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Original Article

The inhibitory effects of xanthohumol, a prenylated chalcone derived from hops, on cell growth and tumorigenesis in human pancreatic cancer

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ABSTRACT

Pancreatic cancer (PC) is one of the most lethal human malignancies worldwide. Here, we demonstrated that xanthohumol (XN), the most abundant prenylated chalcone isolated from hops, inhibited the growth of cultured PC cells and their subcutaneous xenograft tumors. XN treatment was found to induce cell cycle arrest and apoptosis of PC cells (PANC-1, BxPC-3) by inhibiting phosphorylation of signal transducer and activator of transcription 3 (STAT3) and expression of its downstream targeted genes *cyclinD1*, *survivin*, and *Bcl-xL* at the messenger RNA level, which involved in regulation of apoptosis and the cell cycle. Overall, our results suggested that XN presents a promising candidate therapeutic agent against human PC and the STAT3 signaling pathway is its key molecular target.

1. Introduction

Pancreatic cancer (PC) remains one of the most aggressive and deadliest malignancies with overall median survival duration of less than 1 year from diagnosis. Since 1997, gemcitabine has been the first-line therapy for PC patients and is associated with a very low tumor response rate of less than 10% [1]. Adding various cytotoxic and targeted chemotherapeutic agents to gemcitabine has failed to improve overall survival, with the exception of some combination therapies that included erlotinib or the FOLFIRINOX regimen, which demonstrated only a minimal survival benefit [2,3]. For now, the 5-year relative survival rate for PC in the United States is only 7% [4]. Therefore, there is an urgent need to develop new therapeutic strategies with low toxicities to treat PC.

A growing collection of studies has shown that xanthohumol (XN), the most abundant prenylated chalcone found in hops [5], can inhibit proliferation of cancer cells and/or induce apoptosis in hematological malignancies, such as acute leukemia [6,7] and various solid tumors, including breast cancer [5,8], colorectal cancer [9,10], ovarian cancer [11], glioblastoma [12], hepatocellular carcinoma [13,14], prostate cancer [15,16], and fibrosarcoma [17]. Thus, XN may present a promising reagent for cancer therapy.

However, there is no data available regarding the effects of XN in PC. In this study, we aimed for the first time to test the effects of XN on proliferation and tumorigenesis in PC both in vitro and in vivo.

2. Materials and methods

2.1. Cells, reagents and animals

PC cell lines (PANC-1 and BxPC-3) were obtained from American Type Culture Collection (Manassas, VA, USA) and were

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cultured in high-glucose Dulbecco's modified Eagle's medium and Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin), respectively. The adherent cells were detached from the flask surface using a solution of 2.5% trypsin and ethylenediaminetetraacetate. XN (purity, > 98%) was purchased from Shanghai Winherb Medical Science (Shanghai, China). A 50 mM stock solution was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), stored at −20 °C, and then diluted as needed in cell culture medium. Monoclonal or polyclonal antibodies specific for actin, cyclin D1, ki67, total signal transducer and activator of transcription 3 (STAT3), and survivin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p-STAT3-specific monoclonal antibodies were obtained from BD Biosciences (San Jose, CA, USA). Twenty pathogen-free nude mice (4–5 weeks old) were purchased from Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). Five mice each were maintained in an individual ventilated cage. All protocols concerning laboratory animal usage were submitted and validated by Animal Care Ethics Committee of Shanghai Jiao Tong University.

2.2. Cell viability assay

Cell viability of corresponding cells was determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, according to established methods [18]. PANC-1 and/or BxPC-3 cells (5×10^3 cells/well) were treated with various concentrations of XN for 48 h or the indicated times. The Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI, USA) was used to detect cell viability, according to the manufacturer's instructions, and absorption at 490 nm was measured using a spectrophotometer. The morphology of cells treated with XN was recorded with a photo imaging system. Three independent experiments were performed in triplicate.

2.3. 2-D colony formation assay

Cells were trypsinized and seeded at 2000 cells per well in 6-well plates. Cells were allowed to attach overnight and then exposed to different concentration of XN for 7 days. After fixation with 4% paraformaldehyde for 20 min at room temperature, the cells were stained with 0.2% crystal violet. The morphology of cell colonies was recorded with a photo imaging system and the number of cell colonies were calculated and analyzed as the ratio of the number of treated to untreated samples. Triplicate wells were set up for each concentration.

2.4. 3-D colony formation assay

Cells were trypsinized and seeded at a concentration of 2000 cells per well in 6-well plates that were pretreated with a mixture of media and agarose at a ratio of 1:1 (v/v). Additionally, the mixture also contained different concentrations of XN and the cells were cultivated in this media for 3 weeks. After the colonies grew to a suitable volume, the morphologies of the cell colonies were recorded using a photo imaging system and the numbers of cell colonies were calculated and analyzed as the ratio of the number of treated to untreated samples. Triplicate wells were set up for each concentration.

2.5. Apoptosis assay

Apoptosis was assessed using the Apoptosis Detection Kit (BD Biosciences), as described elsewhere [19]. PC cells were treated with XN for 48 h and then collected, washed, and stained with annexin V-fluorescein isothiocyanate and propidium iodide (PI) for 15 min before evaluation by flow cytometry (FACS Calibur; BD biosciences).

2.6. Cell cycle analysis

Cells were initially seeded in 6 cm dishes and after 24 h, the cells were exposed to different concentrations of XN for the indicated times. After washing with phosphate-buffered saline (PBS) and digested with trypsin, adherent and floating cells were collected, washed once with PBS, and fixed in cold 70% ethanol overnight at 4 °C. After ethanol fixation, cells were washed once in PBS and re-suspended in PBS with 200 μg/mL of RNAase and 50 μg/mL of PI in the dark for 30 min. Then, the cells were analyzed by flow cytometry (FACS Calibur; BD biosciences).

2.7. Western blotting

PC cells were treated with XN for the indicated times and then lysed in radioimmunoprecipitation assay (RIPA) buffer. Protein concentrations were determined using a bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, USA). Whole samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis with 8%–12% gels and transferred to polyvinylidene difluoride membranes (Gibco-Life Technologies, Grand Island, NY, USA). The membranes were incubated overnight with specific antibodies. The signals were visualized using the Odyssey Western blotting detection system (LI-COR, Lincoln, NE, USA).

2.8. Real-time polymerase chain reaction (PCR)

RNA was reverse transcribed using the miScript II RT Kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's instructions. The resulting cDNA was used for real-time PCR analysis using the miScript SYBR Green PCR kit (Qiagen, Inc.). Quantification of individual miRNA was performed using the miScript Primer Assay (Qiagen, Inc.). The following primers and probes were used for PCR analysis: Survivin (F: 5′-CGCCATTAACCGCCAGAT-3′, R: 5′-GGTGCCACTTTCAAGACAA-3′), Bcl-xL (F: 5′-ATAGTTCCACAAAGGCATCC-3′, R: 5′-TGGGATGTCAGGTCACTGAA-3′), Cyclin D1 (F: 5′-GCTGCGAAGTGGAAACCATC-3′, R: 5′-CCTCCTTCTGCACACATTTGAA-3′), and β-actin (F: 5′-TCAGGATCCACGTGCTTGTCA-3′, R: 5′-TACCCTTGGACCCAGAGGTTCTTTGA-3′).

All primers were synthesized by Invitrogen Corp. (Carlsbad, CA, USA) and checked for specificity before use.

2.9. Hematoxylin and eosin (HE), immunohistochemical (IHC), and immunofluorescence (IF) staining

PANC-1 cells were grown on chamber slides in the presence or absence of 10 μM XN for 36 h and then stained with anti-p-STAT3 antibody followed by Alexa 488-labeled secondary antibody. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole).

The mice were sacrificed and tumors were removed, treated with 10% buffered formalin, and embedded in paraffin. Histological examination was performed on 5 μm-thick sections by HE staining. Meanwhile, IHC staining for p-STAT3 and Ki-67 was performed on 4 μm-thick sections using the peroxidase-conjugated avidin-biotin method. We randomly chose 10 visual fields (×200) in each section and counted the percentage of positively stained cells for quantitative analysis.

2.10. PANC-1 subcutaneous xenograft animal model

PANC-1 cells (2.5×10^6) were suspended in 1:1 medium fixed with matrigel and then subcutaneously implanted on the right backsides of the nude mice. After the tumors grew to an area of about