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Amentoflavone suppresses hepatocellular carcinoma by repressing hexokinase 2 expression through inhibiting JAK2/STAT3 signaling

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ABSTRACT

Hepatocellular carcinoma (HCC) has become a burden on public health worldwide. Therefore, development of novel therapeutic agents for managing HCC is imperative. Amentoflavone (AF), a flavonoid compound extracted from *Selaginella tamariscina* Spring, reportedly has anti-neoplastic activities. The aim of this study was to investigate the role of AF in HCC and the underlying mechanism of action. We found that AF decreased cell viability in a dose-dependent manner in both HCC cell lines but did not affect the viability of a normal hepatic cell line. The anti-growth effect of AF against HCC was also confirmed in the xenograft model. Standard histological examination of the xenograft tissues also revealed minimal *in vivo* toxicity of AF. The *In vitro* and *in vivo* models also provided evidence of the pro-apoptotic activity of AF. In addition, AF significantly inhibited glycolysis *via* HK2 repression; further dissection of the underlying mechanism revealed that AF downregulated HK2 by generating ROS and hence inhibiting the JAK2/STAT3 signaling pathway. In conclusion, AF induced apoptosis and inhibited glycolysis in HCC by targeting HK2. Our findings provide preliminary experimental data that support further investigation of the therapeutic efficacy of AF in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a global public health problem [1,2], accounting for approximately 782,000 newly diagnosed cases and 745,000 deaths annually [3]. Currently, surgical resection, liver transplantation and radiofrequency ablation remain the mainstream therapeutic regimens of HCC [4]. However, the prognosis of patients who receive surgical resection remains poor, with a five-year survival rate of about 20% [5]. The incidence of recurrence in HCC patients is also high due to high invasiveness and distant metastasis of the tumor cells [6]. Furthermore, the chemo-resistance of HCC cells limits the efficacy of conventional chemotherapy [7]. Therefore, current research on HCC is largely focused on developing novel therapeutic agents.

Aerobic glycolysis or the Warburg effect is a well-documented metabolic process utilized by cancer cells to meet their higher energy demands and generate anabolic precursor for biosynthetic pathways [8]. Since Warburg effect plays a key role in the survival and proliferation of cancer cells, the metabolic enzymes involved in glycolysis have been considered as potential therapeutic targets. Hexokinase II (HK2) catalyzes the initial and rate-limiting step in glycolysis by phosphorylating glucose to glucose-6-phosphate (G-6-P). Although predominantly expressed in insulin-sensitive tissue such as skeletal muscles and adipose tissues [9], aberrantly high HK2 levels have been reported in various cancer tissues compared to normal tissues, further emphasizing its role as a promising therapeutic target. In the context of HCC, HK2 depletion is known to inhibit glycolysis and downregulation of HK2 was associated with increased apoptosis [10,11].

Amentoflavone (AF) is a biflavonoid compound extracted from *Selaginella tamariscina* Spring. As a representative biflavonoid with several pharmacological functions, the bioavailability of AF with intraperitoneal injection was 77.4% ± 28.0%. AF has pharmacological activities including anti-inflammatory [12], anti-microbial [13], anti-oxidative [14], anti-radiation [15], and even anti-neoplastic [16–18]. The pro-apoptotic effect of AF has been observed in melanoma, lung cancer, cervical cancer, breast cancer and colorectal cancer cells [19–23]. In addition, anti-metastatic and anti-angiogenic effects of AF have also been reported in breast cancer [24]. Interestingly, AF has been found to able to modulate metabolic function of cells [25]. Therefore, we postulated whether AF could affect the metabolic process of tumor cells and consequently exhibit anti-cancer activities. In the current study, the suppressive effect of AF on glycolysis and HCC growth was investigated *in vitro* and *in vivo* models. We also examined the role of HK2 in the anti-neoplastic activities of AF and explored the regulatory pathways.

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2. Materials and methods

2.1. Cell culture and treatment

Human Huh7 and Hep3B cells were obtained from Shanghai Cell Bank, China, and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, USA). The normal human hepatic cell line was obtained from Cell Bank of Chinese Academy of Sciences and incubated with same culture medium as above. Cells were grown at 37 °C under 5% CO_2 (v/v) in a humidified atmosphere. HCC cells were incubated with IL-6 (or IL-21) or NAC at indicated concentration for 6 h followed by AF treatment for 24 h.

2.2. Determination of cell viability

Cell viability was determined by the Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) assay. Briefly, cells were plated at 5×10^3 cells onto 12-well plates and were treated with different concentrations of AF for 24 and 48 h. Following treatment, the absorbance of the medium was measured at 450 nm in a spectrophotometer (Tecan Group Ltd, Männedorf, Switzerland).

2.3. DNA fragmentation assay

Apoptosis was analyzed using a Cell Death Detection ELISA^{PLUS} kit (Cat No. 11774425001, Roche Applied Science, Indianapolis, IN), which was designed to for the quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death. Following cell lysis and centrifugation, the lysate was transferred to a specific microplate provided with the kit and incubated with the antibody for 2 h at room temperature according to the manufacturer's instructions. Then the wells were washed before the conjucate solution was added and incubated at room temperature for 90 min. The conjucate solution was removed and the substrate was added for color development. The apoptotic cells were analyzed by measuring the absorbance of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 405 nm with a microplate reader (Tecan Group Ltd, Männedorf, Switzerland).

2.4. Annexin V assay

Following AF treatment, the HCC cells were stained using the FITC Annexin V Apoptosis Detection kit (BD Pharmingen, Franklin Lakes, NJ) as per manufacturer's instructions. The percentage of apoptotic cells was analyzed in a flow cytometer (Beckman Coulter Inc, Miami, FL).

2.5. Western blotting assay

Cytosolic and mitochondrial proteins were separated using isolation buffer (Amresco, Solon, OH) as previously described [26] and their concentration were measured using the BCA protein assay kit (Beyotime). The protein samples were subjected to SDS-PAGE before being transferred onto a PVDF membrane (Thermo Fisher Scientific). Target proteins were probed with specific antibodies following standard protocols. Goat anti-rabbit IgG-HRP (Beyotime, Shanghai, China) was used as the secondary antibody, and β-actin was used as the internal control to normalize the relative levels of protein expression. BandScan software (Glyko, Novato, CA) was used to quantify the blot density.

2.6. Measurement of glucose consumption and lactate production

HCC cells were allowed to grow for 24 h before being trypsinized, and then seeded into 6-well plates at the density of 5×10^5/well. The cells were challenged with AF at the indicated concentrations for 24 h. An Automatic Biochemical Analyzer (Tokyo, Japan) was used to determine the glucose and lactate levels. The relative glucose uptake rate and lactate production rate were normalized to the protein concentration of the samples.

2.7. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cultured HCC cells using TRIzol Reagent (Life Technology, Carlsbad, CA) and reverse transcribed to cDNA with a reverse transcription kit (Promega, Madison, WI) as per the manufacturer's instructions. RT-PCR was performed to analyze target gene expression using Power SYBR Green PCR Master Mix (Carlsbad, CA), with GAPDH as the internal control. Sangon (Shanghai, China) synthesized the forward and reverse primers using previously published sequence [27]. The 2-ΔΔCt method was used to analyze the relative expression of the target genes.

2.8. Immunoprecipitation assay

After treating the cells with AF for 24 h, total cellular protein was isolated using lysis buffer. A total of 500 µg extracted proteins were immunoprecipitated overnight with 4 µg anti-VDAC (Cell Signaling Technology Inc., Beverly, MA) and 4 µg anti-HK2 antibodies (Cell Signaling Technology Inc., Beverly, MA) or irrelevant IgG at 4 °C. The immunoprecipitated protein was pulled down with 20 µl protein G-Sepharose at 4 °C for 4 h, and the beads were then washed four times with lysis buffer before SDS-PAGE.

2.9. Reactive oxygen species (ROS) assay

Fluorescent probe 2′,7,-dichlorofluorescein-diacetate (DCFH-DA) was used to detect ROS generation in the cells. Following AF treatment, the cells were harvested and resuspended in DCFH-DA (10µM) and incubated at 37 °C for 30 min in the dark. The fluorescence signals were examined by microscopy.

2.10. Vector construction and transfection

The human HK2 overexpression vector was constructed as previously described [28]. The HK2 overexpression and empty vectors were respectively transfected into HCC cells by Lipofectamine 3000 (Invitrogen, Grand Island, NY) as per the manufacturer's instruction. The cells were harvested 48 h following transfection, and processed for Western blotting as described to measure HK2 expression levels.

2.11. In vivo tumor growth model

The Institutional Animal Care and Use Committee of the second hospital of Hebei Medical University approved all animal experiments in this study. Eight-week-old male athymic BALB/c nu/nu mice were housed in pathogen-free conditions at 24–26 °C with a 12-hour dark/light cycle with food and water *ad libitum*. The mice were injected with Huh7 cells (5×10^6 cells) in their left flanks and 12 days after injection, 18 mice were randomized into 3 groups (6 mice per group) that received the following injections intraperitoneally: (A) vehicle (0.9% sodium chloride plus 1% DMSO), (B) AF dissolved in vehicle at 40 mg/kg body weight per day, and (C) AF dissolved in vehicle at 80 mg/kg body weight per day [29]. The body weight and tumor volume of the mice were measured twice every week until day 27. Cryostat sections (4 µm/section) of xenograft tumors were stained as per standard IHC and TUNEL assay protocols as previously described [30]. The other organs were also examined by HE staining to determine the general *in vivo* toxicity of AF.