Curculigoside Represses the Proliferation and Metastasis of Osteosarcoma via the JAK/STAT and NF-κB Signaling Pathways

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Received April 27, 2022; accepted June 27, 2022

Curculigoside (Cur) is a natural component from Curculigo orchioides Gaertn, with various bioactivities. The function of Cur in the nervous system and osteoarthritis has been reported. However, its role in osteosarcoma (OS) needs to be investigated. Hence, we focus on probing the impact of Cur on OS. In vitro, cell counting kit 8 (CCK-8), flow cytometry and Transwell assay were used to investigate the effects of Cur on OS cell proliferation, apoptosis, migration and invasion. In vivo, we developed a xenograft model to figure out the effect of Cur on tumor growth in nude mice. Western blotting (WB) was conducted to compare the levels of Cur on apoptosis-related proteins (C-caspase-3, Bax, and Bcl-2), epithelial–mesenchymal transition (EMT)-related proteins (N-cadherin, Snail, and E-cadherin) and the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and nuclear factor-κB (NF-κB) pathways in vitro and in vivo. In-vitro data testified that Cur treatment markedly hampered OS cells’ growth, migration and invasion and intensified their apoptosis compared to that of the control group. In vivo, Cur treatment notably hampered the growth of OS tumors in mice. In addition, both in vitro and in vivo experiments demonstrated that the phosphorylation of JAK2, STAT3, and NF-κB were inhibited through Cur treatment. Furthermore, the inhibition of Cur in OS cells was demonstrated by up-regulating the expression of JAK/STAT and NF-κB pathways protein levels. In summary, the data suggest that Cur curbs OS growth by down-regulating the JAK/STAT and NF-κB pathways, which is an underlying therapeutic option for OS treatment.

Key words osteosarcoma; curculigoside; Janus kinase 2 (JAK2); signal transducer and activator of transcription 3 (STAT3); nuclear factor-κB (NF-κB)

INTRODUCTION

Osteosarcoma (OS), a primary malignant tumor derived from osteogenic mesenchymal stem cells, mainly occurs in childhood and adolescence.1,2) With recent medical advances, the combination of surgery and chemotherapy has been chosen as the primary clinical treatment for OS.3,4) Therefore, the 5-year survival rate for OS patients has been effectively improved.5) However, survival rates for osteosarcoma patients remain low due to the high rate of metastasis and recurrence of osteosarcoma caused by the drawbacks of treatment-drug resistance.6) In recent years, Chinese herbs have been widely used because of their unique effects, which relieve the symptoms of cancer patients as well as control the size of the tumor and prolong the survival of patients. Additionally, some herbal medicines were found to have the ability to target cancer cells directly or exert adjuvant anti-tumor effects by alleviating the side effects of other anti-tumor drugs.7)

Natural herbs have long been a part of Asian culture, especially their application in cancer treatment has spread widely in recent years. Curculigoside (Cur) is a saponin of traditional Chinese medicine Curculigo orchioides Gaertn.8) Cur has diversified pharmacological activities. Cur has been shown in many studies to have bone-protective properties, suggesting that it might be a possible osteoporosis treatment.9,10) In addition, it has been demonstrated that Cur contains antioxidant,11) anti-inflammatory,12) and neuroprotective characteristics.13) However, the function of Cur in OS is little understood.

Janus kinases (JAKs) and signal transducer and activator of transcription (STAT) proteins (especially STAT3) have played essential roles in the occurrence and development of cancer. Studies have documented that this pathway is involved in tumor cells’ proliferation, survival, invasion, and metastasis,14,15) which also participates in the incidence and progression of many malignancies.16–19) This pathway is also critical to the development of OS. It has been confirmed that the abnormality of JAK/STAT pathway in OS can lead to the dysregulation of downstream target genes, thus affecting the disease progression.20) Nuclear factor-κB (NF-κB), as a dimer complex, plays a role in many human diseases.21) Abnormally activated NF-κB has become a participant in the development of cancer.22) As reported, tumor tissues from 75% of OS patients displayed activated NF-κB. Patients with abnormally activated NF-κB had shorter median survival than patients with non-activated NF-κB.23) Besides, NF-κB may function as a downstream gene of JAK/STAT pathway, which affects tumor proliferation and invasion. For example, activation of the JAK/STAT3 pathway facilitates the growth and angiogenesis of esophageal squamous cell carcinoma cells through up-regulation of NF-κB p65, which in turn fosters the progression of esophageal squamous cell carcinoma.24) However, the role and mechanism of the JAK/STAT and NF-κB pathways in OS need to be further investigated.

This study aimed to understand the function and related molecular mechanisms of Cur in OS. The results indicated that Cur restrained OS cells’ proliferation, migration and inva-
sion, and impeded the JAK/STAT and NF-κB pathways activation. Accordingly, we confirmed that the anti-tumor effect of Cur in OS is associated with the inactivation of the JAK/STAT and NF-κB pathways.

MATERIALS AND METHODS

Cell Culture and Treatment OS cell lines Saos-2 and MG63 were bought from the Cell Center of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in the RPMI1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, CA, U.S.A.) at 37 °C in an incubator with 5% CO₂. Thermo Fisher Scientific provided the RPMI1640 medium and FBS (MA, U.S.A.). During the logarithmic growth phase, the cells were sub-cultured by 0.25% trypsin (Thermo Fisher HyClone, UT, U.S.A.). Saos-2 and MG63 cells were treated with Cur (MCE, Shanghai, China) at 37 °C in primary antibodies (Abcam, MA, U.S.A.; 1:1000): anti-JAK2 (ab108596), anti-p-JAK2 (ab32101), anti-STAT3 (ab68153), anti-p-STAT3 (ab267373), anti-NF-κB (ab32536), anti-p-NF-κB (ab76302), anti-N-cadherin (ab76011), anti-Snail (ab216347), anti-E-cadherin (ab40772), anti-C-caspase-3 (ab32042), anti-Bax (ab32503), anti-Bcl-2 (ab32124) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab9485). After washing the membranes three times with TBST, the membranes were incubated with the fluorescein-labeled secondary antibody (Beyotime Biotechnology) for 1.5 h. At last, they were rinsed three times, treated with the enhanced chemiluminescence (ECL) chromogenic agent (D601039, Sangon Biotech, Shanghai, China) and imaged with the membrane scanner (Fusion FX, VILBER LOURMAT, France).

Tumor Formation Experiment in Nude Mice Twenty male BALB/c-nu/nu nude mice (6–8 weeks old; 18–22 g) were obtained from the Experimental Animal Center of Wuhan University. All mice were reared as per the guidelines set by the National Science Council of China. Animal studies were conducted in compliance with the requirements of the Experimental Animal Ethics Committee of Jiangxi University of Chinese medicine. Saos-2 and MG63 cells (1 × 10⁶ cells) were injected subcutaneously into the right axilla of mice, respectively. When the subcutaneous tumors had grown to approximately 50 mm³, mice were randomly divided into Con and Cur groups (1.0 mg/kg). The Cur group was administered daily by tail vein injection (1.0 mg/kg Cur per day), and the con. group received an equal amount of saline daily. After continuous administration for 28 d (with tumor size measured weekly during this period), five nude rats in each group were randomly injected with 200 mg/kg of sodium phenobarbital intravenously to cause respiratory arrest. After euthanasia of mice, tumor tissues were separated and weighed, and tumor length and width were measured.

Immunohistochemistry (IHC) The paraffin-embedded tumor tissue was sliced (4 μM), dehydrated by xylene, and then hydrated with gradient alcohol. After treatment with 3% H₂O₂ for 10 min, the antigenic thermal repair was performed with different antigen repair solutions depending on the type of primary antibodies. Next, 5% bovine serum albumin (BSA) was used to block the sections for 20 min and incubated with the primary antibodies of p-JAK2 (ab195055, Abcam, 1:500), p-STAT3 (ab76315, Abcam, 1:200), p-NF-κB (ab194908, Abcam, 1:200) at 4 °C overnight. The next day, the goat anti-rabbit secondary antibody was incubated at room temperature (r.t.) for 20 min after PBS rinsing. Finally, the sections were developed by dimethylaminobenzene (DAB). The negative control group was given PBS instead of primary antibody. After immunohistochemical staining, 5 sections of each specimen were randomly selected and photographed under 200× light microscopy in 10 non-overlapping fields. Tumor cells with positive expression were analyzed by randomized double-blind cell counting. Positive expression of p-JAK2, p-STAT3 and p-NF-κB was mainly in the form of brown or tan granular staining of the cell membrane and cytoplasm. Five high
magnification fields (×400) were taken at different locations to count 1000 tumor cells in a cell-rich area. The number of positively stained cells in each field of view was recorded. Positive cell rate = positive cells/total number of cells ×100%.

**Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate (dUTP)-Biotin Nick End Labeling (TUNEL) Assay** Mouse tumor tissue was paraffin-embedded, sectioned, dewaxed, hydrated, washed with xylene and ethanol and then immobilized for 15 min using 4% paraformaldehyde. Then, sections were added with 20 µg/mL Proteinase K solution, washed and remobilized using 4% paraformaldehyde for 5 min. The cells were stained by TUNEL kit (Beyotime Biotechnology). Leica (DM2500) fluorescence microscope was used for microscopic observation and take images. Five high magnification fields were randomly picked for each section. Apoptosis rate = apoptotic cells/total cells ×100%.

**Statistics and Analysis** Data were processed using GraphPad Prism 9.0 (GraphPad Software, CA, U.S.A.) for plotting and statistical analysis. Measures are expressed as mean ± standard deviation (X ± S.D.) and compared using ANOVA. t Test was used for comparison between the two groups. p < 0.05 was considered statistically significant.

**RESULTS**

**Cur Hindered OS Cell Viability** To investigate Cur’s antitumor effect on OS cell lines, we cultured Saos-2 and MG63 cells with different concentrations of Cur. As can be shown, the inhibition rate of Saos-2 and MG-63 cells was the highest at a Cur concentration of 64 µg/mL, which indicates that the inhibition effect is the most obvious. The inhibition rate at Cur concentration of 128 µg/mL was not significantly different from that of Cur at 64 µg/mL (Fig. 1B). In addition, we treated Saos-2 and MG63 cells with 8, 16, and 32 µg/mL of Cur, respectively, and observed the changes in cells during a 72-h period. The results showed that Cur strengthened the inhibitory effect of OS cells on survival in a concentration and time-dependent manner (Fig. 1C). That is to say, the higher the concentration and the longer the incubation time, the more effective Cur is in inhibiting cell growth. Therefore, a clear correlation was observed between the concentration of Cur and OS cells.

**Cur Induces Apoptosis in OS Cells** After finding that Cur was able to inhibit OS cells viability, we used flow cytometry to observe the anti-apoptotic effect of Cur on Saos-2 and MG63 cells at 8, 16 and 32 µg/mL of Cur. The data indicated that rate of apoptosis inhibition in OS cells was proportional to the concentration of Cur, which means that Cur increased the apoptosis rate in a dose-dependently (Fig. 2A). Furthermore, the WB results showed that the expression of cleaved caspase-3 and Bax increased with the increase of Cur concentration among the Cur group. In contrast, the expression of anti-apoptotic protein Bcl-2 decreased (Fig. 2B). These data suggested that Cur has the effect of inducing apoptosis in OS cells.

**Cur Curbed OS Cell Migration and Invasion** By co-culturing 8, 16, and 32 µg/mL of Cur with Saos-2 and MG63 cells, we determined the effect of Cur on the migration and invasion ability of OS cells via Transwell assay. As exhibited in Figs. 3A and B, compared with the control group, the higher the concentration of Cur, the less the number of migrating and invading cells. It indicated that Cur treatment dose-dependently hampered Saos-2 and MG63 cell migration and invasion. Moreover, WB results manifested that the expression of E-cadherin protein was significantly up-regulated in Saos-2 and MG63 cells compared to the control group, while the expressions of N-cadherin and Snail were in a downward trend.

![Fig. 1. Cur Hindered OS Cell Viability](image-url)
Fig. 2. Cur Induces Apoptosis in OS Cells
A: Cur (8, 16 and 32 µg/mL) was adopted to treat Saos-2 and MG-63 cells for 36 h, and then flow cytometry was implemented to monitor the ratio of apoptosis. B: Saos-2 and MG-63 were manipulated by Cur (8, 16, and 32 µg/mL) for 36 h, and the apoptosis-related protein expression was compared by WB (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 (vs. control group).

Fig. 3. Cur Abated OS Cell Migration and Invasion
A: The migration of Saos-2 and MG-63 cell was measured by Transwell assay after Cur (8, 16, and 32 µg/mL) treatment. B: Saos-2 and MG-63 cell invasion was checked by WB after Cur (8, 16, and 32 µg/mL) application. C: WB showed the profiles of EMT-related proteins in Saos-2 and MG-63 cells after Cur (8, 16, and 32 µg/mL) application (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 (vs. control group).
Thus, our findings indicated that Cur could inhibit the migration and invasion ability of Saos-2 and MG63 cells.

Cur Impeded the JAK/STAT and NF-κB Pathways Expression in OS Cells After demonstrating that Cur can curb the viability, migration and invasion of OS cells, we also explore the specific molecular mechanisms underlying the effects of Cur in OS. WB was carried out to monitor the JAK/STAT and NF-κB expression in OS cells. The results showed that in Saos-2 cells, the ratios of p-JAK2/JAK2, p-STAT3/STAT3 and p-NF-κB/NF-κB were significantly decreased (Fig. 4A). The same result was also obtained in MG63 cells (Fig. 4B). These above results revealed that Cur could restrained the activity of the JAK/STAT and NF-κB pathways in Saos-2 and MG63 cells.

Activating the JAK/STAT Pathway and NF-κB Weakened the Tumor-Suppressive Effect of Cur To further validate the mechanism of action of Cur in OS cells. Epidermal growth factor (EGF) (10 ng/mL, JAK pathway agonist) was selected to treat Sao-2 cells. The cell was then treated with Cur (32 µg/mL). As the results demonstrated, the activation of the JAK pathway promoted the protein expressions of phosphorylation of JAK2, STAT3 and NF-κB in Saos-2 and MG63 cell-bearing mice after Cur treatment compared with vehicle group (Figs. 5A–D). Then, TUNEL staining was further performed to observe the apoptosis. It was obvious that the apoptotic cells were increased significantly after Cur treatment, as well as the apoptosis rate was significantly higher (Fig. 6E). In addition, we also detected the expression of apoptosis and migration-related proteins in OS tumor tissues. Compared with the vehicle group, the expressions of Bcl-2, N-cadherin and Snail in Cur group were down-regulated, and the expressions of C-caspase-3, Bax and E-cadherin were up-regulated (Figs. 6F, G).

Next, we conducted IHC staining and WB on tumor tissues of OS tumor-bearing mice. Firstly, as it is shown in IHC staining results, both p-JAK2, p-STAT3 and p-NF-κB in Saos-2 and MG63 cells after treatment with Cur (8, 16, and 32 µg/mL) (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 (vs. control group).

Fig. 4. Cur Impeded the JAK/STAT/NF-κB Pathway Expression in OS Cells
A: WB was performed to examine the phosphorylation of JAK2, STAT3 and NF-κB in Saos-2 and MG-63 cells after treatment with Cur (8, 16, and 32 µg/mL) (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 (vs. control group).
DISCUSSION

Although combined surgery with chemotherapy is an effective treatment for OS, the high recurrence and metastasis rate of the disease remains a problem. According to surveys, the prognosis of OS patients with metastasis is not favorable, as well as clinical chemotherapy drugs have their disadvantages. Natural drugs derived from traditional herbs have fewer side effects and higher anti-tumor effects. Cur is an effective pharmacological component in natural herbal medicine, and its pharmacological effects have been reported in several studies (Fig. 1A). Cur plays an immunostimulatory role in the migration of B16F-10 melanoma cell because of its potential cancer cytotoxic ability. Therefore, this study attempted to investigate the role and mechanism of Cur in OS.

First, we examined the effect of Cur on OS cell viability at different concentrations. It can be concluded that the survival rate of Cur on Saos-2 and MG63 cells is the lowest at the concentration of 64 µg/mL. Co-culture at different concentrations for 0–72 h revealed that the inhibition rate of Cur on OS

![Fig. 5. Activating the JAK/STAT Pathway and NF-κB Weakened the Tumor-Suppressive Function of Cur](image-url)

An OS cell (Saos-2) was processed with EGF (10 ng/mL, the JAK/STAT pathway agonist) followed by Cur (32 µg/mL) treatment. A: The JAK/STAT pathway and NF-κB profiles in cells was monitored by WB. B: CCK-8 was performed to examine cell viability. C, D: Transwell assay was implemented to testify cell migration and invasion. E: Flow cytometry was conducted to testify apoptosis. F: The profiles of apoptosis-related were checked by WB. G: WB was implemented to measure EMT-related protein expression. **p < 0.01, ***p < 0.001 (vs. control group), ^p < 0.01, &&p < 0.001 (vs. Cur group).
cells was proportional to the concentration. Flow cytometry was then utilized to determine the effect of Cur on OS cell apoptosis. As our results showed, the concentration of Cur was proportional to the apoptosis rate. In WB experiment, we choose pro-apoptotic protein Bax, anti-apoptotic protein Bcl-2 and cleaved Caspase-3 as detection indicators. Bax and Bcl-2 are typical apoptotic proteins. Caspase-3 mediates tumor apoptosis after being cleaved and activated. Cleaved caspase-3 has been proved to accelerate the process of chemical or radiation-induced tumor. Our results showed that the expressions of Bax and cleaved caspase-3 were up-regulated, whereas the expression of Bcl-2 was down-regulated in OS cells after Cur treatment, which was significantly different from the untreated group. The above results demonstrate that Cur can effectively inhibit the cell viability of Saos-2 and MG63 cells while increasing the apoptosis rate of OS cells.

Fig. 6. Cur Inhibited Tumor Growth in Vivo

Establishment of OS xenograft model in nude mice: subcutaneous injection of OS cells (Saos-2, MG-63) at the right axilla of mice. The Cur group were injected with Cur (1 mg/kg) once daily through the tail vein while mice in the model group were injected with the same amount of saline daily. After 28 d of successive medication, 5 mice were randomly selected from each group and executed by cervical dislocation. Tumor tissues were stripped, weighed and tumor volume and mass measured. A: Tumor images in each group. B: Tumor volume in each group. C, D: Tumor weight in each group. E: TUNEL staining gauged the apoptotic rate in tumor tissues. F: Expression of apoptosis-related protein in tumor tissues was detected by WB. G: EMT-related protein expression in tumor tissues was assessed by WB (n = 5). ***p < 0.001 (vs. vehicle group).
and survival by regulating downstream target genes. The into the nucleus, which affects cell's proliferation, migration activation, followed by STAT3 dimerization and transcription recruitment and phosphorylation of STAT3 through its own is often activated by various cytokines. JAK2 leads to the JAK2, a non-receptor tyrosine kinase superfamily member, ways are known to play significant roles in human diseases. widespread invasion. Our results showed an increase in E-cadherin and Snail were detected by WB. These three genes, inhibited after Cur treatment, and the inhibition rate increased of 32 µg/mL of Cur, the expression of pro-apoptotic proteins Bax and cleaved caspase-3 increased and the expression of phosphorylated JAK2 can result in increased activation of STAT3, whereas induced OS cell migration and proliferation. Moreover, multiple reports have stated that drugs can repress tumor growth in OS by blocking JAK/STAT signaling. On the other hand, mutant NF-κB has been observed in numerous cancers, with the abnormal activation of NF-κB affecting the spread and migration of tumor cells. As the core transcription factor in this pathway, NF-κB is activated in two main ways. That is, it forms a dimer after being stimulated by cytokines. Then, NF-κB dimers enter the nuclear transcription phase and activate or repress downstream target genes. NF-κB signaling pathway plays a pivotal role in OS progression not only by forming crosstalk with JAK/STAT, but also by abnormally activated NF-κB alone. Therefore, inhibiting JAK/STAT and NF-κB pathways contribute to OS. Our results showed the expression of phosphorylated JAK2, STAT3, and NF-κB were in down trends in vitro after Cur treatment, and the ratio of p-JAK2/JAK2, p-STAT3/STAT3 and p-NF-κB/NF-κB were also decreased. These results implied that the activities of JAK2, STAT3, and NF-κB were inhibited after Cur administration compared to the unadministered groups. JAK2 was unable to trigger STAT3 recruitment and reverse transcription by activating itself. At the same time, NF-κB phosphorylation was similarly inhibited, preventing the initiation of the NF-κB pathway for downstream target genes regulation and control of cell migration and invasion.

Next, we added the JAK pathway agonist EGF in Sao-2 cell. First, we verified the effect of the agonist. WB results demonstrated that EGF resulted in an abnormal increase of phosphorylated JAK2, STAT3, and NF-κB compared with the control group, implying that EGF exerts activation effects on JAK/STAT and NF-κB pathways in OS cells. Transwell and Flow cytometry results showed that the inhibitory effects of Cur on migration, apoptosis, and invasion of Sao-2 cells were partially abolished after the addition of EGF. We then examined apoptotic pathway-related proteins, with administration of 32 µg/mL of Cur, the expression of pro-apoptotic proteins Bax and cleaved caspase-3 increased and the expression of anti-apoptotic protein Bcl-2 decreased, indicating that Cur could induce apoptosis in OS cells. Conversely, the expres-
sion of pro-apoptotic proteins Bax and cleaved caspase-3 was decreased, while the expression of anti-apoptotic protein Bel-2 was increased when we added agonist. Similarly, the expression of EMT-associated proteins E-cadherin, N-cadherin and Snail changed accordingly after the addition of JAK pathway agonists. The expression of E-cadherin decreased and the expression of N-cadherin and Snail increased in the EGF group compared with the Cur group. These results suggested that this pathway agonist can eliminate the pro-apoptotic and reduce the EMT effect of Cur.

The above results demonstrated that the activated JAK2/STAT3 pathway and NF-κB were effectively inhibited in OS cells after Cur treatment. Moreover, we explored whether Cur can also exert tumor inhibitory effect in vivo. Consistent with the results of in vitro experiments, Cur exerts tumor suppressive effect in vivo by inhibiting the JAK/STAT and NF-κB pathways. Currently, our study identifies the relationship between the JAK/STAT pathway, NF-κB pathway and Cur. Our findings showed that the JAK/STAT and NF-κB pathways pro-files were restrained by Cur in OS cells, and up-regulating the JAK/STAT and NF-κB pathway choked the tumor-suppressive effect of Cur. These data confirm that Cur could exert an anti-tumor role by inhibiting JAK2/STAT3 and NF-κB signaling pathways.

Overall, this study indicates that Cur can effectively dampen OS cell growth, proliferation, migration and invasion in vivo and in vitro. The inhibitory effect of Cur on OS is at least partially related to the inhibition of the JAK2/STAT3 and NF-κB pathways. Therefore, Cur is a promising adjunct or alternative drug for OS patients.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

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