Am J Cancer Res 2021;11(9):4541-4550
www.ajcr.us /ISSN:2156-6976/ajcr0137052

Original Article

Anti-cancer effects of Hederoside C, a pentacyclic triterpene saponin, through the intrinsic apoptosis and STAT3 signaling pathways in osteosarcoma

Kyung-Ran Park[1,3], Hyun Hee Leem[4], Joonyeop Lee[4], Il Keun Kwon[2,3], Jin Tae Hong[5], Hyung-Mun Yun[1]

[1]Department of Oral and Maxillofacial Pathology, School of Dentistry, Kyung Hee University, Seoul 02453, South Korea; [2]Department of Dental Materials, School of Dentistry, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemun-gu, Seoul 02447, South Korea; [3]Biomedical Science Institute, School of Medicine, Kyung Hee University, Seoul 02447, South Korea; [4]National Development Institute of Korean Medicine, Gyeongsan 38540, South Korea; [5]College of Pharmacy and Medical Research Center, Chungbuk National University, Chungbuk 28160, South Korea

Received July 5, 2021; Accepted August 9, 2021; Epub September 15, 2021; Published September 30, 2021

Abstract: Natural compounds have emerged as an approach in cancer therapy. *Pulsatilla koreana* Nakai is used as a traditional medicinal plant that found throughout China and Korea. However, anti-cancer effects of Hederoside C (HedC) isolated from *P. koreana* has not been investigated in osteosarcoma. The present study aimed to demonstrate anti-cancer functions of HedC against human osteosarcoma cells. Herein, we found that HedC suppressed the proliferation of MG63 cells and U2OS cells in the dose- and time-dependent manner, and caused intrinsic apoptosis pathways as evidenced by morphological changes, TUNEL-positive cells, cleaved-PARP, and cleaved-caspase 9 and 3. HedC increased p53, Bax, and p21, whereas HedC reduced Bcl-2. HedC-mediated apoptosis was accompanied by decreases in the mitogen-activated protein kinases (MAPKs) and STAT3 phosphorylation. Wound healing and Boyden chamber assays also showed the anti-metastatic effects of HedC by suppressing migration and invasion. In addition, the anti-cancer effects of HedC were observed in *in vivo* xenograft mice model, and HedC treatment induced the decreased PCNA and p-STAT3 as well as the increased p53 and cleaved caspase-3. Taken together, our results provide evidence that HedC might be an attractive therapeutic strategy against osteosarcoma.

Keywords: *Pulsatilla koreana Nakai*, HedC, osteosarcoma, proliferation, apoptosis, STAT3

Introduction

The *Pulsatilla koreana* Nakai (*P. koreana*) locally called halmi-kkot is belonging to the Ranunculaceae. The *P. koreana* root has been treated as a traditional medicine in internal hemorrhoids, amoebic dysentery, and malaria [1]. *P. koreanas* revealed the presence of lupane and oleanane type triterpenoid saponins, phenolic acids which are potential source of cytotoxic and cancer chemopreventive agents [2-4]. Hederoside C (HedC) from isolated from *P. koreanas* is a pentacyclic triterpene saponin, which is naturally found in various vegetables, fruits, and medicinal plants as part of the human consumption [5, 6]. Pentacyclic triterpenes and derivatives have also received much attention as therapeutic agents in cancer, inflammatory disease, and other diseases [7].

Osteosarcoma is the most common type in children and teenager that produces immature and abnormal bones [8]. Five-year survival rate of the patients is 5-20%, and the insensitive of chemotherapy drugs is 35-45%, as well as patients with metastatic osteosarcoma have no effective therapy [8-12]. Thus, it is essential to identify novel compounds against osteosarcoma by suppressing cell growth, migration, and invasion.

Cancer is viewed as a result of an imbalance of proliferation and programmed apoptosis, and evasion of apoptosis from normal cells and benign tumors cause malignant transformation [13]. The programmed apoptosis induces cell death via extrinsic and intrinsic pathways, which is accompanied by caspase-dependent proteolysis, membrane blebbing, and chromosomal DNA cleavage [14]. Reduced apoptosis

or its resistance occurs in most types of cancer, and a variety of researches demonstrated that apoptosis is a promising target for treating cancer [15].

In the present study, HedC (> 98% purity), a pentacyclic triterpene, was obtained from *P. koreanas* dried root and we demonstrated its biological activities and anti-cancer effects in human osteosarcoma MG63 cells.

Materials and methods

Plant material

P. koreana dried root was obtained in March 2016 at the commercial herbal medicine market (Cheongmyeongyakcho, South Korea). A voucher specimen has been deposited in the Natural Products Bank, National Institute for Korean Medicine Development (NIKOM).

Hederoside C

White powder, EI-MS *m/z* 749 $[M-H]^-$, Molecular formula $C_{41}H_{66}O_{12}$; ^{1}H-NMR (500 MHz, Pyridine-d_5) δ 6.32 (1H, s, Rha-1), 5.47 (1H, brs, H-12), 5.12 (1H, d, *J*=6.3 Hz, Ara-1), 4.76 (1H, m, Rha-2), 4.74 (1H, dd, *J*=9.4, 6.3 Hz, Rha-5), 4.68 (1H, dd, *J*=9.4, 3.4 Hz, Rha-3), 4.62 (1H, t, *J*=7.7 Hz, Ara-2), 4.32 (1H, m, Rha-4), 4.28 (1H, m, Ara-5b), 4.19 (1H, m, H-23), 4.18 (1H, m, Ara-4), 4.12 (1H, dd, *J*=8.0, 3.7 Hz, Ara-3), 3.75 (1H, d, *J*=10.6 Hz, H-23), 3.71 (1H, brd, *J*=10.3 Hz, Ara-5a), 1.65 (3H, d, *J*=6.0 Hz, Rha-6), 1.23 (3H, s, H-27), 1.09 (3H, s, H-24), 1.03 (3H, s, H-26), 1.00 (3H, s, H-30), 0.94 (3H, s, H-25), 0.93 (3H, s, H-29); ^{13}C NMR (125 MHz, Pyridine-d_5) δ 180.5 (C-28), 145.1 (C-13), 122.9 (C-12), 104.8 (Ara-1), 102.0 (Rha-1), 81.3 (C-3), 76.0 (Ara-2), 75.3 (Ara-3), 74.5 (Rha-4), 72.9 (Rha-3), 72.7 (Rha-2), 70.0 (Rha-5), 69.8 (Ara-4), 66.2 (Ara-5), 64.2 (C-23), 48.4 (C-9), 48.0 (C-5), 46.9 (C-17), 46.7 (C-19), 43.8 (C-4), 42.4 (C-14), 42.2 (C-18), 40.0 (C-8), 39.3 (C-1), 37.2 (C-10), 34.5 (C-21), 33.59 (C-22), 33.58 (C-29), 33.1 (C-7), 31.2 (C-20), 28.6 (C-15), 26.5 (C-2), 26.4 (C-27), 24.17 (C-11), 24.10 (C-30), 23.9 (C-16), 18.9 (Rha-6), 18.4 (C-6), 17.7 (C-26), 16.4 (C-25), 14.3 (C-24).

Cell culture

Human osteosarcomas, MG63 cells and U2OS cells, were obtained from the Korean Cell Line Bank (Seoul, South Korea). Cells were incubated in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1 X Gibco® Antibiotic-Antimycotic (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

MTT cell proliferation assay

Cell growth was measured using an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [16].

TUNEL assay

Terminal deoxynucleotidyl transferase-mediated FITC-dUDP nick-end labeling (TUNEL) assays were performed using an in situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) as previously described [17].

Western blot analysis

20 μg lysates were resolved on sodium dodecyl-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The following procedure was carried out as previously described [18].

Immunocytochemistry

Immunocytochemistry was carried out as previously described [17], and Images were analyzed using a fluorescence microscope.

Boyden chamber assay

The cell invasion of MG63 cells was done using a Boyden chamber assay as previously described [17], and images were analyzed by using a light microscope.

Wound healing assay

The cell migration of MG63 cells was carried out using a wound healing assay as previously described [17], and images were analyzed by using a light microscope.

Animals

Mice were housed in standard cages in an Assessment and Accreditation of Laboratory Animal Care credited specific pathogen-free animal facility. All procedures were approved by

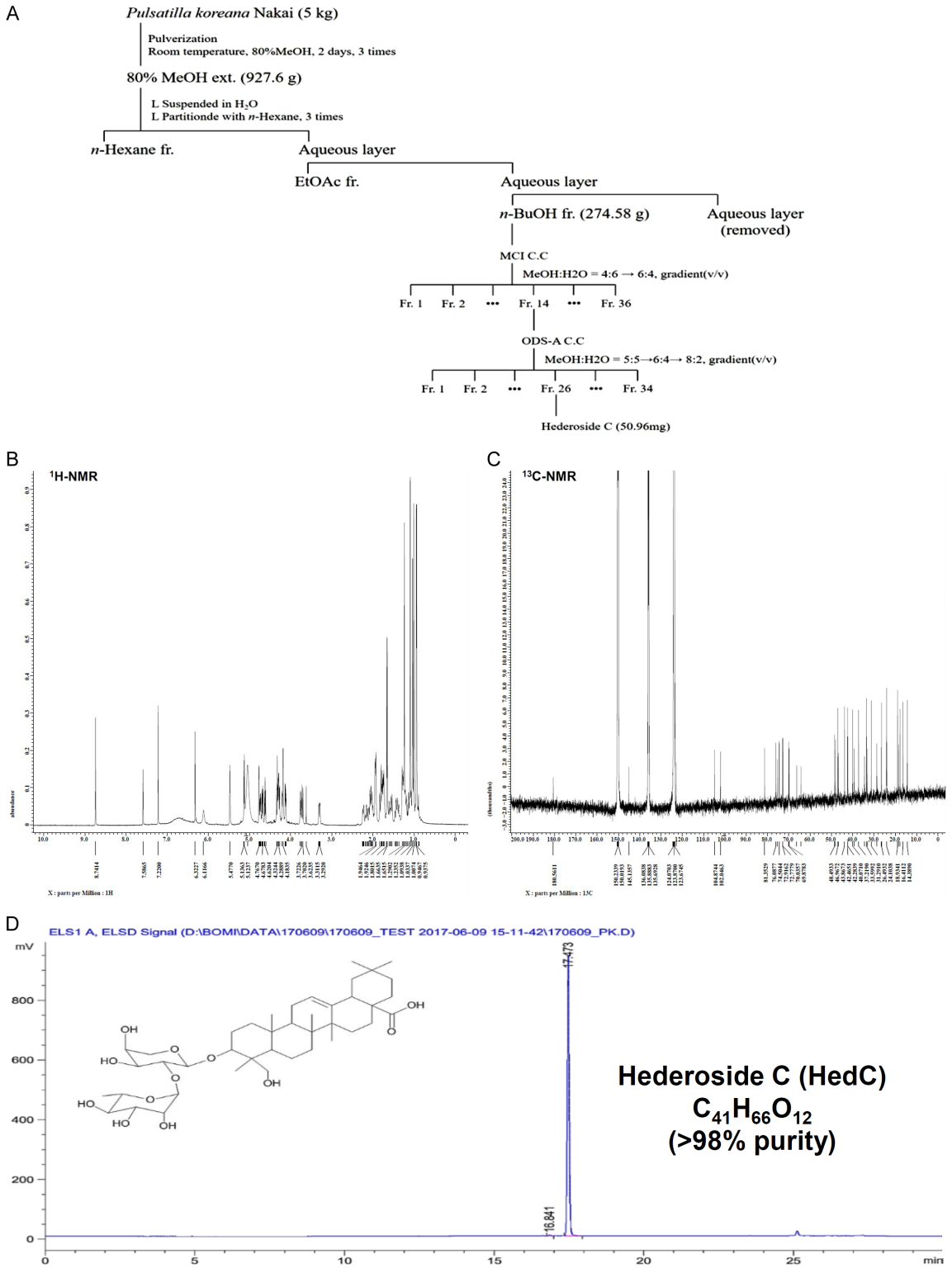

Figure 1. Isolation of HedC from root of *P. koreana*. A. Roadmap of HedC isolated from root of *P. koreana*. B, C. The ^{1}H and ^{13}C nuclear magnetic resonance spectra of HedC. D. The HPLC and structure of HedC.

the Chungbuk National University Institutional Animal Care and Use Committee and complied with Health Guide for the Care and Use of Laboratory Animals (CBNUA-792-15-01).

Xenograft animal model

BALB/c athymic nude mice (Six-week-old male) were obtained from Samtako (Osan, Kyoung

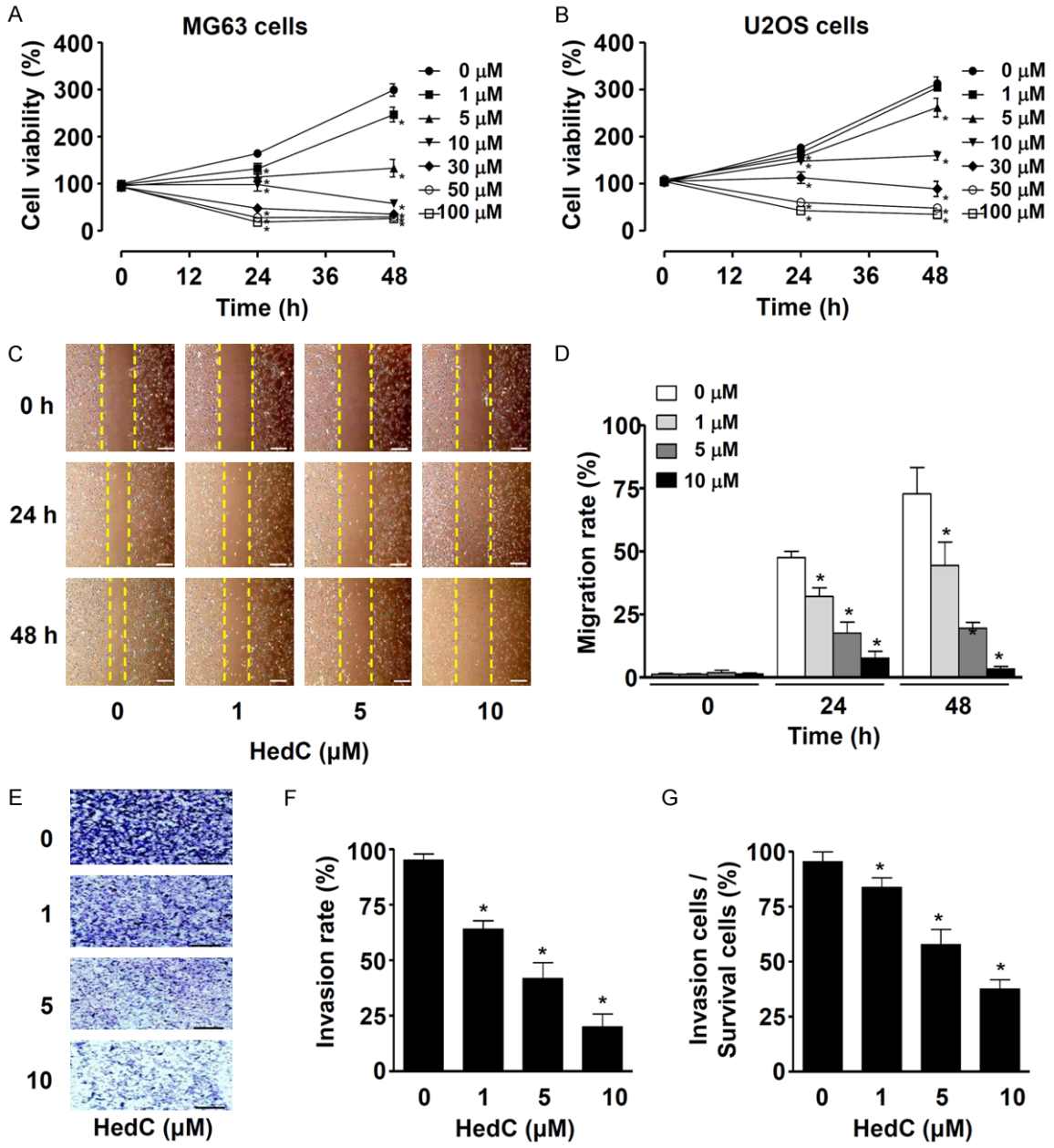

Figure 2. Effects of HedC on cell growth, migration, and invasion in human osteosarcoma MG63 cells. (A, B) MG63 cells (A) and U2OS cells (B) were cultured with HedC for 48 h, and cell proliferation was done using an MTT assay. (C, D) MG63 cells were cultured with HedC for 48 h, and then migration was performed using a wound healing assay and detected under a light microscope (C). The migration was shown as a bar graph (D). Scale bar: 100 μm. (E-G) Cell invasion was analyzed using a Boyden chamber assay and detected using a light microscope (E), and invasion rate was shown as a bar graph (F). Relative invasion rate (%) was calculated and shown as a bar graph (G). Scale bar: 50 μm. * indicates $P < 0.05$ compared to the control. Data are representative of three independent experiments and expressed in mean ± SEM.

Gi-Do, South Korea). Cells (1×10^8 cells) were injected as previously described [19, 20]. After 14 days, HedC (I.p., 7.5 mg/kg) were treated twice a week, and on day 42, mice were sacrificed by cervical dislocation.

Immunohistochemistry

Immunohistochemistry was done as previously described [17], and the sections were analyzed using a light microscope.

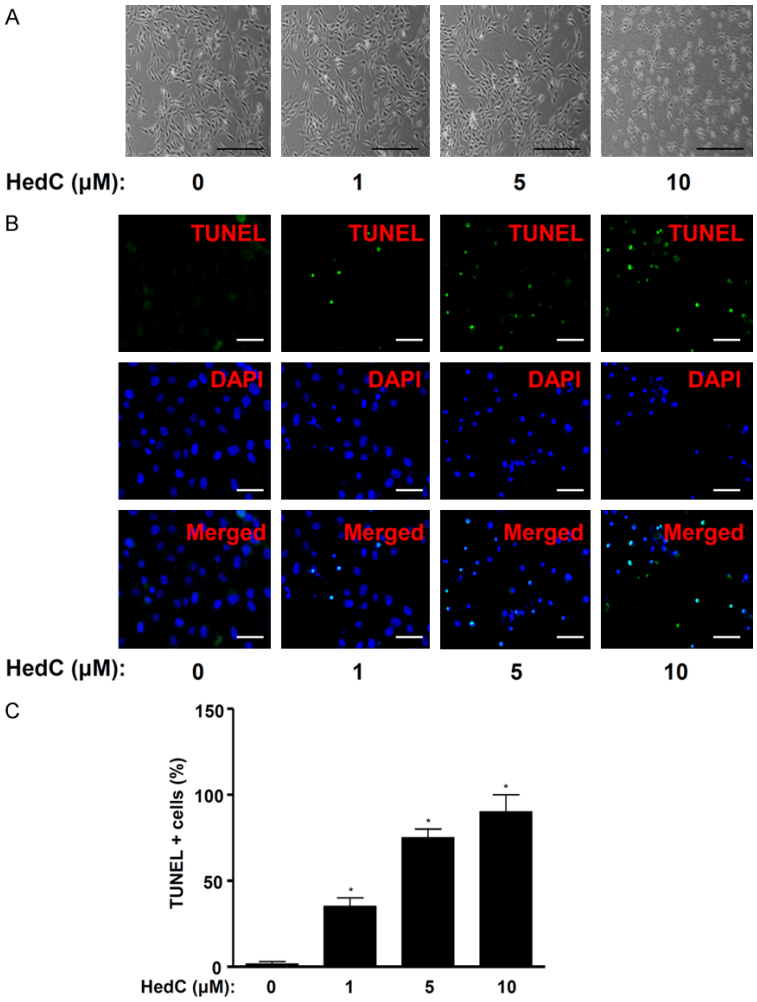

Figure 3. Effects of HedC on apoptotic cell morphology and DNA fragmentation in human osteosarcoma MG63 cells. (A) HedC was treated for 24 h in MG63 cells, and the morphological changes were detected under a light microscope. Scale bar: 100 μm. (B, C) 24 h after the treatment of HedC, apoptotic DNA strand breaks were analyzed by using TUNEL assay and were detected under a fluorescence microscope (B) and shown as a bar graph (C). Scale bar: 50 μm. * indicates $P < 0.05$ compared to the control. Data are representative of three independent experiments and expressed in mean ± SEM.

Statistical analysis

Data were tested using Student's unpaired *t* test (statistical significance, $P < 0.05$) in GraphPad Prism version 5 program (GraphPad Software, Inc., San Diego, CA) and expressed as mean ± S.E.M.

Results

Isolation of HedC from the dried root of P. koreanas

P. koreana (5 kg) dried root was extracted three times with 80% MeOH for 48 h at room temperature. The filtrate was combined and evaporated *in vacuo* to dryness. The crude extract (972.6 g) was suspended in distilled water, and liquid-liquid extraction successively with *n*-Hexane, EtOAc and *n*-BuOH. The *n*-BuOH soluble fraction (274.58 g) was applied to MCI gel column chromatography (CC) eluting with a MeOH-H_2O gradient system (4:6, 6:4, v/v) to give F1-F36 fractions. F14 was purified by ODS-A gel CC eluting with (MeOH-H_2O, 5:5→6:4→8:2, v/v) to give F1-F34 fractions. HedC (50.96 mg) a pentacyclic triterpene, was obtained from F26. ^{1}H- and ^{13}C-NMR spectra, HPLC chromatogram, and structure are shown in **Figure 1B** and **1D**.

HedC suppresses cell proliferation in human osteosarcomas, MG63 cells and U2OS cells

To investigate the activities of HedC on the proliferation of human osteosarcomas, HedC (0, 1, 5, 10, 30, 50, and 100 μM) was treated for 48 h in the MG63 cells and U2OS cells. The MTT assay showed that HedC significantly inhibited cell proliferation in a time- and dose-dependent manner in the MG63 cells and U2OS cells (**Figure 1A** and **1B**). However, HedC strongly suppressed cell proliferation on the MG63 cells compared with the U2OS cells (**Figure 1A** and **1B**). For the following experiments, the MG63 cells were selected and HedC concentrations (0, 1, 5, and 10 μM) were used.

HedC suppresses the migration and invasion of MG63 cells

Next, to find out whether HedC possesses anti-metastatic effects, we examined cell migration using a wound healing assay. Compared to the control, the migration was significantly attenuated by the treatment of HedC during 24 and 48 hours in the MG63 cells (**Figure 2C** and **2D**).

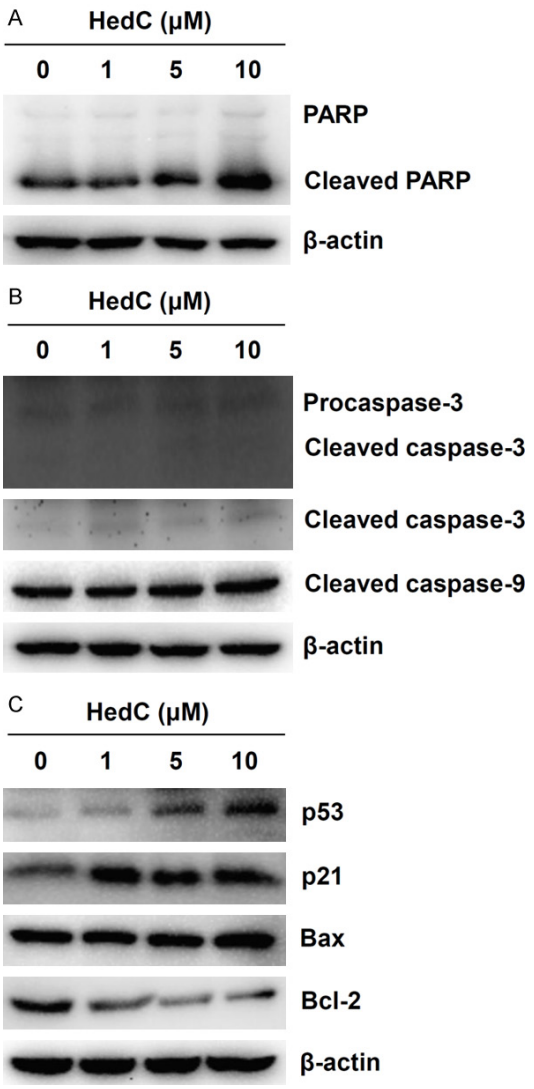

Figure 4. Effects of HedC on the regulatory proteins of apoptosis in human osteosarcoma MG63 cells. (A-C) HedC was treated for 24 h in the cells, and PARP (A), or caspase-3, cleaved-caspase-3, and cleaved-caspase-9 (B), or p53, p21, Bax, and Bcl-2 (C) were subjected to western blot analysis. β-actin was detected as a loading control. Data are representative of three independent experiments.

Since extracellular matrix (ECM) degradation is required for metastasis, we examined cell invasion using a Boyden chamber assay. HedC significantly attenuated the penetration of the MG63 cells through the Matrigel-coated polycarbonate filter compared to control (**Figure 2E** and **2F**). Since the cell proliferation was decreased greatly after HedC treatment, invasion cells were divided by total survival cells and relative invasion rate was calculated. The results showed that HedC (0, 1, 5, and 10 μM) significantly inhibited cell invasion in the MG63 cells (**Figure 2G**).

HedC enhances apoptotic cell death in human osteosarcoma MG63 cells

Next, to examine whether HedC induced apoptotic cell death, HedC (0, 1, 5, and 10 μM) was treated in the MG63 cells, and morphologic changes were observed using a light microscope. The results showed that HedC treatment induced the small-round-single cell shape and reduced cell size, which is the phenotype of apoptotic cell (**Figure 3A**). We also validated the apoptotic cell death by examining DNA fragmentation using the TUNEL assay. The observation showed that HedC treatment significantly enhanced TUNEL-positive cells (green) compared with the control (**Figure 3B** and **3C**).

HedC regulates the regulatory proteins of apoptosis in human osteosarcoma MG63 cells

Next, HedC-mediated apoptosis was examined using western blot analysis for the cleavage of PARP, a hallmark of apoptosis, and active caspase-3 enzymes. HedC (1-10 μM) treatment increased the cleaved-PARP proteins and active caspase-3 as seen by the cleaved-caspase-3 proteins (**Figure 4A** and **4B**). The intrinsic apoptosis pathway activates caspase-9 and caspase-3 is cleaved. Cleaved caspase-9 was induced in response to HedC treatment (**Figure 4B**). We further investigated apoptotic and anti-apoptotic proteins in the MG63 cells. As shown in **Figure 3C**, the treatment of HedC enhanced p53, Bax, and p21 expressions, whereas reduced Bcl-2 expression in the MG63 cells (**Figure 4C**).

HedC inhibits the MAPKs and STAT3 pathways in human osteosarcoma MG63 cells

To find out the mechanisms associated with HedC-mediated apoptosis, the phosphorylation of MAPKs (ERK1/2, JNK, and p38 MAPK) and STAT3 was analyzed in the MG63 cells. The results showed that HedC treatment moderately suppressed the constitutive ERK1/2 and JNK activities, but the treatment significantly inhibited the constitutive p38 MAPK activities in a dose-dependent manner (**Figure 5A**). Moreover, HedC significantly reduced the constitutive STAT3 activities in a dose-dependent manner (**Figure 5B**). Innunocytochemistry

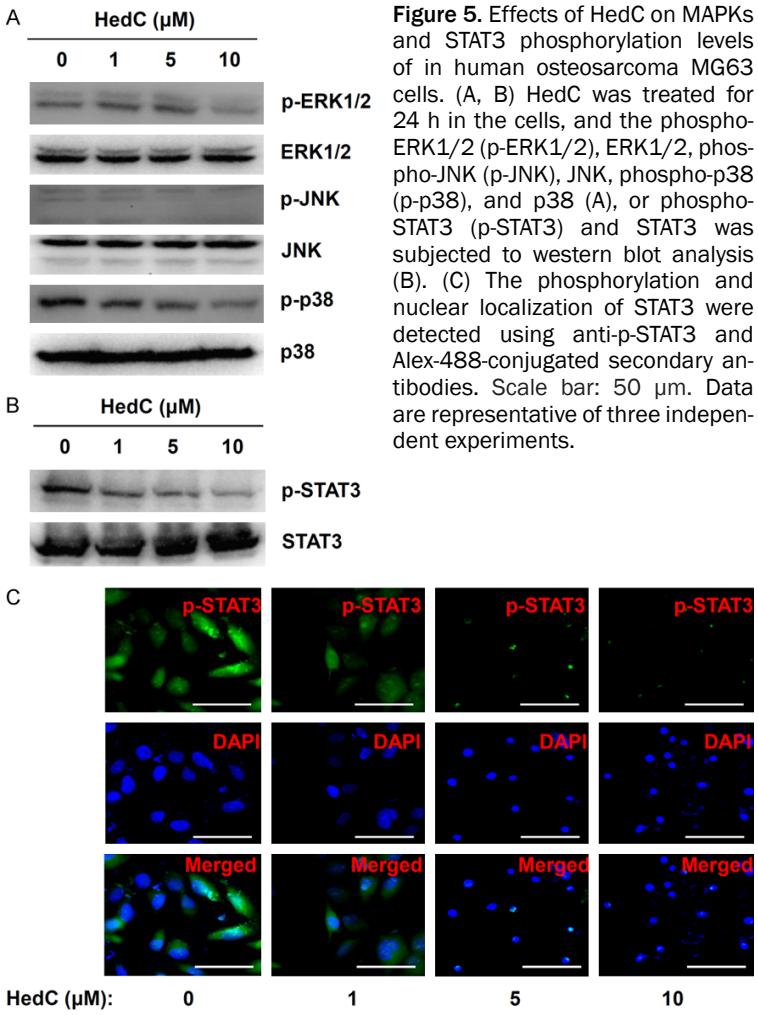

Figure 5. Effects of HedC on MAPKs and STAT3 phosphorylation levels of in human osteosarcoma MG63 cells. (A, B) HedC was treated for 24 h in the cells, and the phospho-ERK1/2 (p-ERK1/2), ERK1/2, phospho-JNK (p-JNK), JNK, phospho-p38 (p-p38), and p38 (A), or phospho-STAT3 (p-STAT3) and STAT3 was subjected to western blot analysis (B). (C) The phosphorylation and nuclear localization of STAT3 were detected using anti-p-STAT3 and Alex-488-conjugated secondary antibodies. Scale bar: 50 μm. Data are representative of three independent experiments.

also validated that HedC treatment reduced the phosphorylation and nuclear localization of STAT3 in a dose-dependent manner in the MG63 cells (**Figure 5C**).

HedC suppresses tumorigenesis in an xenograft nude mouse model

To elucidate the effects of Chi3L1 on the growth of osteosarcoma in vivo, we investigated the effects of HedC an nude mice model. The tumor growth was monitored for 42 days. The observation showed that the tumor volume (mm^3) and weight (g) were significantly attenuated by HedC compared with those of the control (**Figure 6A** and **6B**). Moreover, there was no significant change by HedC treatment in body weight excluding the tumor weight during the experiment period (**Figure 6C**). The immunohistochemistry analysis of tumor section revealed that HedC treatment suppressed the proliferating cell nuclear antigen (PCNA) and p-STAT3 expression compared to those of the control, whereas HedC-treated tumor section showed the increased expression of p53 and cleaved caspase-3 (**Figure 6D**). Moreover, the results were validated by using western blot analysis (**Figure 6E**). Overall, our data suggest that HedC attenuates the tumorigenesis of osteosarcoma.

Discussion

Natural compounds used as traditional medicine for centuries have received great attention in drug development to treat various diseases including cancer because they are relative inexpensiveness and safety compared with chemically synthesized drugs [21-23]. We previously reported evidences on the biological activities of flavonoids, 4-Methoxydalbergione and 4-parvifuran, from *Dalbergia odorifera* heartwoods in osteosarcoma [24, 25]. Pentacyclic triterpene is a natural compound that possesses therapeutic effects against human diseases including various cancers [26, 27].

In the present study, we demonstrated the anti-cancer effects of HedC, a pentacyclic triterpene saponin, isolated from *P. koreanas* in osteosarcoma. We demonstrated the inhibitory effects on cell growth of HedC via MTT assays, and found that HedC induces morphological changes, which represent an apoptotic phenotype. It was well known that endonucleases induce the cleavage of chromatin into about 180-bp oligomers, which is called DNA fragmentation, and thus it is the main feature of apoptosis and is used as a marker of apoptosis [28]. Our results demonstrated that HedC increases the apoptotic DNA fragmentation, as assessed by TUNEL assay. It was reported that caspase-3 causes the DNA fragmentation by inactivating inhibitor of caspase-activated endonucleases and by activating

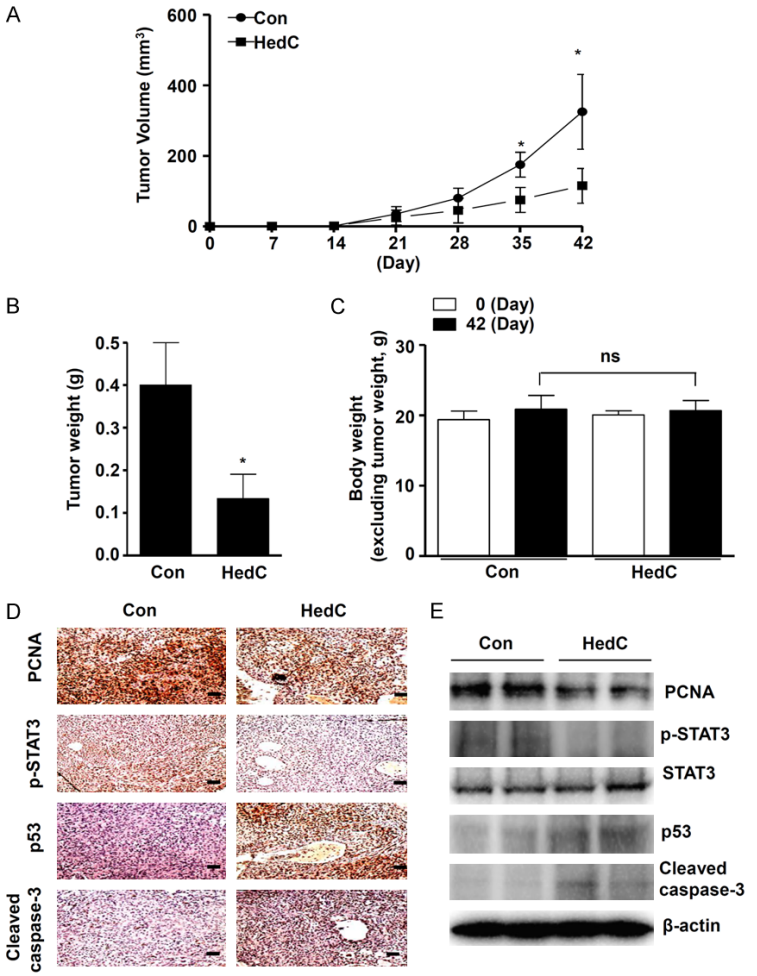

Figure 6. Effects of HedC on *in vivo* tumorigenesis in an xenograft mice model. (A-C) BALB/c athymic nude mice were injected with MG63 cells into and sacrificed 42 days later. The tumor volume (A) and weight (B) of tumors, and body weight (C) were analyzed. (D) The sections were subjected to immunohistochemistry to detect PCNA, p-STAT3, p53, and cleaved caspase-3. Scale bar: 100 μm. (E) Tumor extracts were was subjected to western blot analysis to detect PCNA, p-STAT3, STAT3, p53, and cleaved caspase-3. β-actin was detected as a loading control. * indicates $P < 0.05$ compared to the control. Data are representative of three independent experiments and expressed in mean ± SEM.

caspase-activated endonucleases [29]. Caspase-3 is cleaved by caspase-9 through the intrinsic apoptosis pathway that is controlled by apoptotic regulator proteins such as p53, p21, and Bcl-2 family (Bax and Bcl-2) [30, 31]. In the present study, HedC also induces the caspase-9, caspase-3, and PARP cleavages. HedC-mediated apoptosis was validated by the enhanced expression levels of p53, p21, and BAX, and the reduced expression level of Bcl-2. Therefore, our data suggest that HedC has anti-cancer effects through the intrinsic apoptosis pathway in osteosarcoma.

STAT3 protein is a critical transcription factor which regulates multiple key genes in cell growth, apoptosis, and metastasis, and importantly in malignant transformation [32, 33]. Thus, the regulation of STAT3 is a critical target for tumor therapy. In the previous studies, we found that G721-0282, a ligand of chitinase 3 like 1, attenuates cell proliferation and induces apoptotic cell death via STAT3 downregulation in osteosarcoma [17]. In the present study, we demonstrated that HedC inhibits the nuclear STAT3 phosphorylation in the MG63 cells. One of the main regulators of STAT3 is MAPKs proteins in signal transduction cascades in various cancers [34, 35]. Our data also demonstrated that HedC inhibits the phosphorylation of ERK, JNK, and p38 MAPK. These results suggest that HedC suppresses STAT3 activities via MAPKs signaling cascades, leading to inhibiting cell proliferation and inducing apoptosis in osteosarcoma.

Osteosarcomas tend to invade frequently into surrounding tissues, and the primary site of metastasis is commonly the lung tissues [8-10]. It was clinically reported that five-year survival rate shows about 20% in patients with metastatic osteosarcoma [36, 37]. Many studies suggest that constitutively activated STAT3 has a key role in growth and metastasis, and thus the inhibition of STAT3 has anti-tumor and anti-metastasis effects [38, 39]. It was reported that the inhibitors of STAT3 show potential as therapeutic candidates for patients with osteosarcoma [40, 41]. In the present study, we demonstrated that HedC inhibits migration and invasion in the MG63 cells, and HedC suppresses tumorigenesis in xenograft nude mice. Our *in vivo* observation also showed that HedC reduces tumor proliferation, enhances

apoptosis, and inhibits STAT3, which are consistent with *in vitro* findings, suggesting that HedC has anti-tumor and anti-metastatic effects by targeting the STAT3 signaling pathway in osteosarcoma.

In conclusion, we firstly demonstrated the anti-cancer effects of HedC in osteosarcoma through STAT3 signaling using *in vitro* human osteosarcoma cells and *in vivo* xenograft model. These findings indicate that HedC is a potential candidate as therapeutic agents against patients with osteosarcoma.

Acknowledgements

This work was supported by the National Research Foundation of Korea [NRF] grant funded by the Korea government (MSIP) (MRC, 2017R1A5A2015541). This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MSIT) (No. 2017M3A9E4048170).

Disclosure of conflict of interest

None.

Address correspondence to: Hyung-Mun Yun, Department of Oral and Maxillofacial Pathology, School of Dentistry, Kyung Hee University, Kyunghee-daero, Dongdaemun-gu, Seoul 02453, South Korea. Tel: +82 2-961-0343; Fax: +82 2-960-1457; E-mail: yunhm@khu.ac.kr; Jin Tae Hong, College of Pharmacy and Medical Research Center, Chungbuk National University, Osongsaengmyeong 1-ro 194-31, Osong-eup, Heungduk-gu, Cheongju, Chungbuk 28160, South Korea. Tel: +82 43-261-2813; E-mail: jinthong@chungbuk.ac.kr

References

[1] Ye WC, Ji NN, Zhao SX, Liu JH, Ye T, McKervey MA and Stevenson P. Triterpenoids from Pulsatilla chinensis. Phytochemistry 1996; 42: 799-802.

[2] Kim Y, Bang SC, Lee JH and Ahn BZ. Pulsatilla saponin D: the antitumor principle from Pulsatilla koreana. Arch Pharm Res 2004; 27: 915-918.

[3] Li W, Sun YN, Yan XT, Yang SY, Lee SJ, Byun HJ, Moon CS, Han BS and Kim YH. Isolation of nematicidal triterpenoid saponins from Pulsatilla koreana root and their activities against Meloidogyne incognita. Molecules 2013; 18: 5306-5316.

[4] Li W, Yan XT, Sun YN, Ngan TT, Shim SH and Kim YH. Anti-inflammatory and PPAR transactivational effects of oleanane-type triterpenoid saponins from the roots of Pulsatilla koreana. Biomol Ther (Seoul) 2014; 22: 334-340.

[5] J C Furtado NA, Pirson L, Edelberg H, M Miranda L, Loira-Pastoriza C, Preat V, Larondelle Y and André CM. Pentacyclic triterpene bioavailability: an overview of in vitro and in vivo studies. Molecules 2017; 22: 400.

[6] Siddique HR and Saleem M. Beneficial health effects of lupeol triterpene: a review of preclinical studies. Life Sci 2011; 88: 285-293.

[7] Sheng H and Sun H. Synthesis, biology and clinical significance of pentacyclic triterpenes: a multi-target approach to prevention and treatment of metabolic and vascular diseases. Nat Prod Rep 2011; 28: 543-593.

[8] Kansara M, Teng MW, Smyth MJ and Thomas DM. Translational biology of osteosarcoma. Nat Rev Cancer 2014; 14: 722-735.

[9] Morrow JJ, Bayles I, Funnell APW, Miller TE, Saiakhova A, Lizardo MM, Bartels CF, Kapteijn MY, Hung S, Mendoza A, Dhillon G, Chee DR, Myers JT, Allen F, Gambarotti M, Righi A, DiFeo A, Rubin BP, Huang AY, Meltzer PS, Helman LJ, Picci P, Versteeg HH, Stamatoyannopoulos JA, Khanna C and Scacheri PC. Positively selected enhancer elements endow osteosarcoma cells with metastatic competence. Nat Med 2018; 24: 176-185.

[10] Huang YM, Hou CH, Hou SM and Yang RS. The metastasectomy and timing of pulmonary metastases on the outcome of osteosarcoma patients. Clin Med Oncol 2009; 3: 99-105.

[11] Deng ZP, Ding Y, Puri A, Wang EH, Gulia A, Durban C and Niu XH. The surgical treatment and outcome of nonmetastatic extremity osteosarcoma with pathological fractures. Chin Med J (Engl) 2015; 128: 2605-2608.

[12] Liao YX, Yu HY, Lv JY, Cai YR, Liu F, He ZM and He SS. Targeting autophagy is a promising therapeutic strategy to overcome chemoresistance and reduce metastasis in osteosarcoma. Int J Oncol 2019; 55: 1213-1222.

[13] Hanahan D and Weinberg RA. The hallmarks of cancer. Cell 2000; 100: 57-70.

[14] Carneiro BA and El-Deiry WS. Targeting apoptosis in cancer therapy. Nat Rev Clin Oncol 2020; 17: 395-417.

[15] Tang C, Zhao CC, Yi H, Geng ZJ, Wu XY, Zhang Y, Liu Y and Fan G. Traditional Tibetan medicine in cancer therapy by targeting apoptosis pathways. Front Pharmacol 2020; 11: 976.

[16] Park KR, Kim JY, Kim EC, Yun HM and Hong JT. RANKL-induced osteoclastogenesis is suppressed by 4-O-methylhonokiol in bone marrow-derived macrophages. Arch Pharm Res 2017; 40: 933-942.

[17] Park KR, Yun HM and Hong JT. G721-0282 inhibits cell growth and induces apoptosis in human osteosarcoma through down-regulation of the STAT3 pathway. Int J Biol Sci 2020; 16: 330-341.

[18] Park KR, Kim EC, Hong JT and Yun HM. Dysregulation of 5-hydroxytryptamine 6 receptor accelerates maturation of bone-resorbing osteoclasts and induces bone loss. Theranostics 2018; 8: 3087-3098.
[19] Yun HM, Park KR, Lee HP, Lee DH, Jo M, Shin DH, Yoon DY, Han SB and Hong JT. PRDX6 promotes lung tumor progression via its GPx and iPLA2 activities. Free Radic Biol Med 2014; 69: 367-376.
[20] Jo M, Yun HM, Park KR, Hee Park M, Myoung Kim T, Ho Pak J, Jae Lee S, Moon DC, Park CW, Song S, Lee CK, Bae Han S and Tae Hong J. Lung tumor growth-promoting function of peroxiredoxin 6. Free Radic Biol Med 2013; 61: 453-463.
[21] Sauter ER. Cancer prevention and treatment using combination therapy with natural compounds. Expert Rev Clin Pharmacol 2020; 13: 265-285.
[22] Nobili S, Lippi D, Witort E, Donnini M, Bausi L, Mini E and Capaccioli S. Natural compounds for cancer treatment and prevention. Pharmacol Res 2009; 59: 365-378.
[23] An J, Yang H, Zhang Q, Liu C, Zhao J, Zhang L and Chen B. Natural products for treatment of osteoporosis: the effects and mechanisms on promoting osteoblast-mediated bone formation. Life Sci 2016; 147: 46-58.
[24] Park KR, Yun HM, Quang TH, Oh H, Lee DS, Auh QS and Kim EC. 4-Methoxydalbergione suppresses growth and induces apoptosis in human osteosarcoma cells in vitro and in vivo xenograft model through down-regulation of the JAK2/STAT3 pathway. Oncotarget 2016; 7: 6960-6971.
[25] Yun HM, Park KR, Quang TH, Oh H, Hong JT, Kim YC and Kim EC. 4-parvifuran inhibits metastatic and invasive actions through the JAK2/STAT3 pathway in osteosarcoma cells. Arch Pharm Res 2017; 40: 601-609.
[26] Rodriguez-Rodriguez R. Oleanolic acid and related triterpenoids from olives on vascular function: molecular mechanisms and therapeutic perspectives. Curr Med Chem 2015; 22: 1414-1425.
[27] Patlolla JM and Rao CV. Triterpenoids for cancer prevention and treatment: current status and future prospects. Curr Pharm Biotechnol 2012; 13: 147-155.
[28] Matassov D, Kagan T, Leblanc J, Sikorska M and Zakeri Z. Measurement of apoptosis by DNA fragmentation. Methods Mol Biol 2004; 282: 1-17.
[29] Wolf BB, Schuler M, Echeverri F and Green DR. Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. J Biol Chem 1999; 274: 30651-30656.
[30] Wang C and Youle RJ. The role of mitochondria in apoptosis*. Annu Rev Genet 2009; 43: 95-118.
[31] Tian L, Yin D, Ren Y, Gong C, Chen A and Guo FJ. Plumbagin induces apoptosis via the p53 pathway and generation of reactive oxygen species in human osteosarcoma cells. Mol Med Rep 2012; 5: 126-132.
[32] Hill CS and Treisman R. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 1995; 80: 199-211.
[33] Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C and Darnell JE Jr. Stat3 as an oncogene. Cell 1999; 98: 295-303.
[34] Decker T and Kovarik P. Serine phosphorylation of STATs. Oncogene 2000; 19: 2628-2637.
[35] Gkouveris I, Nikitakis N, Karanikou M, Rassidakis G and Sklavounou A. Erk1/2 activation and modulation of STAT3 signaling in oral cancer. Oncol Rep 2014; 32: 2175-2182.
[36] Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, Zoubek A, Jurgens H and Winkler K. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 2002; 20: 776-790.
[37] Sung L, Anderson JR, Donaldson SS, Spunt SL, Crist WM and Pappo AS. Late events occurring five years or more after successful therapy for childhood rhabdomyosarcoma: a report from the Soft Tissue Sarcoma Committee of the Children's Oncology Group. Eur J Cancer 2004; 40: 1878-1885.
[38] Kamran MZ, Patil P and Gude RP. Role of STAT3 in cancer metastasis and translational advances. Biomed Res Int 2013; 2013: 421821.
[39] Devarajan E and Huang S. STAT3 as a central regulator of tumor metastases. Curr Mol Med 2009; 9: 626-633.
[40] Zuo D, Shogren KL, Zang J, Jewison DE, Waletzki BE, Miller AL 2nd, Okuno SH, Cai Z, Yaszemski MJ and Maran A. Inhibition of STAT3 blocks protein synthesis and tumor metastasis in osteosarcoma cells. J Exp Clin Cancer Res 2018; 37: 244.
[41] Liu Y, Liao S, Bennett S, Tang H, Song D, Wood D, Zhan X and Xu J. STAT3 and its targeting inhibitors in osteosarcoma. Cell Prolif 2021; 54: e12974.