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The flavonoid ampelopsin inhibited cell growth and induced apoptosis and G0/G1 arrest in human osteosarcoma MG-63 cells *in vitro*

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Ampelopsin (AMP), a novel flavonoid, has been shown to effectively inhibit the proliferation and induce apoptosis of some prostate cancer and breast cancer cell lines. Whether AMP has chemopreventive effects on the cell growth and apoptosis of human osteosarcoma MG-63 cells remains unknown. In the present study, MG-63 cells were exposed to different concentrations (0, 25, 50, 75, 100 μmol/L) of AMP for 24, 48, 72 and 96 h and then the cell viability was measured by CCK-8 assay. The AMP-induced apoptotic cells were identified by Hochest33258 staining and quantified by Annexin V-FITC/PI double staining using flowcytometry (FCM). The effect of ampelopsin (AMP) on cell cycle was evaluated using PI staining with FCM. The protein levels of cyclin A, CDK2 and $p21^{CIP1}$ were measured by Western blotting. The cell viability was reduced in a time- and dose-dependent manner after exposure to AMP at a range of 20-100 μmol/L. For the treatment of AMP, increases of apoptotic index and rate were observed in MG-63 cells. The AMP blocked cells in the G0/G1 phase of the cell cycle. Furthermore, AMP increased $p21^{CIP1}$ expression but decreased cyclin A and CDK2 expression after AMP exposure. AMP inhibited cell growth and induced apoptosis and G_0/G_1 phase arrest in MG-63 cells *in vitro*, with the potential mechanism of the negative regulation of cell cycle-related protein.

1. Introduction

Osteosarcoma (OS), characterized by malignant osteoid production and osteoblastic differentiation, is an aggressive sarcoma of the bone. In children and adolescents, it is an infrequent but extremely destructive primary bone tumor (Ottaviani and Jaffe 2009). Despite advances in the understanding and treatment of OS, it still has a poor prognosis after surgical resection. Recent studies have implicated neoadjuvant chemotherapy as a viable therapeutic strategy for OS. Its 5-year survival rate has arrived at approximate 50%-80% under cisplatin, doxorubicin or ifosfamide-based neoadjuvant chemotherapy (Ottaviani and Jaffe 2009; Tan et al. 2009). However, in the last few years, increasing chemoresistance has caused a decrease in the number of OS patients benefiting from chemotherapy (Gorlick 2009). In addition, adverse effects induced by the chemopreventive intervention had a negative impact on survival and quality of life (Longhi et al. 2006). Therefore, the search for chemotherapeutic candidates with moderate adverse effects remains a top priority for OS therapy.

Plant natural products act as major resources of promising anticancer drug candidates (Bishayee et al. 2011; Lo et al. 2011; Chen et al. 2014; Zhou et al. 2013), for example paclitaxel, originally isolated from the bark of the Pacific Yew tree (*Taxus brevifolia*), is widely used for the treatment of breast cancer (Jiang et al. 2013; Peng et al. 2014). *Ampelopsis grossedentata* is widely distributed in China's south regions, and its compounds exert many bioactive functions (Li et al. 2008; Wang et al. 2011; Liao et al. 2014). Ampelopsin (AMP), a novel flavonoid, has been recognized as the major bioactive constituent of *Ampelopsis grossedentata* (Hou et al. 2014; Zhou et al. 2014; Chen et al. 2015). Several studies revealed the biological and pharmacological activities of AMP, involving anti-oxidative, anti-inflammation, anti-HIV infection and hepato-protective effects (Liu et al. 2004; Murakami et al. 2004; Hou et al. 2014; Qi et al. 2012). Recently, AMP was found to exert potent anticancer activities against several types of cancers, including lung, prostate and bladder (Ni et al. 2012; Zhang et al. 2012; Chen et al. 2015). However, its anti-tumor effects on OS have not been explored. This study aimed to evaluate the chemopreventive effect of AMP on the cell growth and apoptosis of human OS cell line MG-63 *in vitro*.

2. Investigations and results

2.1. *Effects of AMP on cell viability of OS cells*

To investigate whether AMP has an anti-tumor role in OS cell lines, the CCK-8 assay was adopted to evaluate the cytotoxic effects of AMP on MG-63, Saos-2 and U-2 OS cells lines. Three common OS cell lines were exposed to different concentrations (0, 25, 50, 75, 100 μmol/L) of AMP for 24, 48, 72 and 96 h, respectively. Compared with the effect of 0 μmol/L AMP, the test concentrations caused strong inhibition on the cell viability of three OS cell lines with significant difference ($P < 0.05$). One way ANOVA analysis showed that AMP decreased the cell

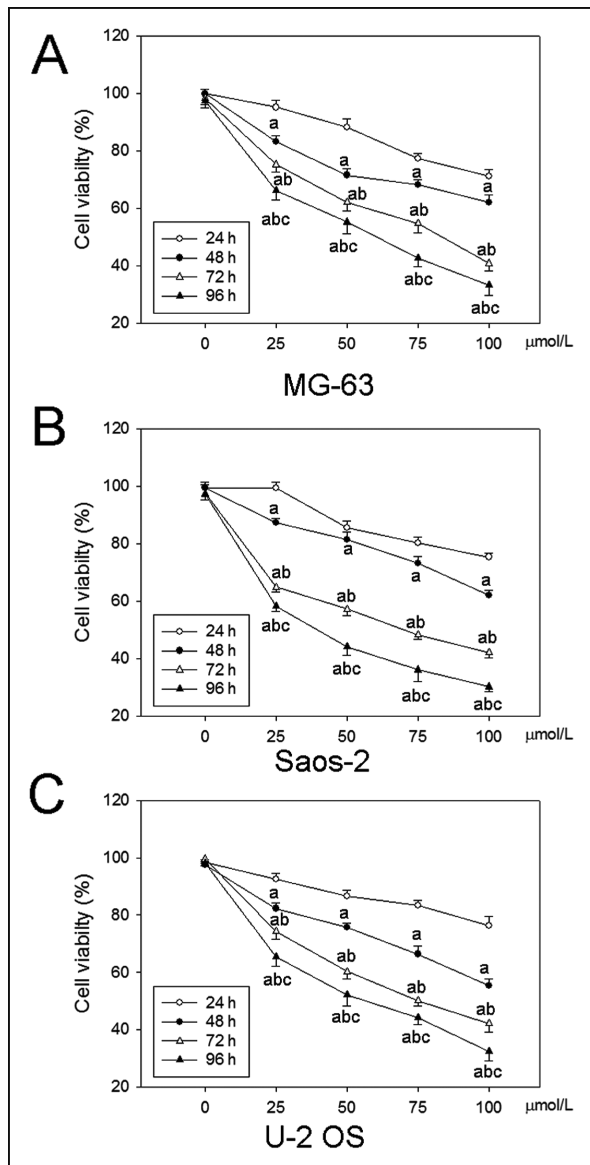

Fig. 1: AMP inhibits cell proliferation in OS cells. Dose- and time- dependent manner of AMP on MG-63 (A), Saos-2 (B) and U-2 OS (C) after exposure to different concentrations (0, 25, 50, 75, 100 µmol/L) of AMP for 24, 48, 72 and 96 h. Values are mean ± SE. Experiments were performed in triplicate and repeated three times. $^{a}P < 0.05$ *vs.* 24 h; $^{b}P < 0.05$ *vs.* 48 h; $^{c}P < 0.05$ *vs.* 72 h.

viability of three OS cell lines in a dose- and time- dependent manner (Fig. 1).

2.2. *Effects of AMP on apoptosis of MG-63 cell*

2.2.1. *Apoptotic index (AI)*

After exposure to different concentrations (0, 25, 50, 75, 100 µmol/L) of AMP for 24 and 48 h, Hoechst staining was adopted to investigate the effect of AMP on AI of MG-63 cells. Compared with the effect of 0 µmol/L AMP, the test concentrations increased AI of MG-63 cell with significant difference ($P < 0.05$). The results in Fig. 2 showed higher AI at 48 h versus 24 h after treatment with AMP (25, 50, 75, 100 µmol/L) with significant difference ($P < 0.05$).

2.2.2. *Apoptotic rate*

After exposure to different concentrations (0, 25, 50, 75, 100 µmol/L) of AMP for 48 h, FCM assay with propidium iodide staining was adopted to investigate the effect of AMP on apoptosis rate of MG-63 cells. Compared with the effect of 0 µmol/L AMP, the test concentrations dose-dependently increased both early and late apoptotic rates of MG-63 cell with significant difference (Fig. 3).

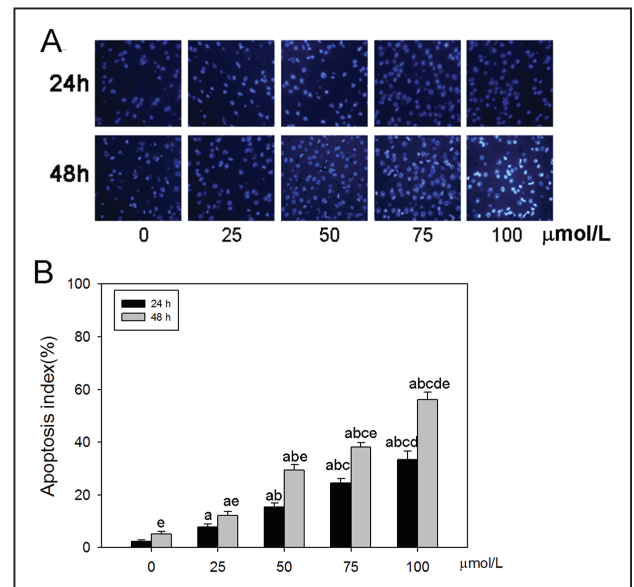

Fig. 2: AMP induced cell apoptosis in MG-63 cells. Representative images of Hoechst staining (A) and AI (B) of MG-63 after exposure to different concentrations (0, 25, 50, 75, 100 µmol/L) of AMP for 24 and 48 h. Values are mean ± SE. Experiments were performed in triplicate and repeated three times. $^{a}P < 0.05$ *vs.* 0 µmol/L; $^{b}P < 0.05$ *vs.* 25 µmol/L; $^{c}P < 0.05$ *vs.* 50 µmol/L; $^{d}P < 0.05$ *vs.* 75 µmol/L; $^{e}P < 0.05$ *vs.* 24 h.

2.3. *Effects of AMP on cell cycle of MG-63 cell*

After exposure to different concentrations (0, 25, 50, 75, 100 µmol/L) of AMP for 48 h, Annexin V-FITC/PI double staining with FCM was adopted to investigate the effect of AMP on cell cycle of MG-63 cells. Compared with the effect of 0 µmol/L AMP, the test concentrations dose-dependently increased the proportion of G0/G1 cells but decreased the proportions of S and G2/M cells with significant difference (Fig. 4).

2.4. *Effects of AMP on expression levels of cell cycle-related protein*

After exposure to different concentrations (0, 25, 50, 75, 100 µmol/L) of AMP for 48 h, Western blotting was adopted to investigate the effect of AMP on expression levels of cell cycle related protein, including cyclin A, CDK2 and $p21^{CIP1}$. Compared with the effect of 0 µmol/L AMP, the test concentrations dose-dependently increased the protein level of $p21^{CIP1}$ but decreased protein levels of cyclin A and CDK2 with significant difference (Fig. 5).

3. Discussion

In the present study, we examined the effect of ampelopsin (AMP), a novel flavonoid, on the cytological behaviors of human OS cell lines *in vitro*. The primary novel findings in this study are that AMP dose-dependently and strongly decreased the cell viability of three common OS cell lines, suggesting a wide anti-proliferation role of AMP on OS. Furthermore, AMP induced cell apoptosis and cell cycle arrest at the G0/G1 phase on MG-63 cells in a dose-dependent manner. Besides, AMP prevented the accumulation of cyclin A and CDK2 as well as the con-

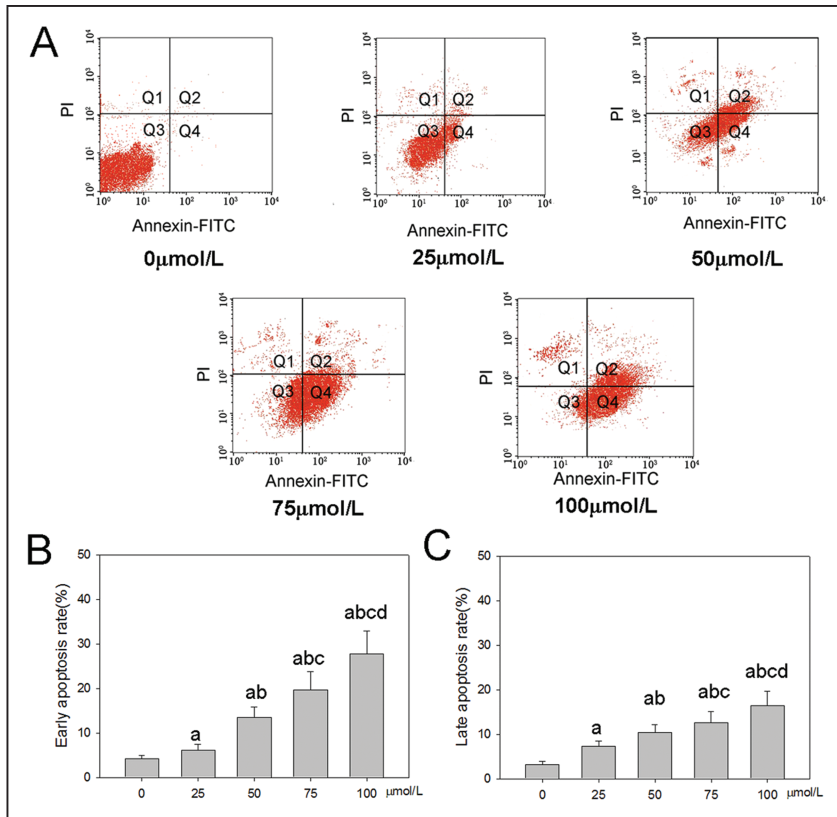

Fig. 3: AMP induced cell apoptosis in MG-63 cells. (A) The apoptotic status was evaluated by Annexin V-FITC binding assay. The lower right part (Annexin V-FITC$^+$/PI$^-$) and top right part (Annexin V-FITC$^+$/PI$^+$) were considered as early stage and late stage of apoptotic cells, respectively. Early apoptotic rate (B) and lateapoptotic rate (C) after exposure to different concentrations (0, 25, 50, 75, 100 μmol/L) of AMP for 48 h. Values are mean ± SE. Experiments were performed in triplicate and repeated three times. $^aP < 0.05$ *vs.* 0 μmol/L; $^bP < 0.05$ *vs.* 25 μmol/L; $^cP < 0.05$ *vs.* 50 μmol/L; $^dP < 0.05$ *vs.* 75 μmol/L.

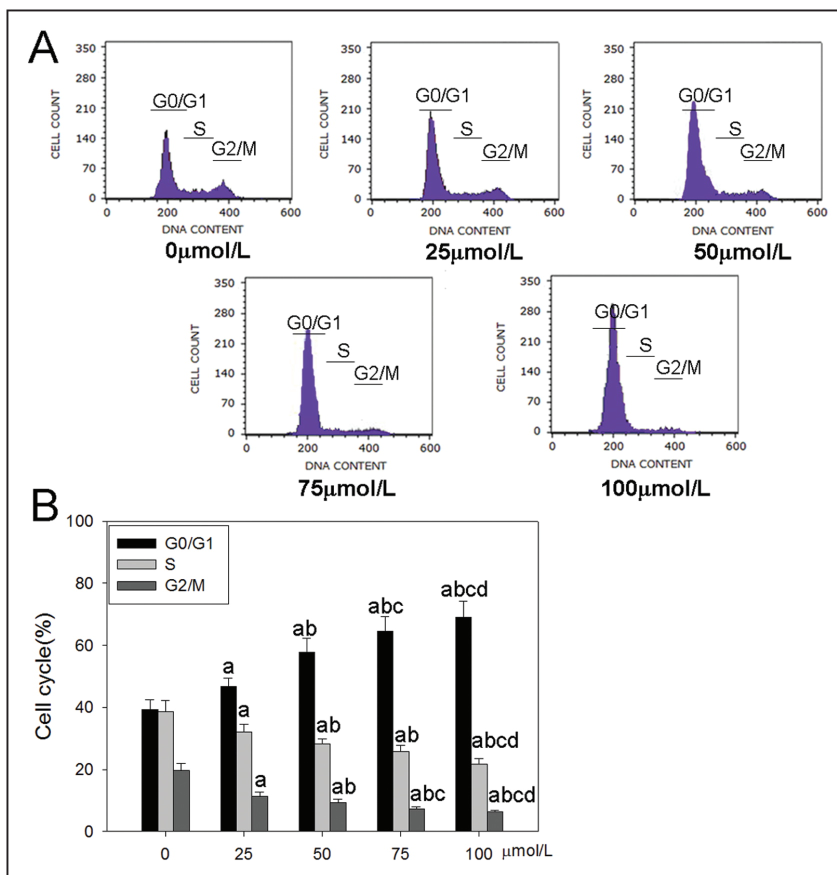

Fig. 4: AMP induced G0/G1 arrest in MG-63 cells. (A) The cell cycle was assessed by PI staining with FCM. (B) Proportions of cell cycle phases after exposure to different concentrations (0, 25, 50, 75, 100 μmol/L) of AMP for 48 h. Values are mean ± SE. Experiments were performed in triplicate and repeated three times. $^aP < 0.05$ *vs.* 0 μmol/L; $^bP < 0.05$ *vs.* 25 μmol/L; $^cP < 0.05$ *vs.* 50 μmol/L; $^dP < 0.05$ *vs.* 75 μmol/L.

sumption of p21^{CIP1}. To our knowledge, this is the first study to show that AMP exerts potent anti-cancer activities against OS by inhibiting cell growth and induction of apoptosis and G0/G1 phase arrest in MG-63 cells *in vitro*.

In the clinical treatment of OS, achieving favorable outcome is still a challenge. The therapeutic strategy for OS is limited due to the poor prognosis after surgical resection and increasing chemoresistance (Zhao et al. 2013). Moreover, chemopreventive intervention with a negative impact on survival and quality of life is not recommended (Longhi et al. 2006). It is urgent to exploit chemotherapeutic candidates with curative effect and moderate adverse effects. AMP is a novel flavonoid, and one of the bioactive constituents of *Ampelopsis grossedentata* (Hou et al. 2014; Zhou et al. 2014; Chen et al. 2015). Recent studies revealed the anti-cancer activities of AMP against lung, prostate and bladder cancers (Ni et al. 2012; Zhang et al. 2012; Chen et al. 2015). Continuous excessive growth is a characteristic feature of more advanced tumors. In the present study, we observed that AMP dose-dependently inhibited the growth of MG-63, Saos-2 and U-2 OS cells lines, suggesting AMP is cytotoxic to OS cells. The results were consistent with the growth inhibition effect of AMP on MCF-7 and MDA-MB-231 breast cancer cells (Zhou et al. 2014), and LNCaP and PC-3 human prostate cancer cell lines (Ni et al. 2012). The above evidence revealed high potencies and broad-spectrum anticancer activities of AMP. Moreover, Zhou et al. (2014) found that AMP had no cytotoxic effect on normal breast cells, and Ni et al. (2012) observed much less inhibiting effect on the growth of normal prostate epithelial cells versus prostate cancer cell lines. Similarly, we observed that AMP caused no cytotoxic activity to a normal human fetal osteoblastic cell line hFOB 1.19 (data not shown). These observations demonstrate that AMP is a promising and prospective chemotherapeutic candidate with moderate adverse effects for OS therapy. A previous report from Zhang et al. (2012) showed that AMP exhibited anticancer effects against bladder carcinoma in orthotopic xenograft models, confirming its potent anticancer effect *in vivo*. However, it is necessary to further investigate the *in vivo* anti-cancer effects of AMP against OS.

Excessive or misregulated cellular proliferation is usually accompanied with the decrease of apoptosis in cancers. The intrinsic inability of cells to undergo apoptosis may harbour an enhanced malignant potential and advance cancer development (Forones et al. 2005). Recent studies have observed the apoptosis-inducing activity of AMP in A549 human lung adenocarcinoma epithelial cells (Chen et al. 2015) and breast cancer cells (Zhou et al. 2014). As noted earlier for PC-3 human prostate cancer cells, *in vivo* anti-growth and anti-metastasis activities of AMP were associated with induction of apoptosis (Ni et al. 2012). These studies led us to hypothesize that AMP may be effective to induce the apoptosis of OS cells, so we employed two methods to evaluate the apoptotic status of MG-63 cells. Consistent with the previous studies, both the AI and apoptotic rate of MG-63 cells were elevated after exposure to AMP, indicating its capability to induce apoptosis. Interesting, Ye et al. (2008) demonstrated that AMP can prevent the H_2O_2-induced apoptosis of T lymphocytes. This appears to contradict our data about its apoptosis-inducing action. Involvement of different pathways modifying AMP activity towards apoptosis under physiological and pathological conditions may explain these intriguing findings. One example was recently provided by Zhou et al. (2014), which showed that AMP induced cell apoptosis in breast cancer cells through ROS generation and endoplasmic reticulum stress pathway. The same situation may apply to the present study. In addition, a preliminary report by Kou et al. (2012) found ERK and Akt signaling pathways mediating the inhibition effect of AMP on H_2O_2-induced apoptosis. Combined with data from studies showing anti-growth activity of AMP in lung and breast cancer cells (Zhou et al. 2014; Chen et al. 2015), we postulated that the AMP-mediated cell growth inhibition might be partly owing to the induction of apoptosis.

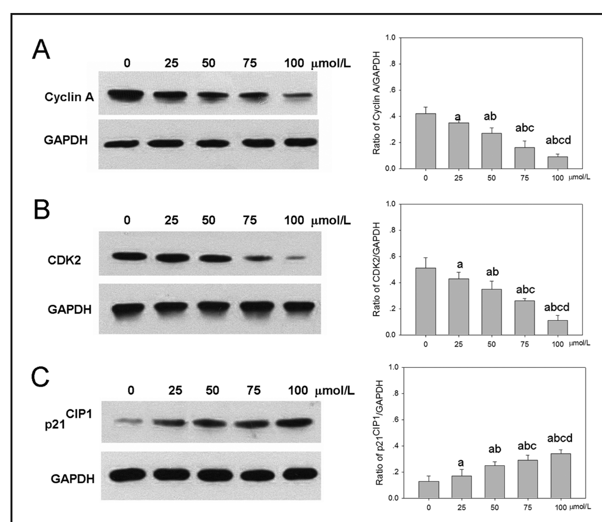

Fig. 5: AMP induced expression changes of cell cycle-related protein in MG-63 cells. Left panel showed the representative images of Western blotting, and right panel indicated the relative expression of cell cycle-related protein. Relative protein levels of Cyclin A (A), CDK2 (B) and p21^{CIP1} (C) after exposure to different concentrations (0, 25, 50, 75, 100 μmol/L) of AMP for 48 h. All experiments were performed in triplicate and repeated three times. $^{a}P < 0.05$ *vs.* 0 μmol/L; $^{b}P < 0.05$ *vs.* 25 μmol/L; $^{c}P < 0.05$ *vs.* 50 μmol/L; $^{d}P < 0.05$ *vs.* 75 μmol/L.

Cell cycle dysfunction is one of the main characteristics of tumors, and target for chemotherapy (You et al. 2014; Xiao et al. 2015). Unfortunately, the effect of AMP on the cell cycle has rarely been reported. In the present study, AMP dose-dependently increased the proportion of G0/G1 cells but decreased the proportions of S and G2/M cells, suggesting the induction of cell cycle arrest by AMP at the G0/G1 phase. An earlier study using *in vivo* orthotopic xenograft models also showed that AMP caused cell cycle arrest in tumor cells (Zhang et al. 2012). The inhibition of cell cycle progression was correlated with up-regulation of Cdk inhibitory proteins and decreased levels of Cdk2 activity (Xu et al. 2010). In order to verify whether AMP exhibited blocking effects on cell cycle *via* influencing the cell-cycle gene expression, we also measured the expression levels of cell cycle related protein, involving cyclin A, CDK2 and p21^{CIP1}. CDK2 is required for the transition from G1 to S phase of the cell cycle that determines cell division (Donato et al. 2002). CDK2 binding with cyclin A is required to progress through the S phase (Chinami et al. 2005). However, p21^{Cip1} is found to prevent cell cycle progression by inhibiting Cdk2-cyclin A kinases (Kumari et al. 2014). The above proteins play an important role in the regulation of cell cycle. It was likely that AMP induced G0/G1 arrest *via* the changes of certain factors involved in the regulation of cell cycle. Our study demonstrated that AMP dose-dependently increased the protein level of p21^{CIP1} but decreased protein levels of cyclin A and CDK2, consistent with the hypothesis.

Although AMP exerts anti-cancer effects in many cell lines of different malignant tumors, the poor aqueous solubility limit its use. Solanki et al. (2012) developed a microemulsion as novel delivery system to enhance the dissolution rate and bioavailability of AMP. Besides, 5-fluorouracil-substituted AMP derivatives, more effective than AMP (Zhou et al. 2010), may facilitate a better application of AMP anti-cancer properties both *in vivo* and *in vitro*.

In summary, we have demonstrated that AMP as a novel flavonoid can inhibit cell growth and induce apoptosis and G_0/G_1 phase arrest in MG-63 cells *in vitro*, with the potential mechanism of the negative regulation of cell cycle-related protein. Our findings may provide a new drug candidate as an alternative therapy for human OS in the future.

4. Experimental

4.1. Antibodies and reagents

AMP (purity≥ 98%) was purchased form Chengdu Must Biotechnology CO., LTD. Antibodies against cyclin A (sc-571), CDK2 (sc-163) and $p21^{CIP1}$ (sc-6246) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Cell Signaling Technology (Denver, Colo., USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Co. (Gibco BRL, NY). Annexin V-FITC Apoptosis Detection kit (ab14085) was purchased from BD Pharmingen (San Diego, California, USA). Hochest 33258, trypsin, thapsigargin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell counting kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology (China).

4.2. Cell cultures

Three human OS cell lines (MG-63, Saos-2 and U-2 OS) were obtained from the Shanghai Institute of Cell Biology (Shanghai, China). The cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under the atmosphere of 5% CO_2 in humidified incubator. When cells grew to approximately 80%-90% confluence, they were collected and prepared for the following protocols.

4.3. Cell viability

Cell viability assay was performed according to a procedure previously reported (Zhou et al. 2014). Briefly, cells were plated in 96-well microplates at a density of 1×10^5 cells per well in DMEM for 24 h. Then, the cells were exposed to different concentrations of AMP (0, 25, 50, 75, 100 μmol/L) for 24, 48, 72 and 96 h. DMSO diluted in culture medium at the final concentration of 0.1% without AMP was designated as 0 μmol/L. The cells were treated with 0.1% DMSO and used as control. For the cell viability assay, CCK-8 was used according to the manufacture's instructions. Cells were collected and washed with PBS following addition of 10 μl CCK-8 reagent to each well. After 2-h incubation, the optical density (OD) values of each well were assessed at the wavelength of 450 nm with an Elx808 absorbance microplate reader (BioTek, Winooski, VT, USA). Cell viability was expressed as percentage of the control. All experiments were performed in triplicate and repeated three times to ensure reproducibility.

4.4. Hoechst staining

Cells were plated in 24-well plates, incubated for 24 h and exposed to different concentrations (0, 25, 50, 75, 100 μmol/L) of AMP for 24 and 48 h. After being washed three times with PBS, the MG-63 cells were stained with Hoechst 33258 (1 mg/l) for 20 min at 37 °C. Images of the Hoechst 33258 fluorescence were captured using fluorescence microscope (Nikon, Japan). The apoptotic index was calculated as follows: apoptosis cells/total cells × 100%.

4.5. Annexin V-FITC/PI double staining

Annexin V-FITC/PI double staining was used for quantifying the AMP-induced apoptosis of MG-63 Cells by flow cytometry (FCM) (Cai et al. 2011). Briefly, cells were cultured overnight in 6-well plates and then exposed to different concentrations (0, 25, 50, 75, 100 μmol/L) of AMP for 48 h. DMSO diluted in culture medium at the final concentration of 0.1% without AMP was designated as 0 μmol/L. According the manufacturer's instructions of an Annexin V-FITC apoptosis detection kit, cells were washed with ice-cold PBS, detached by trypsinization and then centrifuged at 2000 rpm (4 °C) for 5 min. After being resuspended in 1 × Annexin binding buffer (200 ml), the cells were harvested and stained with propidium iodide (5 ml) and Annexin V-FITC (2.5 ml) for 15 min at room temperature. The apoptotic rates of cell samples were acquired using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

4.6. Cell cycle

The FCM was performed to detect the cell cycle changes induced by AMP as previously reported (Liu et al. 2009). Briefly, after incubation with different concentrations (0, 25, 50, 75, 100 μmol/L) of AMP for 48 h, cells were detached in trypsin, washed twice with ice-cold PBS and fixed in 70% ice-cold ethanol, followed by treatment with 0.25 mg/mL RNase at 37 °C. The propidium iodide staining was carried out and then the cellular DNA content was assessed accordingly.

4.7. Western blotting

AMP-induced changes of cell cycle–related proteins of MG-63 cells were detected by Western blotting as previous reported (Fan et al. 2012). Briefly, cells were harvested and homogenized under ice-cold conditions after exposure to different concentrations (0, 25, 50, 75, 100 μmol/L) of AMP for 48 h. The supernatants containing the cytoplasmic protein fraction were collected and subjected to protein assay *via* Bradford assay. Equivalent amounts of proteins were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane (Pall, Pensacola, FL, USA) and immunoblotted with primary antibody against cyclin A, CDK2 and $p21^{CIP1}$ (dilution 1:200 for cyclin A and $p21^{CIP1}$, 1:100 for CDK2). The secondary antibody, biotinylated goat anti-rabbit IgG, was used in a dilution of 1:5000. After incubation with enhanced chemiluminescence (Pierce, Rockford, IL, USA) and visualized by exposing to BioMax films (Kodak, Rochester, NY, USA), the membranes were stripped and sequentially immunoblotted with a mouse monoclonal GAPDH primary antibody and rabbit anti-mouse secondary antibody. The results were expressed as the ratio of the optical density value of the interested band to GAPDH band.

4.8. Statistical analysis

The data analyses were performed with SPSS software, version 18.0 (SPSS, Chicago, IL). The values were expressed as mean ± SE. ANOVA followed by *post hoc* Bonferroni test was applied when multiple comparisons between different concentrations or different time points of the same concentration were made. The significance level of P was set at 5%.

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