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Sulforaphane promotes apoptosis, and inhibits proliferation and self-renewal of nasopharyngeal cancer cells by targeting STAT signal through miRNA-124-3p

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ABSTRACT

Sulforaphane (SF) exhibits an anti-tumor effect in a variety of cancers, but little is known about its function in nasopharyngeal carcinoma. SF could decrease the expression of stem cell markers, β-catenin, Nanog, c-Myc, Oct3/4 and Sox2 in nasopharyngeal cancer cells (HONE1 and SUN1), and inhibit the formation of tumor spheres. Moreover, SF inhibits proliferation and induces apoptosis decreasing the stemness of nasopharyngeal cancer cells through a mechanism related to STAT3 signaling in vitro. We found that SF inhibits total STAT3 expression level and STAT3 phosphorylation (troy 704 and troy 705) by upregulation of miRNA-124-3p. Our results provide the evidence for discovering the novel drugs against nasopharyngeal carcinoma, and potential drugs targeting STAT3 signaling pathway.

1. Introduction

Sulforaphane (SF) abundantly exists in the Cruciferae plants, such as broccoli. Subsequent studies have shown that SF exhibits anti-tumor effects, inhibits both proliferation and cell cycle mechanisms, promotes apoptosis, and protects cells against methylation in cancer cells [1]. However, its role in attenuating cancer stem cells remains poorly understood [2].

Signal Transduction and Activator of Transcription (STAT) is a family of proteins that include STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. This group of proteins can be activated by different cytokine receptors. Upon phosphorylation, they translocate into the nucleus and bind with the promoter region of targeted genes to regulate the expression of related proteins [3]. STAT3 is the most important member of this family, as it recently has been implicated in tumorigenesis and exhibits sustained expression and activity in a variety of tumor tissues [4,5]. Inhibition of STAT3 impedes cancer cell growth, proliferation, cell cycle mechanisms, while overexpression promotes apoptosis [6]. As such, more studies have focused on the role of STAT 3 in different tumors [4]. While there are many phosphorylation sites at the two termains, the tow most important sites are Tyr 705 and Ser 727, with most studies focused on Tyr 705 [7].

STAT3 targeting drugs are a hot topic in cancer studies. STAT3 expression has been shown to be abnormal in nasopharyngeal carcinoma [8]. However, the role of SF in regulating the STAT3 signaling pathway in nasopharyngeal carcinoma is still unknown. Expression of the tumor suppressor miRNA-124, which targets STAT3 to inhibit tumor growth and metastasis, is absent in many tumors; as such, abnormal expression of miRNA-124 is an initiator for tumor development [9–11].

In this study, we investigated the effect of SF in affecting the biological behavior of nasopharyngeal carcinoma, including stemness, STAT3 expression and phosphorylation. Moreover, we studied the effect of SF on miRNAs, upstream of STAT3, and investigated the potential molecular mechanism of SF in nasopharyngeal carcinoma, thus providing the theoretical basis for the development of drugs against nasopharyngeal carcinoma.

2. Materials

2.1. Cell lines

Human nasopharyngeal cancer cell lines Hone1, CNE1, CNE2 and

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Sune1 were provided by the specimen bank of Cancer Institute in the Southern Medical University in China.

2.2. Reagents

The reagents used in this study include cell culture medium and fetal bovine serum (Hyclone, USA), SF (LKT Laboratories, Inc. USA), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, USA), Annexin-V FITC apoptosis analysis kit (BestBio, Shanghai, China), GAPDH monoclonal antibodies (Bioss, Beijing, China), mouse anti-human β-catenin, Nanog, c-Myc, Oct3/4, Sox2, STAT3, p-STAT3 (Tyr705) and p-STAT3 (Ser727) monoclonal antibodies (Satan Cruz, USA), goat anti-mouse IgG conjugated horse radish peroxidase (IgG-HRP, proteintech, USA) and lipo2000 (Invitrogen, USA). Primers were synthesized by Invitrogen, and miR-124-3p mimic and inhibitor were synthesized by Shanghai GenePharma Co., Ltd, China.

2.3. Equipments

Vertical slab electrophoresis tank, electrophoresis apparatus and electrical transfer instrument were the products of Bio-Rad Laboratories, Inc. USA. Optical microscope, fluorescence microscope, inverted microscope and camera equipment were purchased from Nikon (Japan). Flow cytometry was the product of BD Biosciences, USA.

3. Methods

3.1. Sulforaphane preparation

SF was dissolved in DMSO at a concentration of 10 mmol/L, and stored in a −20 C refrigerator.

3.2. The analysis of the inhibition effect of sulforaphane on nasopharyngeal cancer cell growth

A MTT assay was applied to the Hone1 and CNE1 lines of human nasopharyngeal cancer cells to test their viability; they were then treated with SF at various concentrations. The Optical Density (OD) value was measured by a Bio-Tek microplate at 490 nm, and then the inhibitive effects of SF on Hone1 and CNE1 cell growth was analyzed. The cell viability was analyzed using the following formula: Cell viability (%) = (OD value of treated well –OD value of the blank)/(OD value of control–OD value of the blank) × 100%. 50% inhibitory concentration (IC50) of the two cell lines was calculated by software, and the diagram was made by Excel software.

3.3. The analysis of the effect of sulforaphane on nasopharyngeal cancer cell apoptosis

After the treatment with different concentrations of SF (20, 10 and 5 μmol/L) or DMSO (control group) for 48 h, the Hone1 and CNE1 cells were digested with trypsin (preheated at 37 ℃, without ethylenediaminetetraacetic acid). Following resuspension, the cells were incubated at 4℃ for 15 min after adding in 5 μl Annexin-V-FITC, avoiding light. Finally, 5 μl PI was added before the analysis of apoptosis by flow cytometry.

3.4. Investigation of the formation and growth of tumor spheres

To analyze tumor sphere formation, cells were cultured in a non-serum DMEM/F12 medium supplemented with B27, endothelial growth factor (EGF), and bFGF (termed tumor sphere culture medium). Hone1 and CNE1 cells were dissociated into a single cell suspension, and then suspended in tumor sphere culture medium. After the establishment of Hone1and CNE1 spheres (including 3 ml tumor sphere culture medium), SF at was added at different concentrations (0, 5, 10 and 20 μmol/L) every other day for 10 days. At each time point, tumor spheres were gently collected, cultured with fresh tumor sphere culture medium, and the inhibitive effects on tumor sphere formation of SF was observed for analysis. The diameter and volume of the tumor was measured and analyzed under the microscope.

3.5. Western blot

The protein was extracted by a lysis buffer containing protease inhibitors. The protein in the loading buffer was resolved on 10% SDS-PAGE gels (using 80 V constant voltage electrophoresis for 30 min, and then 120 V constant voltage electrophoresis for 90 min) and subsequently transferred into PVDF membrane (wetransfer 250 mA for 1 h). Membranes were either incubated with primary antibodies STAT3, Nanog and β-catenin(1:500), or GAPDH antibodies (1:1000) and horseradish peroxidase-conjugated goat anti-mouse antibody (1:3000).

3.6. The co-treatment with EGF and sulforaphane

SF at various concentrations in the presence or absence of EGF (5 ng) was added to the logarithmic phase Hone1 and CNE1 cells. The cells were divided into 5 groups, including blank control group (DMSO), control group (EGF + DMSO), treatment groups (EGF + SF 5, 10 or 20 μmol/L). After treatment for 48 h, the cells were collected and subjected to protein extraction and Western blot analysis.

Table 1
The sequences of specific reverse primer and RT-qPCR primer of miRNAs.

primer	sequence
miR-17-3p RT	5`-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTACAAGT-3`
miR-17-3p Reverse	5`-ACACTCCAGCTGGGACTGCAGTGAAGGCACTTGTAG-3`
miR-124-3p RT	5`-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGCATTCA-3`
miR-124-3p Reverse	5`-ACACTCCAGCTGGGTAAGGCACGCGGTGAATGCC-3`
miR-125a-5p RT	5`-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCACAGGT-3`
miR-125a-5p Reverse	5`-ACACTCCAGCTGGGTCCCTGAGACCCTTTAACCTGTGA-3`
miR-21-5p RT	5`-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAACATC-3`
miR-21-5p Reverse	5`-ACACTCCAGCTGGGTAGCTTATCAGACTGATGTTGA-3`
miR-130a-5p RT	5`-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCAGACAG-3`
miR-130a-5p Reverse	5`-ACACTCCAGCTGGGTTCACATTGTGCTACTGTCTGC-3`
miR-301a-3p RT	5`-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCTTTGAC-3`
miR-301a-3p Reverse	5`-ACACTCCAGCTGGGCAGTGCAATAGTATTGTCAAAGC-3`
All Forward	5`-TGGTGTCGTGGAGTCG-3`
U6 RT and Reverse	5`-AACGCTTCACGAATTTGCGT-3`
U6 Forward	5`-CTCGCTTCGGCAGCACA-3`