced bax and p53, and significantly inhbited cyclin D1 protein expression levels compared with the control. Honokiol treatment led to increased caspase-3 activity of MG-63 cells, compared with the control group (0 μg/ml group).

Honokiol promoted LC3, PI3K, p-Akt and p-mTOR protein expression of osteosarcoma cells. In order to test the role of autophagy in the anticancer effects of honokiol on osteosarcoma cells, LC3II protein expression of MG-63 cell was measured. Western blotting revealed that 10 and 20 μg/ml honokiol significantly promoted LC3 protein expression of MG-63 cell, compared with the control group (0 μg/ml group; Fig. 5A and B). The authors then examined the autophagy mechanism of anticancer effects of honokiol on osteosarcoma cells. As presented in Fig. 5A and C-E, PI3K, p-Akt and p-mTOR protein expression levels of MG-63 cells was significantly suppressed by 10 and 20 μg/ml honokiol in MG-63 cells, compared with the control group (0 μg/ml group).

Discussion

Osteosarcoma is the bone malignancy most commonly occurring in adolescents (15). Neoadjuvant chemotherapy significantly improves the survival rate of patients with osteosarcoma, and reduces the rate of recurrence and metastasis, but it has a poor chemotherapy effect in some patients, easy leading to recurrence and metastasis (15,16). Therefore, revealing the mechanism of the incidence and development of osteosarcoma from a new perspective to fundamentally cure tumor has always been the focus and challenge in the study of orthopedics (16). The present study demonstrated that honokiol significantly inhibited cell proliferation and induced apoptotic rate of MG-63 cells.

Cell autophagy removes excessive and harmful proteins or organelles with long half-life from cells, to protect the components of cells and provide raw materials for the reconstruction, regeneration and repair of cells, thus realizing recycle and reuse of components in cells (17). Cell autophagy is closely associated with cancers through multiple signaling pathways, such as the PI3K/Akt/mTOR signaling pathway, the LKB1/AMPK/mTOR signaling pathway, the p53 signaling pathway and the BCL-2 pathway, as well as endoplasmic reticulum stress (18). In the present study, honokiol significantly induced Bax and p53 protein expression, increased caspase-3 activity and suppressed cyclin D1 of MG-63 cells.

Cell autophagy removes excessive and harmful proteins or organelles with long half-life from cells to protect the components of cells (5). Under normal circumstances, autophagy is conducive to the survival of cells, so the dysfunction of autophagy causes many diseases, such as neurodegenerative diseases, bacterial infection, intestinal inflammation, aging and cancers (19,20). In normal cells, autophagy pathways are regulated by the signaling network, which centers on mTOR (21). Nutrients and growth factors can activate the PI3K/Akt/mTOR signaling pathway, thereby inhibiting the activity of the autophagy pathway, to promote the growth and proliferation of cells (22). When cells are lacking in nutrients and growth factors, or under the condition of hypoxia and other stresses, PI3K/Akt/m TOR signaling pathways will be inhibited and thus, the autophagy pathway be activated, inhibiting cell growth and proliferation (23). The autophagy pathway basically serves the same role in tumor cells as it does in normal cells, only the PI3K/Akt/mTOR signaling pathway is damaged and interacts with other signaling pathways, which makes the autophagy pathway in tumor cells more complex (24). The current study demonstrated that honokiol significantly promoted LC3 protein expression of MG-63 cells. Lv *et al* (19) revealed that honokiol increased the expression of p62 and LC3 I in the A549 and H460 cells.

The PI3K-AKT pathway is improperly activated in various types of cancer. PI3K-AKT can be activated through two major mechanisms, such as the activation of specific nodes in pathway and the activation of receptor tyrosine kinase, so understanding the activation mechanisms of PI3K is vital for the development of effective therapies and PI3K inhibitors (10,18). It is often

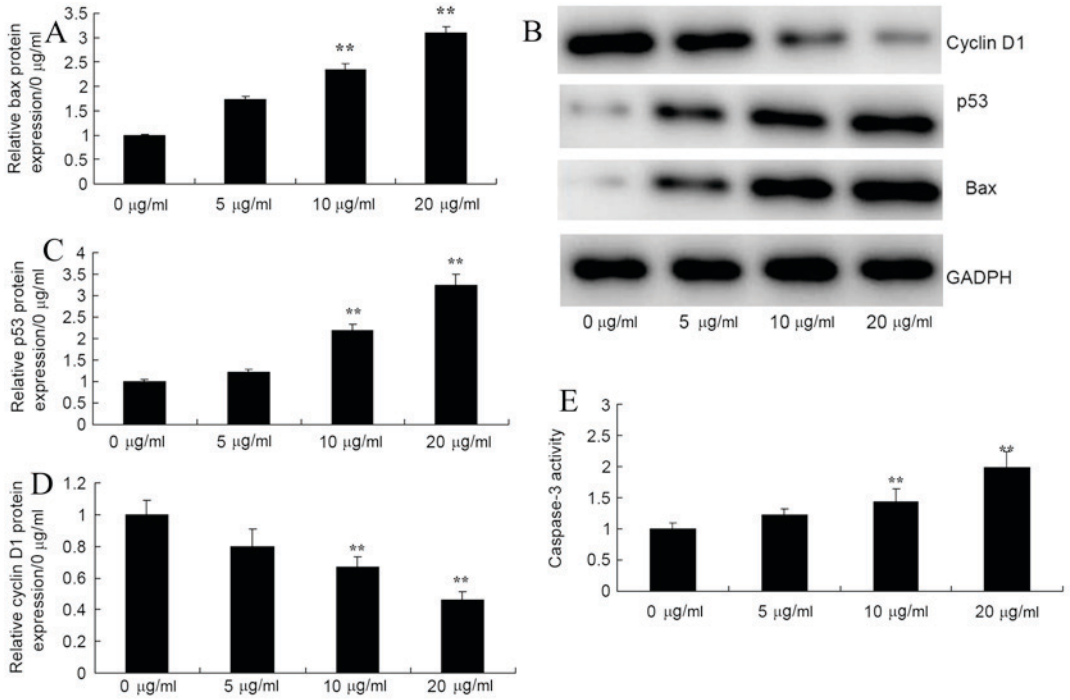

Figure 4. Honokiol induced Bax, p53 and cyclin D1 protein expression and caspase-3 activity of osteosarcoma cell. Honokiol induced (A and B) Bax, (B and C) p53 and (B and D) cyclin D1 protein expression by western blotting and statistical analysis. (E) caspase-3 activity of osteosarcoma cells. 0 μg/ml, 0 μg/ml honokiol; 5 μg/ml, 5 μg/ml honokiol; 10 μg/ml, 10 μg/ml honokiol; 20 μg/ml, 20 μg/ml honokiol. **P<0.01 vs. 0 μg/ml honokiol group. Bax, B-cell lymphoma-2-associated X protein.

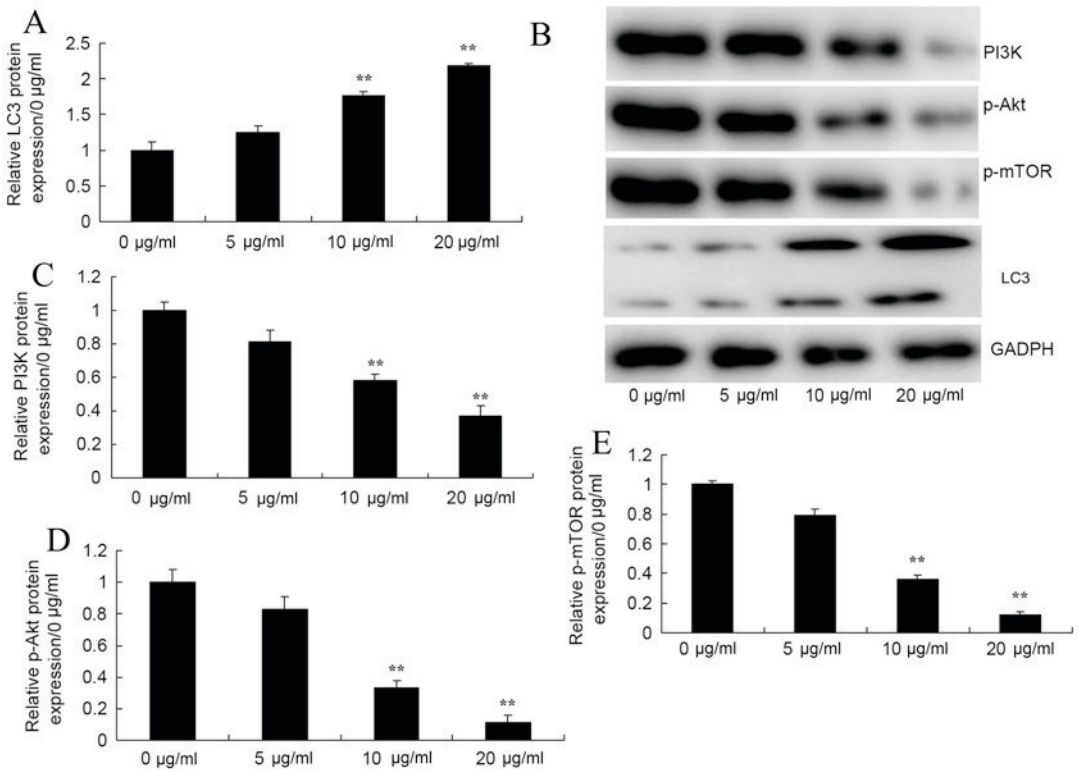

Figure 5. Honokiol promoted LC3, PI3K, p-Akt and p-mTOR protein expression of osteosarcoma cells. Honokiol induced (A and B) LC3, (B and C) PI3K, (B and D) p-Akt and (B and E) p-mTOR protein expression of osteosarcoma cells by western blotting and statistical analysis. 0 μg/ml, 0 μg/ml honokiol; 5 μg/ml, 5 μg/ml honokiol; 10 μg/ml, 10 μg/ml honokiol; 20 μg/ml, 20 μg/ml honokiol. **P<0.01 vs. 0 μg/ml honokiol group. PI3K, phosphoinositide 3-kinase; p-Akt, phosphorylated protein kinase B; p-mTOR, phosphorylated mammalian target of rapamycin.

reported that the PI3K/Akt/mTOR signaling pathway works abnormally in cancer cells, and even being constitutively activated, which promotes the growth and proliferation of tumor cells (18). This may be due to one or more cellular events

closely related to the incidence and development of cancers, such as mutation or deletion of PTEN, mutation of TSC1/2, tyrosine kinase receptor growth factor and type I PI3K, overexpression of Akt, inhibition of mTOR, exposure to carcinogens, which may lead to the activation of PI3K/Akt/mTOR, thereby inhibiting the autophagy of cells (25,26). The results demonstrated that honokiol significantly suppressed PI3K, p-Akt and p-mTOR protein expression of MG-63 cell. Lin *et al* (27) reported that honokiol induces autophagic cell death via regulation of the p53/PI3K/Akt/mTOR signaling pathway in malignant glioma (27).

In summary, the present study demonstrates, honokiol significantly inhibited cell proliferation, induced apoptotic rate, and induced Bax and p53 protein expression, increased caspase-3 activity and suppressed cyclin D1 of MG-63 cells, which may induces autophagy through the PI3K/Akt/mTOR signaling pathway. In summary, the current study indicated that honokiol may be a promising strategy for osteosarcoma therapy or clinical applications.

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