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Apigetrin inhibits gastric cancer progression through inducing apoptosis and regulating ROS-modulated STAT3/JAK2 pathway

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ABSTRACT

Apigetrin (APG), as a flavonoid, has many cellular bioactivities, including regulation of oxidative stress, and induction of apoptosis. However, the means by which APG suppresses human gastric cancer are still little to be understood. In the present study, the anti-cancer effects of APG on human gastric cancer cells were investigated. The results indicated that APG could suppress the proliferation and induce apoptosis in gastric cancer cells. Its role in apoptosis induction was through reducing Bcl-2, and enhancing Bax, Caspase-9/-3 and poly ADP-ribose polymerase (PARP) cleavage. In addition, APG incubation resulted in the generation of intracellular reactive oxygen species (ROS) in cells. Meanwhile, APG suppressed constitutive and interleukin-6 (IL-6)-stimulated signal transducer and activator of transcription 3 (STAT3), Janus kinase 2 gene (JAK2) and Src activation. However, ROS scavenger, N-acety-l-cysteine (NAC), diminished apoptosis induced by APG. And APG-triggered de-phosphorylation of STAT3/JAK2 was rescued by NAC pre-treatment. *In vivo*, APG administration significantly inhibited the gastric cancer cell xenograft tumorigenesis through inducing apoptosis and inhibiting STAT3/JAK2 pathways. Taken together, the findings above illustrated that APG might be used as a promising candidate against human gastric cancer progression.

1. Introduction

Gastric cancer is a frequently occurring cancer with high mortality every year in the world [1,2]. The majority of patients admitted to hospital are in the advanced stage of gastric cancer and less than one-half of these patients survive for>5 years [3]. Therefore, finding new and effective therapeutic strategies against human gastric cancer is urgently required.

Accumulating evidences suggest that flavonoids might be helpful in reducing cancer risk by various pathways [4,5]. Apigetrin (APG) is a flavonoid commonly found in many fruits and vegetables, and might be also a promising candidate against gastric cancer progression [6]. Recently, APG has been focused for its bioactivities, including anti-cancer by regulating oxidative stress, and inflammation both *in vitro* and *in vivo* [7,8].

Apoptosis is one of the main mechanisms by which the cell death was induced in tumor treatment [9]. Its deregulation may lead to a variety of pathological conditions, such as the initiation, augment, and development of tumor [10]. Apoptosis has become a preferable choice of cancer cell death to prevent cancers [11]. Reactive oxygen species (ROS) play an important role in biological systems [12,13]. And excessive ROS lead to irreversible cellular damage, including apoptosis induction in several types of cancer cells [14]. STAT3, belonging to a family of cytoplasmic latent transcription factors, functions as both signal transducers and activators of transcription, which is associated with tumor progression and worse survival in cancer [16].

In the present study, APG could reduce cell viability and induce apoptosis in human gastric cancer cell lines. Intracellular ROS was significantly induced by APG. Meanwhile, APG blocked constitutive and IL-6-stimulated STAT3/JAK2 expression, which was reversed by NAC pre-treatment. *In vivo*, APG reduced the growth of tumor samples. Notably, there was no toxicity was observed in APG-treated normal cells and organ tissues. We supposed that APG might be used as a promising candidate against human gastric cancer progression.

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2. Materials and methods

2.1. Cells and culture

Human gastric cancer cell lines, SGC-7901 and MGC-803, human normal glia cell of HEB were purchased from American Type Culture Collection (ATCC, USA). Human gastric cancer cell lines of BGC-823 and MKN-45, and mouse normal gastric cell line of HL-7702 were obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Human normal gastric cell line of GES-1, namely a gastric epithelial cell line, was purchased from the Beijing Research Institute of Cancer Prevention and Treatment (Beijing, China). BGC-823, MKN-45, SGC-7901 and MGC-803 were cultured in RPMI-1640 medium (Gibco Life Sciences, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA). GES-1 and HL-7702 were cultured in DMEM (Gibco Life Sciences) containing 10% FBS. All cells were cultured in a humidified atmosphere with 5% CO_2 and 95% humidity at 37 °C in an incubator. Apigetrin (>98% purity, CAS: 578-74-5), purchased from Pure-one Bio Technology, CO. LTD. (Shanghai, China) used for the treatment of gastric cancer is dissolved in DMSO and stored at −20 °C, and then diluted in RPMI-1640 or DMEM medium for experimental treatment. IL-6 was purchased from R and D Systems (USA). N-acety-l-cysteine (NAC) was obtained from Sigma Aldrich (USA). For siRNA transfection, cells were transfected with scrambled control siRNA or Bax, or PARP siRNA (Generay Biotech, Shanghai, China) for 24 h with Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. z-VAD-fmk, Stattic and LY2784544 were purchased form Selleck Chemicals (USA).

2.2. Cell viability analysis

After various treatments, the cell viability was determined by MTT assay (KeyGen Biotech, Nanjing, China) at 570 nm following the manufacturer's instruction.

2.3. Hoechst33258 analysis

The cells were collected after various treatments for 24 h. Next, the cells were stained with Hoechst 33258 solution (Beyotime, Shanghai, China) following the manufacturer's instructions.

2.4. Flow cytometry assays

The Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was purchased from KeyGen Biotech to evaluate apoptosis. The cells after different treatments were harvested and washed with cool PBS, then incubated in a darkroom for Annexin V-FITC and PI for 15 min. Next, the cells were analyzed using flow cytometry (BD Biosciences, USA).

2.5. TUNEL analysis in vitro

The gastric cancer cells after different transfections were planted onto 12-well plates. Then, One Step TUNEL Apoptosis Assay Kit (Beyotime Biotech) was used to evaluate apoptosis following the manufacturer's instructions. The staining intensity was measured by a fluorescence microscopy.

2.6. ROS assessments

Intracellular ROS production was measured using the Reactive Oxygen Species Assay Kit (Keygen Biotech) following the manufacturer's protocols.

2.7. Western blot analysis

The total protein of the gastric cancer cells and tumor tissue samples was extracted using a lysis buffer for 50 min at 4 °C. Then, the lysates were centrifuged at 12,000 rpm at 4 °C for 15 min. The detailed protocol was accordance with previous reports [16]. The primary antibodies used in our study were shown in Supplementary Table 1. The staining intensity of the blotting bands was quantitated by densitometry with imageJ software (National Institute of Health, USA).

2.8. Athymic nude model experiment

The 40 male, 4–5 weeks old, nude mice, were obtained from the Animal Center of Shanghai (Shanghai, China). Single-tumor cell of SGC-7901 suspensions (1×10^6) were injected into the left flank of each mouse subcutaneously to establish gastric cancer xenografts. The mice were divided into four groups (n = 10/group) 3 days after cell implantation. Mice in the experimental groups were intraperitoneally injected with APG (10, 20 and 30 mg/kg body weight per day). Mice in the control group (Con) received an equal volume of normal saline. The tumor volume was measured every two days, and the tumor size was assessed as followings: tumor volume = $\text{width}^2 \times \text{length} \times 0.4$. Mice were sacrificed after 30 days. The body weight of mice was recorded. The liver samples and the tumor tissues were weighed and fixed in 10% formalin for further experiments. The lung and heart samples were fixed in 10% formalin for H&E staining.

2.9. Immunohistochemical (IHC) analysis

For the immunohistochemistry, the fresh tumor tissue samples were fixed in formalin for 48 h. Then the tissue sample was put in paraffin and next cut into the desired thickness (4 μm), and was then fixed into a slide. Primary tumor sections, liver, heart and lung tissues were stained with hematoxylin and erosion (H&E). After washing, the sections were prepared for blocking and incubating with antibodies, including KI-67, and p-STAT3, at 4 °C overnight. Sections were then incubated with diluted streptavidin-peroxidase HRP conjugates at room temperature. The tumor sections were then stained with hematoxylin for 3 min and mounted and analyzed by a phase-contrast microscope. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was performed using FragEL™ DNA Fragmentation Detection Kit (Colorimetric, USA) following the manufacturer's instruction.

2.10. Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using GraphPad PRISM (version 6.0; Graph Pad Software) by ANOVA with Dunnet's least significant difference post-hoc tests. A p-value less than 0.05 will be considered significant.

3. Results

3.1. Apigetrin reduces proliferation and induces apoptosis in human gastric cancer cell lines

As shown in Fig. 1A, the cell viability of human gastric cancer cell lines was all significantly reduced after treatment with APG in a dose-dependent manner. Fig. 1B indicated that normal gastric cells were not as sensitive as cancer cells to APG treatment. As shown in Fig. 1C and Supplementary Fig. 1A, APG exposure induced filament formation and nucleus condensation exhibited with the large

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