Huaier Extract Inhibits Proliferation and Promotes Apoptosis via the MAPK Pathway in Human Osteosarcoma Cells

Xin Guan

Shandong University

Yaxi Zhai

Shandong University

Rong Tang

Shandong University

Panpan Yang

Shandong University

Dongfang Li

Shandong University

Yuying Kou

Shandong University

Ziqi Yue

Shandong University

Yi Zhang

Shandong University

Lin Sun

Shandong University

Sen Zhang

Shandong University

Hongrui Liu

Shandong University

Jie Guo

Shandong University

Tomoka Hasegawa

Hokkaido Daigaku

Minqi Li (✉ liminqi@sdu.edu.cn)

Department of Bone Metabolism, School and Hospital of Stomatology, Cheeloo College of Medicine, Shandong University & Shandong Key Laboratory of Oral Tissue Regeneration & Shandong Engineering Laboratory for Dental Materials and Oral Tissue Regeneration, Jinan 250012, China <https://orcid.org/0000-0001-9980-1382>

Research

Keywords: Huaier extract, proliferation, apoptosis, MAPK pathway, osteosarcoma cells

Posted Date: January 4th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-136202/v1>

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Abstract

Background: Osteosarcoma is a common pediatric bone malignancy. Huaier, a traditional Chinese medicine, attracts increasing attention for its antitumor effect. The aim of this study is to investigate the inhibitory effect and molecular mechanisms of Huaier in osteosarcoma cells.

Methods: Bioinformatics was performed to determine the biological processes and pathways connected to Huaier. The CCK-8 method and flow cytometry were used to detect the cell viability, cell cycle distribution and apoptosis of osteosarcoma cells (MG-63 and MNNG/HOS). Western blot was applied to assess the expression of proteins involved in apoptosis, cell cycle and the MAPK pathway.

Results: Huaier could inhibit osteosarcoma cells proliferation by arresting cells in the G0/G1 phase. The extract also suppressed invasion and migration, while promoting the apoptosis of osteosarcoma cells in a time- and dose-dependent manner. Under Huaier stimulation, the expression of p-ERK/ERK, Cyclin D1 and Bcl-2 decreased, while the expression of p-JNK/JNK, p-P38/P38, P21, P27, Bax and Caspase3 increased in osteosarcoma cells.

Conclusions: Our findings demonstrated for the first time that Huaier extract could inhibit proliferation and promote apoptosis in osteosarcoma cells *via* the MAPK pathway *in vitro*, suggesting that Huaier may be developed as a chemopreventive medicine for the treatment of osteosarcoma.

Background

Osteosarcoma (OS) is the most common primary malignant tumor of bone in the young adults and children (Ottaviani and Jaffe 2009). Only 65% of patients who present with localized osteosarcoma can be expected to be cured by surgery and routine chemotherapy (Miller 1981). Osteosarcoma has a high potential for metastasis. Only 20% of patients with metastasis will be cured. These survival trends have remained virtually unchanged over the past 20 years. Although conventional chemotherapy drugs are effective enough to increase survival rate, their current administration suffers from several side effects (Kubo et al. 2015). Consequently, it is imperative to develop new therapeutic agents, especially chemopreventive medicines produced by less harmful, natural substances.

Recently, more attention has been attached on the medicinal herbs and plants as a potential source of novel chemotherapy anticancer drugs for their low-toxic effects but more effectiveness, among that traditional Chinese medicine (TCM) has got more popularity for its new effect in killing tumor cells more naturally and less intensively. Huaier attracts increasing attention due to its anti-tumor effects as a traditional Chinese medicine. The active ingredient of Huaier is extracted from the fermented fungus extract which is considered to be a proteoglycan containing the following components: polysaccharides, 41.53%; water, 8.72%; and amino acids, 12.93% (Song et al. 2015; Wang et al. 2013). However, the anticancer effect of proteoglycan is less effective than that of the Huaier extract (Lee et al. 2007; Yang et al. 2017). Huaier has been confirmed to be effective against various cancers through some types of mechanisms, such as inhibition of tumor-induced angiogenesis, inhibition of cell growth, induction of apoptosis, and epithelial-mesenchymal transition (Ji et al. 2017; Pan et al. 2019). However, to the best of our knowledge, research on the effects of Huaier on OS cells is scarce.

The BATMAN-TCM is a traditional Chinese medicine database that collects detailed information on various drugs (<http://bionet.ncpsb.org/batman-tcm/>). The target genes of Huaier were found using Chinese medicine database and they showed MAPK pathway was involved in the antitumor effect by Huaier. Mitogen-activated protein kinases (MAPKs), which include extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK, are signal transduction molecules highly conserved in all eukaryotic cells (Chang and Karin 2001). Members of the MAPK family regulate many cellular functions, such as cell proliferation, apoptosis, and transforming signals from upstream extracellular stimulation into downstream effectors which are located in the nucleus (Dhillon et al. 2007; Wagner and Nebreda 2009). Abnormal expression of MAPK leads to the development and progression of cancer through various mechanisms (Hobbs et al. 2016; Kroemer et al. 2007; Zhang et al. 2013). As the most importantly mutated signaling pathway in human cancers, the MAPK pathway has been recognized as a promising target of cancer therapy for several years.

In this study, we observed the effects of Huaier extract on two widely used OS cell lines, MG-63 and MNNG/HOS, and demonstrated for the first time that Huaier extract could inhibit proliferation and promote apoptosis in OS cells *via* the MAPK pathway *in vitro*.

Methods

Cell culture and reagents

Human fibroblast cell line MRC-5 and human MG-63 and MNNG/HOS osteosarcoma cells, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) that supplemented 1% penicillin streptomycin and 15% fetal bovine serum (Gibco, Grand Island, NY, USA) and at a temperature of 37 °C. The humidity is 5% CO₂. The non-essential amino acid (NEAA) was also added to the medium of MG-63. Huaier extract was friendly gifted by Gaitianli Medicine Co. Ltd. (Jiangsu, China). Two grams of the ointment were dissolved in 20 ml of the complete medium and sterilized with the 0.22 micron filter. The 100 mg/ml solution was stored at -20 °C for a long time. Antibodies against Antibodies to P-JNK(ab76572), JNK(ab179461), P38(ab170099), P-P38(ab178867), ERK(ab184699), P-ERK(ab76299), Bcl-2(ab185002), Caspase3(ab184787), P21(ab109199), Cleaved Caspase-3(ab2302), Cyclin D1(ab134175), P27(ab32034) were purchased from Abcam (Shanghai, China). Anti-GAPDH (10494-1-AP) was obtained from Proteintech (Wuhan, Hubei, China).

Colony formation assay

MNNG/HOS cells were plated at a density of 700 cells per well in 6-well plates and MG-63 cells were plated at a density of 1000 cells per well in triplicate. 24 h later, the cells were treated with various concentrations of Huaier (0, 2 and 4 mg/mL) and then incubated at 37 °C in a 5% CO₂ incubator for 10 days. Washing with PBS was followed, then fixing with methanol and staining with 1% crystal violet. Finally, we counted the colonies which contained more than 50 cells.

Cell viability assay

Cell Counting Kit-8 assay was performed to evaluate the cell viability activity according to the manufacturer's instructions. MNNG/HOS, MG-63 and MRC-5 cells were harvested into 96 - well plates at a density of 5×10^3 cells per well with 200 μ L culture medium. After 24 hours of incubation, cells were treated with various concentrations of Huaier for indicated times. Then the cell viability was measured using cell counting kit – 8(CCK8, solarbio, Beijing, China). Finally, the absorbance of plate was read using the automated microplate spectrophotometer (Bio-Rad Laboratories, USA) at 450 nm. The data was repeated three times.

Invasion assay

Transwell invasion assay was performed to detect the effect of Huaier on the invasion ability of MNNG/HOS and MG-63 cells. We put 8 μ m pore-sized Transwell inserts which were covered by matrigel into 24-well plate full of 700 μ L of medium including 20% FBS. We added complete medium without FBS to the upper layer of the well plate. Cells (2×10^4) were inoculated with or without Huaier for 24 h in an incubator that was maintained at 37 °C and 5% CO₂. After 24 h, we washed the cells twice with PBS, carefully detached the cells from the upper layer plate with a cotton swab and then we fixed the cells with 100% methanol for 30 min. Finally cells were stained with 1% crystal violet for 15 min and were counted by using a light microscope (Olympus BX53, Tokyo, Japan).

Scratch assay

The migration ability of the cells was tested using a scratch test. The MNNG/HOS and MG-63 cells were seeded into 6-well plates at 5×10^5 per well. After 12 h, the cells were cultured in complete medium to a confluent state. Next, the cells were scraped vertically with a 20 μ L pipette tip. Then, the cells were washed three times with PBS. Finally, we treated cells with various concentrations of Huaier for 24 h in Serum-free medium. After 24 h, the width of the scratches was observed under a microscope (Olympus BX53, Tokyo, Japan) and statistical analysis was performed.

Cell cycle analysis

MG-63 and MNNG/HOS cells were seeded into 6-well plate at a density of 2×10^5 /well and treated with different concentrations of Huaier (0, 2, 4 mg/mL) for 24 h. Osteosarcoma cells were harvested by EDTA-free trypsinization and then washed with pre-chilled PBS. Then, we placed it overnight at 7 °C in 75% alcohol. The next day, we stained the cells with propidium iodide (PI) and RNase A according to the instructions of the KGI Biocycle Kit (Nanjing, China). Since the stain is a photosensitizer, we stain in the dark. After

incubation for 30 min, we analyzed the stained cells by BD Accuri C6 Plus (Becton - Dickinson, San Jose, CA) software. All experiments were repeated three times.

Apoptosis flow-cytometry assay

Cell apoptosis was detected by Annexin-V-FITC and PI staining. Osteosarcoma cells were plated at 6-well plate and treated with various concentration (0, 2, 4 mg/mL) of Huaier for 24 h. Next, we collected cells with EDTA-free trypsin followed by Annexin V FITC Apoptosis Assay Kit (Hanbio, Shanghai, China) instructions for operation. For the reason phosphatidylserine can bind to Annexin V-FITC, we determined the proportion of cells that were early apoptotic; PI was a DNA-binding dye which has excitation and emission wavelengths of 617 nm and 536 nm, respectively. It can produce red fluorescence but no membrane permeability, not through the living cell membrane. So, we use it to determine the proportion of cells with late apoptosis/death. Annexin V-FITC and PI are photosensitizers and we use them in the dark. We immediately analyzed stained cells using BD Accuri C6 Plus (Becton - Dickinson, San Jose, CA) software.

Western blot analysis

We scraped the cells into EP tubes with cell scraping and lysed the supernatant proteins with RIPA lysis buffer (Beyotime Institute of Biotechnology, Beijing, China). All operations were performed on ice. We measured the protein concentration of the sample using the BCA assay kit (Beyotime Biotechnology Institute). The remaining protein was boiled at 100 ° C for 5 min, and stored at minus 20 degrees. We separated the total protein by 10–15% SDS-PAGE gel electrophoresis then transferred it to the polyvinylidene fluoride (PVDF) membrane by electrolysis on ice. After blocking the PVDF membrane with 5% BSA in TBST for 45 minutes, the primary antibody of the target protein was incubated for 2 h, and then the membrane was washed three times with TBST for ten minutes each time. Next, goat anti-rabbit secondary antibody cultured with horseradish peroxidase were incubated for 1 h. The membrane was still washed three times with TBST for ten minutes each time. Finally, the immune response zone was detected using an ECL detection system (SmartChemi 420, Beijing, China). Every experiment was performed three times.

Drug-target search and functional enrichment analysis

The BATMAN-TCM is a traditional Chinese medicine database which contains detailed information on various drugs and their related information. In this study, the main target genes of Huaier were found using Chinese medicine database. Next, we use BATMAN-TCM to perform pathway enrichment analysis and gene ontology. BATMAN-TCM provides researchers with a comprehensive and systematic functional annotation tool to discover the biological implications behind the list of submitted genes. Gene ontology analysis includes molecular functions (MF), cellular components (CC), and biological processes (BP). $P < 0.05$ was considered statistically significant. The terms of GO results were also ranked by P value. We used the same manner to carry out the KEGG pathway.

Statistical analysis

Statistical analysis was performed using Graphpad prism 7 software and Student's t-test was carried out to analyze statistical differences. $P < 0.05$ was considered as a statistically significant difference. The result is shown as the mean $\pm$ SD. All experimental data was repeated at least three times.

Results

Drug-target search, enrichment analysis and Gene Ontology

Huaier was input into the Chinese medicine database BATMAN. Then, the main corresponding target genes of Huaier were detected (Table 1). Subsequently, we performed enrichment analysis on pathways and GO functions. To assess the functional characteristics of the Huaier-mediated genome, we carried out GO annotations on all genes. The top 12 items of GO and KEGG enrichment analysis were then selected based on the P -value ranking (Figs. 1a, b). Among the top 12 KEGG terms and 12 GO terms, we subsequently selected some terms of interest (Table 2). Functional analysis showed that the genes associated with Huaier are mainly related to cell growth. In addition, the KEGG results showed that the MAPK signaling pathway is involved in the antitumor effect mediated by Huaier.

Table 1
The corresponding target genes of Huaier extract using BATMAN

Predicted targets [Gene Symbol] ranked according to the decreasing score
CACNA2D1 (26.373) SRD5A1 (26.373) CACNA1H (26.373) ESR2 (26.373) ESR1 (26.373) CACNA2D2 (26.373) CACNA1S (26.373) PGR (26.373) AR (26.373) CACNA1C (26.373) CACNA1D (26.373) NR1I2 (26.373) HSD17B1 (26.373) CACNB2 (26.373) CACNG1 (26.373) HSD17B1 (26.373) CRYZ (23.000) VKORC1 (23.000) NQO1 (23.000) CRYZ (23.000) VKORC1 (23.000) NQO1 (23.000)
BATMAN-TCM: bioinformatics analysis tool for molecular mechanism of traditional Chinese medicine

Table 2
Notable pathways/gene ontology enrichment terms in 12 top KEGG/GO

KEGG term	P-value	Count	Genes
MAPK signaling pathway	0.0000000833	8	CACNA1C;CACNA1D;CACNA1H;CACNA1S;CACNA2D1;CACNA2D2;CACNB2;CACNG1;
GO term	P-value	Count	Genes
GO:0040007~ growth	0.00765	4	AR;CACNA2D2;ESR1;ESR2;
KEGG: "Kyoto Gene and Genomic Encyclopedia", GO: "Gene Ontology".			

Huaier inhibits the proliferation of MG-63 and MNNG/HOS cells in a dose- and time-dependent manner

In order to test the cytotoxicity effects of Huaier on OS cells, MG-63 and MNNG/HOS cells were cultured and treated with various concentrations of Huaier for 24 h, followed by using CCK-8 assay to analyze the cell viability. The concentration is selected basing on the concentration of Huaier acting on other tumors. The CCK-8 assay showed Huaier could inhibit the proliferation of MG-63 and MNNG/HOS cells in a dose- and time-dependent manner. The IC₅₀ (the half maximal inhibitory concentration) of MG-63 and MNNG/HOS cells treated with Huaier for 24 h were 2.89 mg/mL and 2.48 mg/mL, respectively (Figs. 2a, b). In the subsequent experiment, we selected 2 mg/mL and 4 mg/mL as representative doses for the in vitro treatment. Next, we determined the cytotoxic effects of Huaier on normal human fibroblast cells in comparison with osteosarcoma cells. As shown in Fig. 2c, the cytotoxicity of Huaier for the normal cells showed much lower levels than for the osteosarcoma cells tested under the same conditions.

Colony formation assay was carried out to detect the anti-proliferative effect of Huaier on the MG-63 and MNNG/HOS cell lines (Figs. 2d-g). Similar results from the colony formation assay confirmed Huaier-treated groups exhibited lower and fewer colonies compared to the untreated cells.

Huaier induces cell cycle arrest in MG-63 and MNNG/HOS cells

OS cells were treated with 2 and 4 mg/mL Huaier for 24 h and tested by flow cytometry. As shown in Figs. 3a-d, Huaier induced cell cycle arrest in both OS cell lines in a dose-dependent manner, as evidenced by the increased percentage of cells in the G₀/G₁ phase. MG-63 cells in G₀/G₁ phases (control 63.6%):79.5% at 2 mg/mL and 86.4% at 4 mg/mL. MNNG/HOS cells in G₀/G₁ phases (control 58.6%):63.4% at 2 mg/mL and 73.4% at 4 mg/mL. On the other hand, the percentage of OS cells in the G₂/M phase was decreased definitely. MG-63 cells in G₂/M phases (control 24.2%):12.7% at 2 mg/mL and 7.3% at 4 mg/mL. MNNG/HOS cells in G₂/M phases (control 26.3%):20.5% at 2 mg/mL and 15.2% at 4 mg/mL. In order to gain insight into the underlying mechanisms of the cell cycle arrest, the expression of cell-cycle-associated proteins were carried out by Western blotting. The Huaier treatment decreased the expression of Cyclin D1 and increased the expression of P21 and P27 (Figs. 3e-g).

Huaier induces apoptosis in MG-63 and MNNG/HOS cells

Annexin V-FITC/PI staining was carried out to test apoptosis and the death rate of OS cells under treatment with Huaier. Huaier induced the apoptosis of MG-63 and MNNG/HOS cells in a dose-dependent manner (Figs. 4a, b). Treatment with Huaier for 24 h resulted in nearly 40% apoptosis and death in MG-63 cells, while the apoptosis and death rate of the control cells was only 18%

(Fig. 4c). Similar effects were observed for the MNNG/HOS cells (Fig. 4d). To further understand the mechanisms of cell apoptosis, we examined the changes of apoptosis-associated regulatory proteins using Western blotting. Results showed that Huaier could increase the expression of Cleaved-Caspase 3 and Bax, and decrease the expression of Bcl-2 (Figs. 4e, f). Similar effects were observed in the MNNG/HOS cell line (Figs. 4e, g).

Huaier inhibits the migration and invasion of MG-63 and MNNG/HOS cells

The migration and invasion of MG-63 and MNNG/HOS cells under treatment with 2 and 4 mg/mL Huaier for 24 h were respectively tested by wound-healing detection and the Transwell invasion test *in vitro*. As shown in Figs. 5a-b, the relative wound closure under 2 mg/mL and 4 mg/mL of Huaier was 38.55% and 10.92%, respectively, in MG-63 cells. In MNNG/HOS cells, the relative wound closure under 2 mg/mL and 4 mg/mL Huaier was 27.73% and 7.61%, respectively. Moreover, we performed the Transwell invasion test by adding 20% FBS in the lower wells with or without 2 mg/mL Huaier. As shown in Figs. 5c-d, the number of Huaier-treated OS cells invading through a Matrigel-coated membrane was obviously lower than that of untreated cells. Since under 2 mg/mL of Huaier almost no cells passed through the Matrigel, we did not repeat the test with 4 mg/mL Huaier. Collectively, our results showed, for the first time, that Huaier is involved in the migration and invasion of OS cells.

Huaier may suppress OS cells via the MAPK signaling pathway

To determine whether the MAPK signaling pathway is involved in the OS cancer cell proliferation and apoptosis induced by Huaier, the expression levels of ERK, JNK and P38-MAPK were tested by Western blot. We treated MG-63 and MNNG/HOS cells with Huaier for 24 hours. The total ERK content was basically unchanged while the P-ERK/ERK content was significantly reduced. Furthermore, both P-JNK/JNK and P-P38/P38 levels increased, which was consistent in both cell lines (Figs. 6a-c).

Discussion

Huaier extract, a natural, traditional Chinese medicine, has got growing attention in recent years for its anti-tumor effect. Previous studies on Huaier extract showed that a variety of cancer cells are suppressed by Huaier via inhibition of proliferation and promotion of apoptosis. In addition, multiple signaling pathways and biological processes were found to be involved in the anticancer effect of Huaier, such as JAK2/STAT3 (Yang et al. 2016), autophagy (Wang et al. 2015), Wnt signaling (Zhang et al. 2013), and the PIK3/AKT signaling pathway (Xie et al. 2015), among others. However, the effect of Huaier on OS and the associated mechanisms are yet to be elucidated. In this study, we observed that Huaier could inhibit OS cell proliferation by blocking G0/G1 into S phase. It could also suppress invasion and migration and promote apoptosis of OS cells in a time- and dose-dependent manner. The MAPK pathway, among the most frequently mutated signaling pathway in human cancers, has been recognized as a promising target of cancer therapy (Bao et al. 2016). Furthermore, we found the MAPK pathway is involved in the anti-OS effect of Huaier.

We analyzed the target genes of Huaier extract using bioinformatics. The results showed that Huaier is closely related to cell growth. Moreover, the MAPK pathway plays an important role in the biological effects of Huaier. Therefore, we used the CCK-8 and clone formation assays to show that Huaier can reduce the viability and inhibit the proliferation of OS cells. Previous studies have shown that numerous anticancer medicines can arrest the cell cycle in the G0/G1, S, and G2/M phases (Lee et al. 2007). Flow cytometry was performed to detect the cell cycle of MG-63 and MNNG/HOS cells under treatment with Huaier. In contrast to results obtained by other study (Matsumoto et al. 2008; Yan et al. 2015), Huaier increased the percentage of OS cells in the G0/G1 phase, while decreasing the cell percentage in the G2/M phase. Similar medicine-induced of G0/G1 arrest has also been detected in other cell type, such as human breast cancer cells (Zhou et al. 2018), and rheumatoid arthritis fibroblast-like synoviocytes (Zhang et al. 2018a). Cyclin D1 is a cell-cycle-associated protein which is involved in the transition from the G1 phase to the S phase (Wang et al. 2018). Overexpression of Cyclin D1 is linked to the progression and development of cancer (Cai et al. 2012). Multiple studies have shown that the expression of Cyclin D1 is decreased when the cell cycle is arrested (Zhang et al. 2018b). P21 and P27 are Cyclin-dependent kinase inhibitors, which always increase when cancer cell proliferation is induced (Zhou et al. 2008). To further explore the mechanism of Huaier-induced cell cycle arrest, Western blotting was performed to examine the expression levels of cell-cycle-associated proteins. Under treatment with Huaier, the expression of P21 and P27 increased, while Cyclin D1 expression decreased. This indicates that Huaier suppressed the proliferation of OS cells by regulating the expression of cell-cycle-associated proteins. However, the underlying mechanism should be explored further.

Multiple studies have shown that Huaier suppresses tumor development by inducing cell apoptosis. Consequently, flow cytometry was used to detect the apoptotic rate of Huaier-treated OS cells. As expected, the rate of apoptosis and death of OS cells increased significantly after exposure to Huaier. Apoptosis is a crucial biological process in the regulation of cancer development and treatment response. There are two distinct apoptotic pathways, namely, the extrinsic membrane death-receptor pathway and the intrinsic mitochondrial pathway. Many chemopreventive medicines promote cancer cell apoptosis through the mitochondrial pathway (Liu et al. 2012). Bcl-2 and Bax are two key proteins involved in the intrinsic mitochondrial pathway (Li et al. 2009). Bax can induce mitochondria to release cytochrome C, which enhances cell apoptosis. In contrast, the function of Bcl-2 is to suppress apoptosis by blocking the release of cytochrome C (Walensky 2006). Cytochrome C activates the down-regulation of the caspase cascade, while Caspase3 ends this process (Kroemer et al. 2007). By assessing the expression of apoptosis-related proteins, we showed that Bcl-2 decreased while Cleaved-Caspase3 and Bax increased significantly under treatment with Huaier. These findings suggest that Huaier promoted apoptosis in OS cells by triggering the mitochondrial-signaling pathway.

To detect the migration and invasion ability of OS cells under the Huaier treatment, we performed the wound healing test and Transwell assay. Obtained results showed that Huaier can inhibit the migration and invasion of OS cells. The low five-year survival rate of OS is closely related to its strong metastatic ability. Therefore, Huaier could be a potential drug for the treatment of OS metastasis. Based on the abovementioned results, Huaier could inhibit the proliferation and promote the apoptosis of OS cells. To explore the underlying mechanisms, we used Western blotting to detect the expression of major proteins in the MAPK signaling pathway which was predicted to be involved in the antitumor effect of Huaier by bioinformatics analysis. Results showed that P-ERK/ERK decreased, while P-JNK/JNK and P-P38/P38 increased. Numerous studies have shown that ERK is involved in cell proliferation, migration and invasion (Chen et al. 2018; Liu et al. 2018; Reddy et al. 2003). These results illustrated that Huaier can inhibit the proliferation and motility of OS cells by downregulating P-ERK. The JNK protein kinases include JNK1, JNK2, and JNK3, of which JNK1 and JNK2 are widely present in various cells (Bode and Dong 2007). P38 is generally divided into four subtypes, of which the most widely studied is P38 α (Ono and Han 2000). Bcl2 is a downstream protein of JNK1 and JNK2, while P38 α can affect Bax expression (Weston and Davis 2007). Therefore, JNK and P38 are closely related to the mitochondrial apoptosis pathway. P38 α can also down-regulate the content of Cyclin family proteins, thereby negatively regulating the cell cycle (Thornton and Rincon 2009). Consequently, it may be considered that Huaier can affect proliferation and apoptosis in OS cells by influencing MAPK pathway.

Conclusions

In this study, we showed that Huaier is a potential drug for the treatment of OS. Huaier inhibited the proliferation, suppressed the invasion and migration, and promoted the apoptosis of OS cells in a time- and dose-dependent manner. Furthermore, the MAPK pathway was shown to be involved in the anticancer effect of Huaier (Fig. 7). Further studies should assess the effects of Huaier on OS *in vivo*, possibly using a xenograft mouse model of OS.

Abbreviations

OS: Osteosarcoma

TCM: Traditional Chinese medicine

MAPKs: Mitogen-activated protein kinases

ERK: Extracellular signal-regulated kinase

JNK: C-Jun N-terminal kinase

DMEM: Dulbecco's modified Eagle's medium

NEAA: Non-essential amino acid

CCK8: Cell counting kit – 8

PI: Propidium iodide

PVDF: Polyvinylidene fluoride

MF: Molecular functions

CC: Cellular components

BP: Biological processes

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All available data and material can be accessed.

Competing interests

Not applicable

Funding

This work was partially supported by the National Nature Science Foundation of China (Grant No. 81972072) to Li M; the National Nature Science Foundation of China (Grant Nos. 81771108, 81970964) to Guo J; and the National Natural Science Foundation of China (Grant No. 81800982) to Liu H.

Authors' contributions

XG performed the experiment, and was a major contributor in writing the manuscript, YZ and PY analyzed the data, RT, DL, YK, HL and ML critically revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable

References

1. Bao H, Liu P, Jiang K, Zhang X, Xie L, Wang Z, Gong P (2016) Huaier polysaccharide induces apoptosis in hepatocellular carcinoma cells through p38 MAPK Oncology Letters 12:1058-1066 doi:10.3892/ol.2016.4686
2. Bode AM, Dong Z (2007) The functional contrariety of JNK Molecular Carcinogenesis 46:591-598 doi:10.1002/mc.20348
3. Cai Q et al. (2012) Hedyotis diffusa Willd Inhibits Colorectal Cancer Growth in Vivo via Inhibition of STAT3 Signaling Pathway International Journal of Molecular Sciences 13:6117-6128 doi:10.3390/ijms13056117
4. Chang LF, Karin M (2001) Mammalian MAP kinase signalling cascades Nature 410:37-40 doi:10.1038/35065000
5. Chen Y et al. (2018) Huaier Granule extract inhibit the proliferation and metastasis of lung cancer cells through down-regulation of MTDH, JAK2/STAT3 and MAPK signaling pathways Biomedicine & Pharmacotherapy 101:311-321 doi:10.1016/j.biopha.2018.02.028
6. Dhillon AS, Hagan S, Rath O, Kolch W (2007) MAP kinase signalling pathways in cancer Oncogene 26:3279-3290 doi:10.1038/sj.onc.1210421

7. Hobbs GA, Der CJ, Rossman KL (2016) RAS isoforms and mutations in cancer at a glance *Journal of Cell Science* 129:1287-1292 doi:10.1242/jcs.182873
8. Ji X et al. (2017) *Trametes robiniophila* may induce apoptosis and inhibit MMPs expression in the human gastric carcinoma cell line MKN-45 *Oncology Letters* 13:841-846 doi:10.3892/ol.2016.5517
9. Kroemer G, Galluzzi L, Brenner C (2007) Mitochondrial membrane permeabilization in cell death *Physiological Reviews* 87:99-163 doi:10.1152/physrev.00013.2006
10. Kubo T, Shimose S, Fujimori J, Furuta T, Ochi M (2015) Quantitative (201)thallium scintigraphy for prediction of histological response to neoadjuvant chemotherapy in osteosarcoma; systematic review and meta-analysis *Surgical Oncology-Oxford* 24:194-199 doi:10.1016/j.suronc.2015.06.009
11. Lee C-H, Lim H, Moon S, Shin C, Kim S, Kim B-J, Lim Y (2007) Novel anticancer agent, benzyldihydroxyoctenone, isolated from *Streptomyces* sp causes G(1) cell cycle arrest and induces apoptosis of HeLa cells *Cancer Science* 98:795-802 doi:10.1111/j.1349-7006.2007.00473.x
12. Li X et al. (2009) Inhibitor of growth 4 induces apoptosis in human lung adenocarcinoma cell line A549 via Bcl-2 family proteins and mitochondria apoptosis pathway *Journal of Cancer Research and Clinical Oncology* 135:829-835 doi:10.1007/s00432-008-0519-7
13. Liu F, Yang X, Geng M, Huang M (2018) Targeting ERK, an Achilles' Heel of the MAPK pathway, in cancer therapy *Acta Pharmaceutica Sinica B* 8:552-562 doi:10.1016/j.apsb.2018.01.008
14. Liu Z, Li D, Zhao W, Zheng X, Wang J, Wang E (2012) A Potent Lead Induces Apoptosis in Pancreatic Cancer Cells *Plos One* 7 doi:10.1371/journal.pone.0037841
15. Matsumoto S, Arakawa Y, Ohishi M, Yanaihara H, Iwanaga T, Kurokawa N (2008) Suppressive action of pituitary adenylate cyclase activating polypeptide (PACAP) on proliferation of immature mouse Leydig cell line TM3 cells *Biomedical Research-Tokyo* 29:321-330 doi:10.2220/biomedres.29.321
16. Miller RW (1981) Contrasting epidemiology of childhood osteosarcoma, Ewing's tumor, and rhabdomyosarcoma *National Cancer Institute monograph*:9-15
17. Ono K, Han JH (2000) The p38 signal transduction pathway - Activation and function *Cellular Signalling* 12:1-13 doi:10.1016/s0898-6568(99)00071-6
18. Ottaviani G, Jaffe N (2009) The Epidemiology of Osteosarcoma. In: Jaffe N, Bruland OS, Bielack SS (eds) *Pediatric and Adolescent Osteosarcoma*, vol 152. *Cancer Treatment and Research*. pp 3-13. doi:10.1007/978-1-4419-0284-9_1
19. Pan J, Yang C, Jiang Z, Huang J (2019) *Trametes robiniophila* Murr: a traditional Chinese medicine with potent anti-tumor effects *Cancer Management and Research* 11:1541-1549 doi:10.2147/cmar.s193174
20. Reddy KB, Nabha SM, Atanaskova N (2003) Role of MAP kinase in tumor progression and invasion *Cancer and Metastasis Reviews* 22:395-403 doi:10.1023/a:1023781114568
21. Song X, Li Y, Zhang H, Yang Q (2015) The anticancer effect of Huaier *Oncology Reports* 34:12-21 doi:10.3892/or.2015.3950
22. Thornton TM, Rincon M (2009) Non-Classical P38 Map Kinase Functions: Cell Cycle Checkpoints and Survival *International Journal of Biological Sciences* 5:44-51 doi:10.7150/ijbs.5.44
23. Wagner EF, Nebreda AR (2009) Signal integration by JNK and p38 MAPK pathways in cancer development *Nature Reviews Cancer* 9:537-549 doi:10.1038/nrc2694
24. Walensky LD (2006) BCL-2 in the crosshairs: tipping the balance of life and death *Cell Death and Differentiation* 13:1339-1350 doi:10.1038/sj.cdd.4401992
25. Wang J, Li X-m, Bai Z, Chi B-x, Wei Y, Chen X (2018) Curcumol induces cell cycle arrest in colon cancer cells via reactive oxygen species and Akt/ GSK3 beta/cyclin D1 pathway *Journal of Ethnopharmacology* 210:1-9 doi:10.1016/j.jep.2017.06.037
26. Wang X et al. (2015) Huaier Extract Induces Autophagic Cell Death by Inhibiting the mTOR/S6K Pathway in Breast Cancer Cells *Plos One* 10 doi:10.1371/journal.pone.0131771
27. Wang X, Zhang N, Huo Q, Sun M, Lv S, Yang Q (2013) Huaier aqueous extract suppresses human breast cancer cell proliferation through inhibition of estrogen receptor a signaling *International Journal of Oncology* 43:321-328 doi:10.3892/ijo.2013.1947
28. Weston CR, Davis RJ (2007) The JNK signal transduction pathway *Current Opinion in Cell Biology* 19:142-149 doi:10.1016/j.ceb.2007.02.001

29. Xie H-X, Xu Z-Y, Tang J-N, Du Y-A, Huang L, Yu P-F, Cheng X-D (2015) Effect of Huaier on the proliferation and apoptosis of human gastric cancer cells through modulation of the PI3K/AKT signaling pathway *Experimental and Therapeutic Medicine* 10:1212-1218 doi:10.3892/etm.2015.2600
30. Yan L, Liu X, Yin A, Wei Y, Yang Q, Kong B (2015) Huaier aqueous extract inhibits cervical cancer cell proliferation via JNK/p38 pathway *International Journal of Oncology* 47:1054-1060 doi:10.3892/ijo.2015.3094
31. Yang A, Fan H, Zhao Y, Zha X, Zhang H, Hu Z, Tu P (2016) Huaier aqueous extract inhibits proliferation and metastasis of tuberous sclerosis complex cell models through downregulation of JAK2/STAT3 and MAPK signaling pathways *Oncology Reports* 36:1491-1498 doi:10.3892/or.2016.4969
32. Yang L, Song Z, Wang X, Yang W, Wang M, Liu H (2017) Huaier extract enhances the treatment efficacy of paclitaxel in breast cancer cells via the NF-kappa B/I kappa B alpha pathway *Oncology Reports* 38:3455-3464 doi:10.3892/or.2017.6024
33. Zhang T et al. (2013) Huaier aqueous extract inhibits colorectal cancer stem cell growth partially via downregulation of the Wnt/beta-catenin pathway *Oncology Letters* 5:1171-1176 doi:10.3892/ol.2013.1145
34. Zhang X et al. (2018a) Aspirin promotes apoptosis and inhibits proliferation by blocking G(0)/G(1) into S phase in rheumatoid arthritis fibroblast-like synoviocytes via downregulation of JAK/STAT3 and NF-kappa B signaling pathway *International Journal of Molecular Medicine* 42:3135-3148 doi:10.3892/ijmm.2018.3883
35. Zhang X, Feng H, Li Z, Guo J, Li M (2018b) Aspirin is Involved in the Cell Cycle Arrest, Apoptosis, Cell Migration, and Invasion of Oral Squamous Cell Carcinoma *International Journal of Molecular Sciences* 19 doi:10.3390/ijms19072029
36. Zhou Q et al. (2018) 3-Nitroacridine derivatives arrest cell cycle at G0/G1 phase and induce apoptosis in human breast cancer cells may act as DNA-target anticancer agents *Life Sciences* 206:1-9 doi:10.1016/j.lfs.2018.05.010
37. Zhou Y et al. (2008) Lactotransferrin: A candidate tumor suppressor - Deficient expression in human nasopharyngeal carcinoma and inhibition of NPC cell proliferation by modulating the mitogen-activated protein kinase pathway *International Journal of Cancer* 123:2065-2072 doi:10.1002/ijc.23727

Figures

a

b

Figure 1

The functional enrichment analysis of Huaier BATMAN-TCM was used to analyze the KEGG pathway and GO (biological processes) annotation of all genes. The top 12 items of GO and KEGG enrichment analysis were selected based on the P-value ranking (Figs. 1a, b). BATMAN-TCM: bioinformatics analysis tool for molecular mechanism of traditional Chinese medicine, KEGG: Kyoto Gene and Genomic Encyclopedia, GO: Gene Ontology.

Figure 2

Anti-proliferative effect of Huaier on MG-63 and MNNG/HOS cell lines The cell viability of OS cells and normal human cells was determined by cell counting kit 8 assay. Cells were treated with different Huaier for 12-72h. Huaier inhibits cell viability in a dose- and time- dependent manner. Data are presented as means $\pm$ SD of three independent experiments. Set the cell viability of control group as 100% (Figs. 2a-c). Clone forming assay was performed on MG-63 and MNNG/HOS cell. MG-63 and MNNG/HOS cells were treated with increasing concentrations of Huaier for 10 days (Figs. 2d, f). The colony formation numbers of MG-63 and MNNG/HOS was reduced significantly treated with increasing concentration of Huaier. The data analysis was from three independent experiments (Figs. 2e, g). * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

Figure 3

Huaier induced cell cycle arrest in MG-63 and MNNG/HOS cells by increasing the percentage of cells in G0/G1 phase

Figure 4

Huaier induced apoptosis in a dose-dependent manner in MG-63 and MNNG/HOS cells

Figure 5

Huaier is involved in migration and invasion of MG-63 and MNNG/HOS cells

Figure 6

Huaier targeted ERK, JNK and P38 kinases in MG-63 and MNNG/HOS cells

Figure 7

The MAPK pathway is involved in the anticancer effect of Huaier