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3,3′-Diindolylmethane induces anti-human gastric cancer cells by the miR-30e-ATG5 modulating autophagy

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ABSTRACT

3,3′-Diindolylmethane (DIM), a class of relatively non-toxic indole derivatives from cruciferous vegetables, has been reported as a promising anticancer phytochemical, but the underlying molecular mechanism is not completely elucidated. In the present study we report a novel regulation of autophagy by DIM in human gastric cancer cells. We found that DIM dose-dependently inhibited the growth of gastric cancer cells *in vitro* and *in vivo*. Moreover, ATG5 and LC3 were activated by DIM in gastric cancer cells. Furthermore, miR-30e was down-regulated by DIM and miR-30e targeted the 3′-UTR of ATG5 to inhibit its translation. Overall, these results suggest that DIM may through the miR-30e-ATG5 modulating autophagy inhibit the proliferation of gastric cancer cells.

1. Introduction

Worldwide, gastric cancer is one of the most common malignancies and remains the second leading cause of carcinomaassociated mortality. Despite advancements in the diagnosis and treatment, the five-year survival rate for gastric cancer patients remains low [1]. Thus, the use of phytochemicals presented in the diet could be a promising strategy for their chemoprevention of cancer. 3,3′-Diindolylmethane (DIM), a class of relatively non-toxic indole derivatives from cruciferous vegetables, has been reported as a candidate anticancer phytochemical in various cancer cells including gastric cancer [2–5]. However, the mechanism by which DIM inhibits proliferation of gastric cancer cell has not been fully elucidated.

Autophagy, a dynamic catabolic process whereby long-lived cytosolic proteins and damaged organelles are engulfed by autophagosomes, cleared in autolysosomes, and recycled to maintain cellular homeostasis, has dual roles in cancers [6,7]. Some evidence suggest that autophagy is classified as an antitumor mechanism [8], while accumulating data support the idea that autophagy may promote tumorigenesis and protect cancer cells from death [9]. In the process of autophagy, several autophagy-related genes (ATG) have been reported. Among them, ATG5, which is conjugated with ATG12 to form ATG5–ATG12 complex contributes to closure and elongation of autophagosomes in the generation of the microtubule-associated protein light chain 3 (LC3) family proteins, is essential for autophagy process though the mechanism still needs further investigation [10].

MiRNAs are ~22 nucleotide-long noncoding RNAs which are expressed in various mammals [11]. MiRNAs can regulate the biological events of their target mRNAs, by recognizing the specific miRNA-response elements (MREs) in the 3′ UTR of target mRNAs. Recent reports provided evidence that miRNAs may play important role in modulating some key autophagy-related proteins [12–15]. MiR-30e is one of the members in miR-30 miRNA family, which was amplified in multiple types of tumors [16,17].

Given the efficacy of DIM in treating gastric cancer, the study was undertaken in order to investigate the mechanisms orchestrating the effects of DIM. Here, we demonstrate a novel role

Abbreviations: DIM, 3,3′-diindolylmethane; LC3, microtubule-associated protein light chain; ATG, autophagy-related gene; mRFP, monomeric red fluorescent protein; GFP, green fluorescent protein; siRNA, small interfering RNA; mRNA, messenger RNA; miRNA, microRNA; MREs, miRNA-response elements; 3-MA, 3-methyladenine; BafA1, bafilomycin A1; DMSO, dimethyl sulfoxide; PBS, phosphate buffer saline; RT-PCR, reverse transcription PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI3K, phosphatidylinositol 3-kinase; UTR, untranslated regions; IFN-γ, interferon-γ.

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of action for DIM which involves the inhibition of autophagy flux in gastric cancer via miR-30e-ATG5-LC3 regulatory axis.

2. Materials and methods

2.1. Cell culture and regents

The human gastric cancer cell lines BGC-823 and SGC-7901 were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Tianhang Biological Technology Co., Ltd., Hangzhou, China). All cells were maintained in a 5% CO_2 atmosphere at a temperature of 37 °C. The regents were purchased from the following commercial sources: DIM, 3-MA, BafA1, DMSO (Sigma, St. Louis, MO, USA).

2.2. siRNA and plasmid constructs

ATG5 siRNA, miR30e-mimic or control (both from Ruibo Biotechnology, Guangzhou, China) was transfected using Lipofectamine 2000 (Invitrogen, Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. For the dual luciferase assay, the MRE found in the 3′ UTR region of the ATG5 mRNA and its mutated version were cloned into the pGL3-control vector (Generay Biotechnology, Shanghai, China) at the 3′ region of the luciferase gene using following linker primers: ATG5 wildtype primers: 5′-tctagaAACGAAAATTTCCTATGTTTACAtc taga-3′ and ATG5 mutant primers: 5′-tctagaAACGAAAATTTCCTAa caaatgAtctaga-3′. XbaI restriction sites between the stop codon and polyadenylation signal were used.

2.3. Cell growth assay

To determine cell growth, a colorimetric water-soluble tetrazolium salt assay (Cell Counting Kit 8; Dojindo Laboratories, Kumamoto, Japan) was performed. This allowed the number of viable cells to be evaluated following treatment with various agent combinations.

2.4. Clonogenic assay

BGC-823 or SGC-7901 cells were treated with DIM at indicated concentrations for 2 h; and the cells were then trypsinized, seeded in six-well plates (300 cells per well) and further cultured for 2 weeks. For scoring colonies, the cells were fixed in 1 ml of methanol for 15 min and stained with Giemsa (Beyotime Biotechnology, Nantong, China) for 10 min. The numbers of cloning were expressed as mean ± S.D. from at least three independent experiments.

2.5. In vivo human tumor xenograft system

Female nude mice (6 weeks old) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China) and maintained under specific pathogen-free conditions. After subcutaneously injecting with 5×10^6 BGC-823 cells one week, the mice were divided randomly into two groups: the control group ($n = 5$, DMSO in 50 μl PBS once daily) and DIM-treated group ($n = 5$, DIM 10 mg/kg in 50 μl PBS once daily). Tumor volume was examined every 3 days for the whole duration of the experiment. Tumors were harvested and weighed at the end of experiments, and photos of the tumor were taken at the same time. The Student t test was performed in the groups at each time-point data. Nude-mice xenograft experiments for miR-30e inhibitor and control cells were performed the same as above. All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised 1996.

2.6. Western blotting and real-time quantitative RT-PCR

Protein was extracted by whole cell extract protocols from cell pellets in protein lysis buffer containing protease and phosphatase inhibitors (Halt Protease Inhibitor Cocktail, Pierce). Western blotting analysis was performed by means of standard procedures. Blots were incubated with primary antibodies against LC3, ATG5, Beclin-1 (Cell Signaling Technology, Inc., Beverly, MA, USA), and β-actin or GAPDH (Beyotime Biotechnology, Nantong, China). Total RNA was extracted from cells or tissues with TRIzol (invitrogen). Total RNA (2 μg) was reverse transcribed with oligo (d18T) primer using the M-MLV reverse transcriptase for RT-PCR. cDNA was used a template for quantitative real-time PCR analysis, preformed using SYBR Premix Ex Taq Mix (TaKaRa) with ABI Prism 7300 sequence detection system. Reactions were in triplicate for each sample and data were normalized to GAPDH levels.

2.7. Tandem mRFP-GFP fluorescence microscopy

A tandem monomeric RFP-GFP-tagged LC3 (tfLC3) was used to monitor autophagy flux as previous reported. For evaluating tandem fluorescent LC3 puncta, 48 h after tfLC3 transfected alone, or with siRNAs/plasmid cotransfected, cells were washed with 1 × PBS, and then observed with confocal microscope directly. Samples were examined under the Zeiss LSM 700B confocal microscope system (Carl Zeiss, Germany) and images were processed with ZEN LE software (Carl Zeiss, Germany).

3. Results

3.1. DIM exerts a deleterious effect on gastric cancer cells in vitro and in vivo

To investigate the effect of DIM on gastric cancer cell, we treated the BGC-823 and SGC-7901 cells with various concentrations of DIM for 48 h and assessed the cellular viability by CCK-8 assays. As shown in Fig. 1A, B, DIM concentration-dependently decreased cellular viability of BGC-823 and SGC-7901 cells. Moreover, the long-term clonogenic cell growth assay supported the data from the above short-term cell growth CCK-8 assay (Fig. 1C, D), indicating that DIM exerts an anti-proliferation effect on gastric cancer cell. To evaluate the effects of DIM on tumor growth *in vivo*, immunodeficient mice were inoculated BGC-823 gastric cancer cells in right flank. Mice were randomly divided into two groups and were daily subcutaneously treated with either DIM (10 mg/kg) or vehicle. Tumor volume and weight were recorded once every three days. Tumor growth was significantly inhibited in the DIM-treated group compared to the control group (Fig. 2A–C). After checking the ratio of tumor volume and tumor weight, we did not find significant difference between the DIM treatment group and control (Fig. 2D), thus it may be implied that DIM did not shrink the tumors and just slow their growth. Furthermore, more necrosis was found in tumor sections of DIM-treated mice by histological examination (Fig. 2E, F). Taken together, DIM inhibited the proliferation of gastric cancer cells *in vitro* and significantly slowed tumor growth *in vivo*.

3.2. DIM induced LC3 expression on gastric cancer cells in vitro and in vivo

We next investigated whether DIM affected the autophagy pathway. After DIM treatment, increased LC3-II expression was

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