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Andrographolide inhibits proliferation and induces apoptosis of nasopharyngeal carcinoma cell line C666-1 through LKB1-AMPK-dependent signaling pathways

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Background: Andrographolide (Andro) belongs to the main bioactive ingredients of *Andrographis paniculata*. Many studies have shown that andro has a variety of pharmacological activities such as anti-inflammatory, anti-bacterial, anti-virus, anti-oxidant, immune regulation and liver protective effects. Moreover andro has been reported to have anticancer activity in multiple types of cancer, including gastric cancer, breast cancer, lung cancer and so on. However, there is no report about the effect of andro on the human NPC cell line C666-1 and the molecular mechanisms of andro-mediated apoptosis in C666-1 cells remain to be clarified. *Methods:* Cell proliferation was measured by a CCK8 assay, cell apoptosis rate was evaluated by flow cytometric analysis, and the protein expression of LKB1/AMPK signaling pathways was detected by Western blotting. *Results:* Treatment with andro inhibited cell proliferation and induced apoptosis of C666-1 cells. Moreover, andro could activate LKB1-AMPK signaling. We also demonstrated that Ca^{2+}/calmodulin-dependent protein kinase kinaseβ (CaMKKβ) was not involved in the regulation of andro on AMPK activation in C666-1 Cells. *Conclusions*: Andro suppressed proliferation and induced apoptosis of C666-1 cells through regulating the LKB1/AMPK/mTOR signal pathway.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a multifactorial malignancy associated with both genetic and environmental factors. The cancer arises from the epithelium of the nasopharynx. It has a remarkably distinctive ethnic and geographic distribution (Lo et al. 2004). It is more common in males (sex ratio of 2.3:1) with very distinct geographic areas of high risk. In South-Eastern Asia, Micronesia/Polynesia, Eastern Asia and Northern Africa, China, the NPC have a high incidence (Ferlay et al. 2015). The main treatment is radiotherapy, but the increased resistance of surviving cells after radiotherapy for NPC is the basis for the recurrence and metastasis of NPC. Therefore, it appears urgent to develop an effective anti-cancer drug to increase the sensitivity of tumor cells to radiotherapy.

The use of natural products for cancer treatment has stimulated increasing attention in recent years (Seca and Pinto 2018). There is a demand for naturally-derived anticancer agents which can be regarded as a good choice for anti-cancer drugs. *Andrographis* is known as the "king of bitter", which is not only a traditional medicine in China, but is also commonly used in Southeast Asia. Andrographolide (andro) belongs to the main bioactive ingredients of *Andrographis paniculata*. Many studies have shown that andro has a variety of pharmacological activities such as anti-inflammatory, anti-bacterial, anti-virus, anti-oxidant, immune regulation and liver protective effects (Tan et al. 2017). Moreover andro has been reported to have anticancer activity in multiple types of cancer, including gastric cancer, breast cancer, lung cancer and so on (Li et al. 2017). However, up to now, little is known about the effect of andro on NPC C666-1 cells.

In this present study, we aimed to investigate the anticancer ability of andro in NPC C666-1 cells. We further evaluated the effect of andro on LKB1/AMPK-dependent signal pathways in NPC C666-1 cells. This is the first evidence linked to the AMPK with the anticancer effects of andro on NPC C666-1 cells.

2. Investigations and results

2.1. Andro inhibited proliferation of C666-1 cells

We first evaluated the antiproliferative effects of andro on C666-1 cells, the cells were cultured with various concentrations of Andro (25, 50, 75, 100 μM) for 48 h or with 100 μM andro for different times (0, 12, 24, 48 h). As shown in Fig. 1A and B, andro inhibited the proliferation of C666-1 cells in a dose- and time-dependent manner.

2.2. Andro induced apoptosis of C666-1 cells

To clarify whether andro induced the apoptosis in C666-1 cells, flow cytometry analysis and western blot have been performed. As shown in Fig. 2A and B, andro could significantly induce cell apoptosis of human NPC C666-1 cells. We also found that andro increased the protein expression of bax and decreased the level of bcl-2 (Fig. 3A-C). Moreover, andro caused caspase-3 cleavage (Fig. 3D). These results further indicated the pro-apoptosis effect of andro on C666-1 cells.

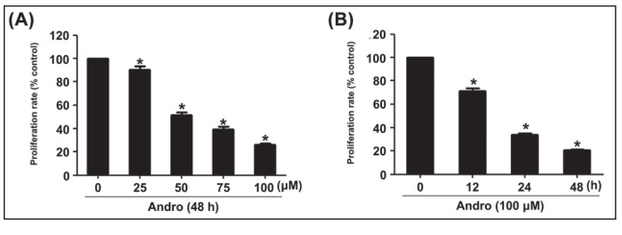

Fig. 1: Andro inhibited proliferation of C666-1 Cells. (A and B) C666-1 cells were treated with various concentrations of andro for 48 h or with 100 μM andro for indicated time points. The cell viability was detected by CCK8 assay. *P<0.05 vs control. n=3.

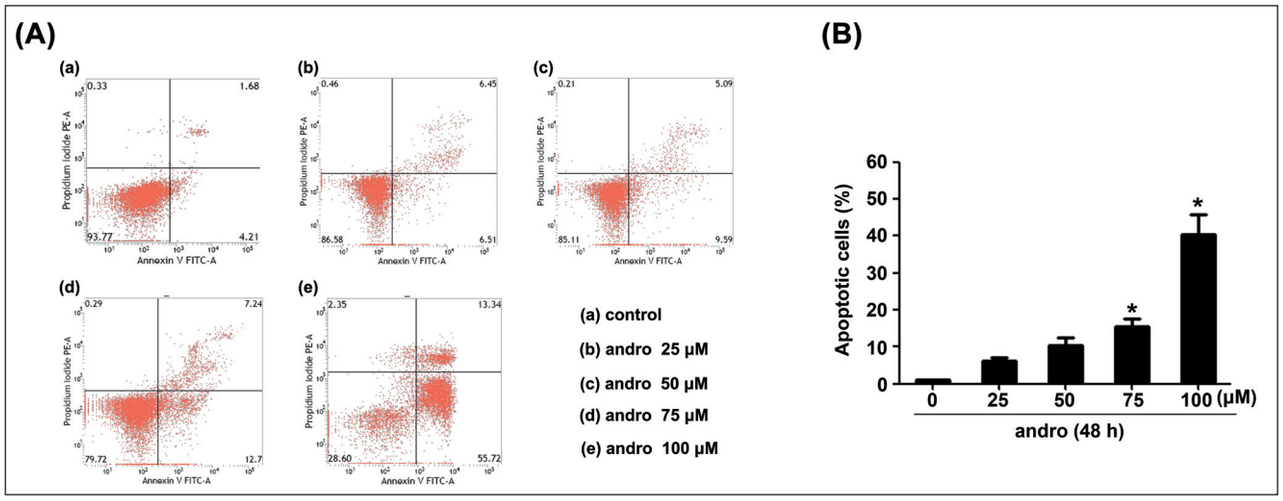

Fig. 2: Andro induced apoptosis of C666-1 Cells. (A and B) C666-1 cells were treated with indicated doses of Andro for 48 h. Cell apoptosis was then detected by Flow Cytometric Analysis. *P<0.05 vs control. n=3.

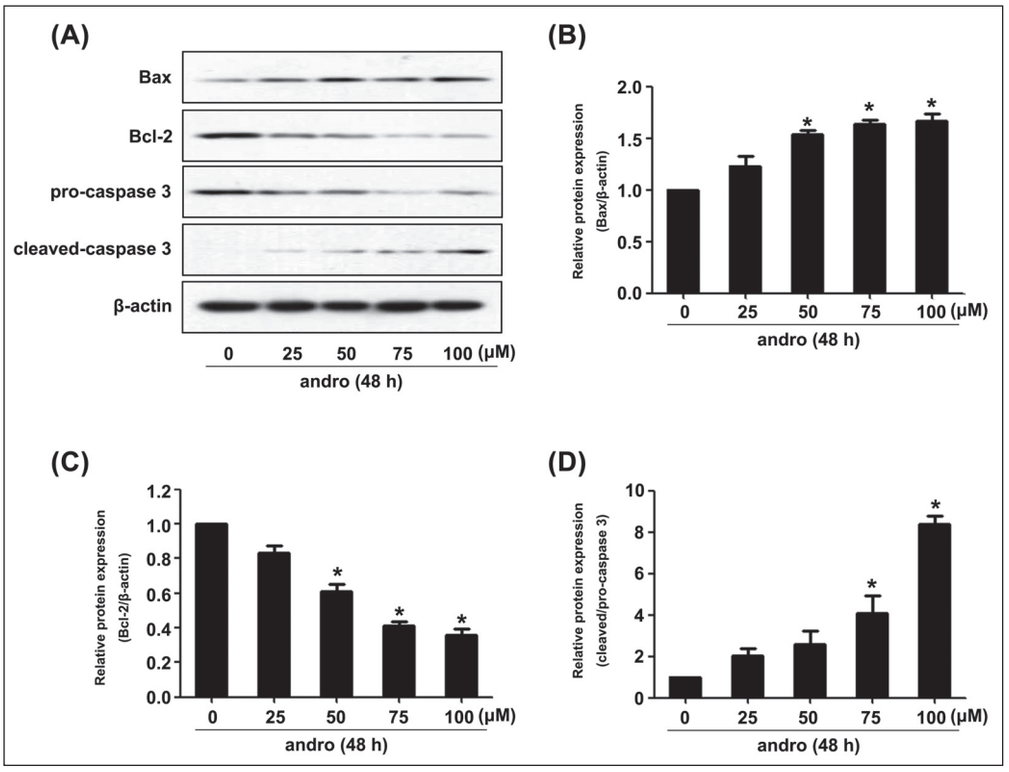

Fig. 3: Effect of andro on the protein expression of bax/bcl-2 and casepase-3. (A-D) C666-1 cells were treated with increasing concentrations of Andro for 48 h and the expression of bax, bcl-2 and caspase-3 were analyzed by western blot. *P<0.05 vs control. n=3.

2.3. Andro activated LKB1/AMPK-dependent signaling pathway

To elucidate the potential molecular mechanisms of andro on C666-1 cells, we detected the phosphorylation level of LKB1 and AMPK. As shown in Fig. 4A-C, andro could significantly enhance the protein expression of p-AMPK and p-LKB1 in a dose-dependent manner. Andro also decreased the protein level of p-P70S6K and p-S6, the main downstream of mTOR signaling (Fig. 4D).

2.4. CaMKKβ was not involved in the regulation of andro on AMPK activation

In order to investigate whether CaMKKβ was also involved in the regulation of andro on AMPK activation, we inhibited CaMKKβ by pretreating STO-609, a specific inhibitor of CaMKKβ. As shown in Fig. 5, pretreatment with STO-609 had no effect on AMPK activation induced by andro, suggesting that CaMKKβ was not involved in the regulation of andro on AMPK activation.

3. Discussion

Andro, the main bioactive ingredient of *Andrographis*, is a popular medicinal plant and widely cultivated in South East Asian countries for treating various disorders, due to its antioxidant, anti-inflammatory and cardiovascular protective activities (Tan et al. 2017). Recent studies have shown that andro is also a powerful anti-cancer agent, which works by promoting the apoptosis of cancer cells (Li et al. 2017). Different mechanisms of andro regarding apoptosis induction in cancer cells have been reported. Andro has been found

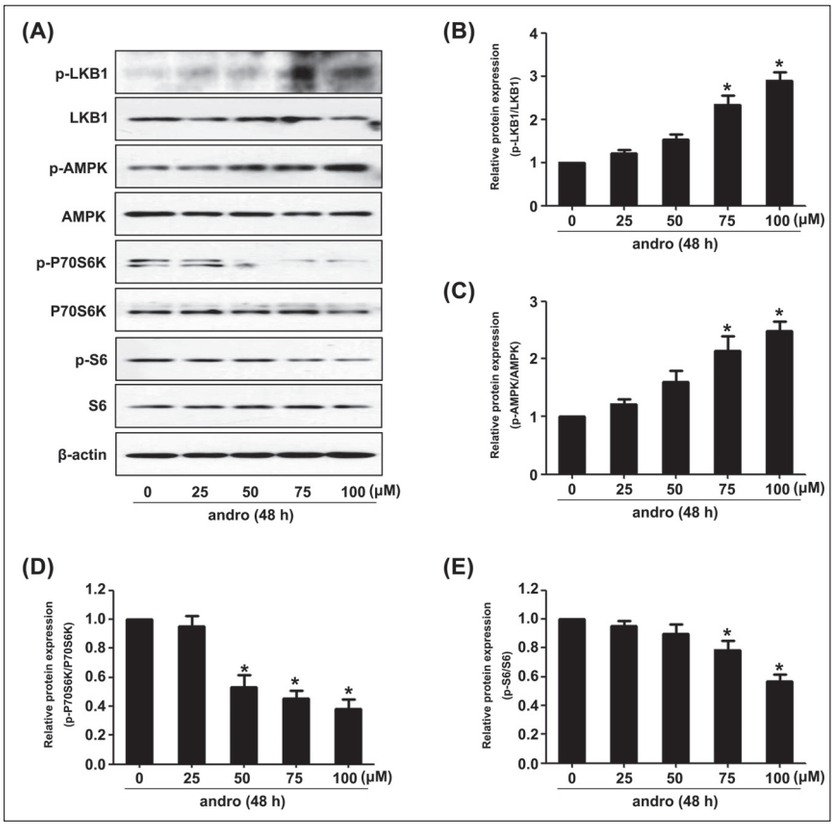

Fig. 4: Andro activated LKB1/AMPK-dependent signaling pathway in C666-1 cells. (A-E)C666-1 cells were treated with different concentrations of Andro for 48 h. The expression of p-LKB1, LKB1, p-AMPK, AMPK, p-P70S6k, P70S6K, p-S6, S6 and β-actin were detected by western blot. *P<0.05 vs control. n=3.

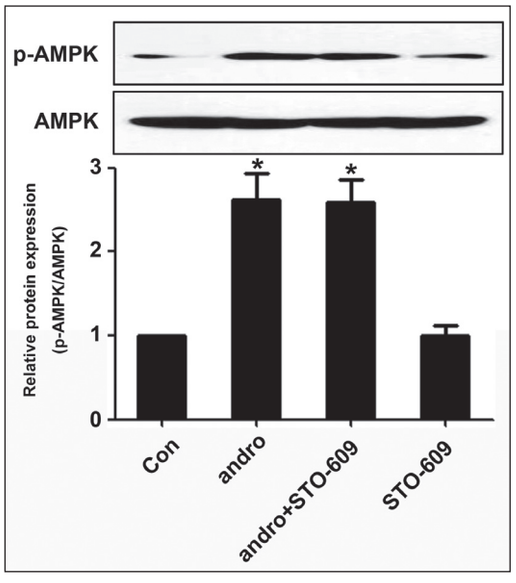

Fig. 5: CaMKKβ was not involved in the regulation of Andro on AMPK activation. Cells were pretreated with 1 μM STO-609 for 1 h before being treated with 100 μM andro for 48 h, and the expression of AMPK and p-AMPK were detected by western blot. *P<0.05 vs control, #P<0.05 vs. andro group, n=3.

to induce cell cycle arrest and apoptosis of pancreatic cancer cells by inhibiting STAT3 and Akt activation (Bao et al. 2013). Moreover, andro enhanced cisplatin-mediated anticancer effects in lung cancer cells through blockade of autophagy (Yuwen et al. 2017). Similarly, recently studies showed that andro increased 5-fluorouracil-induced antitumor effects in colorectal cancer *via* inhibition of the c-MET pathway (Su et al. 2017). However, to the best of our knowledge, there is no report about the pro-apoptotic effect of andro on NPC C666-1 cells. In this study, we demonstrated that andro suppressed proliferation and induced apoptosis of human NPC cell line C666-1.

The mechanism by which andro mediates its pro-apoptotic effects remains unclear. AMPK, a crucial regulator of energy metabolic homeostasis, was demonstrated to play a vital role in tumor formation (Cai et al. 2015c). Activated AMPK on its turn can phosphorylate TSC2 at multiple serine and threonine sites leading to inactivation of mTOR. The inhibition of mTOR can dephosphorylate S6 and p70S6K, which are critical factors in promoting protein synthesis and inducing cell apoptosis (Cai et al. 2015d; Huang et al. 2014). Metformin, an AMPK activator, could significantly inhibit ovarian cancer growth, which was greatly associated with mTOR inhibition following AMPK activation (Gwak et al. 2017). Moreover, previous studies also showed that AICAR could induce mitochondrial apoptosis in human osteosarcoma cells through an AMPK-dependent pathway (Morishita et al. 2017). In this study, we showed that andro is able to activate AMPK and to inhibit mTOR signaling, as manifested by dephosphorylation of S6K1 (T389) and S6 (S235/236) in human C666-1 cells.

The activity of AMPK is mainly regulated by two upstream kinases, namely LKB1 and the calcium-dependent kinase CaMKKβ. LKB1 has been considered as a constitutively active serine/threonine protein kinase that is ubiquitously expressed in mammalian cells and phosphorylates the catalytic a subunit of AMPK by an increase in the AMP:ATP ratio in cells. In contrast to LKB1, the activation of AMPK *via* CaMKKβ is independent of changes in the AMP:ATP ratio and is initiated by agonists, which leads to a receptor-coupled increase of intracellular Ca^{2+} (Ma et al. 2012). Previous studies demonstrated that etoposide induced AMPK activation in prostate C4-2 cancer cells *via* the modulation of kinase LKB1 (Luo et al. 2013). Moreover, another study reported that CaMKKβ-mediated AMPK activation is required by adiponectin-induced secretion in rat submandibular glands (Ding et al. 2018). In this present study, we showed that andro could activate the LKB1/AMPK-dependent signaling pathway.

However, it is currently unknown whether CaMKKβ is also involved in the regulation of andro-mediated AMPK activation. In this paper, we showed that pretreatment of the cells with STO-609 did not alter AMPK phosphorylation, indicating that CaMKKβ is not involved in andro-induced AMPK activation in C666-1 cells. Taken together, these data indicated that andro-induced activation of AMPK signaling in C666-1 cells is likely to be mediated by LKB1.

In summary, this is the first report that andro could inhibit proliferation and induce apoptosis of human C666-1 cells. This effect is linked to the activation of LKB1/AMPK-dependent signaling pathway. The finding provides experimental evidence for considering the application of andro as an adjuvant in NPC therapy.

4. Experimental

4.1. Chemicals and reagents

Andro (purity>90%) was purchased from Aladdin Reagent Co., Ltd (Shanghai, China). Dulbecco's Mofified Eagle Medium (DMEM) was purchased from Gibco (Logan, UT, USA). Fetal Bovine Serum (FBS) purchased from Yeasen Biotechnology Co., Ltd. Antibodies against Bax, Bcl-2, p-AMPK, p-S6, S6, p-P70S6K, P70S6K and Caspase 3 were purchased from Cell Signaling Technology. Antibodies against AMPK and β-actin were purchased from Santa Cruz Biotech (Santa Cruz Biotechnology). STO-609 was purchased from Selleck Chemicals. Cell counting kit 8 (CCK8) was purchased from Dojindo Molecular Technologies, Inc. Dimethyl sulfoxide (DMSO, purity>90%) was purchased from Sigma-Aldrich.

4.2. Cell culture

C666-1 cells were maintained in DMEM medium supplemented with 10% FBS in humidified incubator of 5 % CO_2 at 37 °C. Experiments were performed using cells in the logarithmic growth phase of growth. Before treating with Andro, cells were cultured in DMEM with free FBS for 12 h.

4.3. Cell viability assay

Cell viability was measured by CCK8 assay. C666-1 cells were seeded into 96 well microplates at a density of 8×10^3 cells/well. At 24 h following seeding, C666-1 cells were treated with various concentrations of andro for 48 h or with 100 μM andro for indicated time points. Subsequently, cells were incubated for additional 1 h with 10 μl of CCK8 at 37 °C. Finally, absorbance of each well was read at 450 nm wavelength using a microplate reader (BioTek Instruments Inc.).

4.4. Cell apoptosis assay

Cell apoptosis analysis was performed as previously described (Cai et al. 2015a) and was measured using an Annexin V-FITC/PE Apoptosis Detection Kit (Bio Vision) together with flow cytometry. Briefly, after treating with various concentrations of Andro (0, 25, 50, 75, 100 μM), C666-1 cells were harvested and washed with phosphate buffer solution (PBS) buffer and resuspended in 100 μL binding buffer. Staining was performed with Annexin V-PI labeling solution for 5 min at room temperature in the dark. The samples were analyzed with the flow cytometer (BD FACSVerse™) and BD FASCuite software analysis.

4.5. Western blot analysis

Western blot analysis was performed as previously described (Cai et al. 2015b). Briefly, equal amounts of proteins from each sample were separated by 10 % or 12 % SDS-PAGE gel electrophoresis and then transferred to PVDF membranes (Millipore). After incubating in primary and secondary antibodies, immunoreactive bands were detected with the Super-Signal West Pico Chemiluminescent Substrate (Pierce), and molecular band intensity was determind by densitometry.

4.6. Statistical analysis

Data are expressed as the mean±standard deviation. Student's *t*-test was used to statistical analyzed between two groups. One-way analysis of variance (ANOVA) tested the differences among groups. $P < 0.05$ was considered as significant for all analyses. Source of Founding: This study was supported by the Education Administration Research Foundation of Guangzhou City (NO. 1201410511), Guangdong Provincial Key Laboratory of allergic reaction and immune fund project (NO. 15KAL10), Natural Science Foundation of Guangdong Province (NO. 2017A030313571). Moreover, we also appreciated Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University.

Conflicts of interest: None declared.

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