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miR-19 targeting of GSK3β mediates sulforaphane suppression of lung cancer stem cells☆

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Abstract

Cancer stem cells (CSCs) play a central role in the development of cancer. The canonical Wnt/β-catenin pathway is critical for maintaining stemness of CSCs. Phytochemicals from dietary compounds possess anti-CSCs properties and have been characterized as promising therapeutic agents for the prevention and treatment of many cancers. To date, the involvement and function of miR-19, a key oncogenic miRNA, in regulating Wnt/β-catenin pathway and lung CSCs has not been defined. Meanwhile, the effect of sulforaphane (SFN) on lung CSCs also remains to be elucidated. Here, we reported that lung CSCs up-regulated miR-19a and miR-19b expression. Overexpression of miR-19a/19b enhanced the ability of tumorsphere formation, up-regulated the expression of lung CSCs markers, increased Wnt/β-catenin pathway activation and β-catenin/TCF transcriptional activity in lung CSCs. In contrary, down-regulation of miR-19 suppressed lung CSCs activity and Wnt/β-catenin activation. We further revealed that miR-19 activated Wnt/β-catenin pathway by directly targeting GSK3β, the key negative modulator of this pathway. Moreover, we showed that SFN exhibited inhibitory effect on lung CSCs through suppressing miR-19 and Wnt/β-catenin pathway. Taken together, these data illustrate the role of miR-19 in regulating lung CSCs traits and miR-19/GSK3β/β-catenin axis in SFN intervention of lung CSCs. Findings from this study could provide important new insights into the molecular mechanisms of lung CSCs regulation as well as its target intervention.

Keywords: Lung cancer stem cells; Wnt/β-catenin pathway; miR-19; GSK3β; Sulforaphane

1. Introduction

Lung cancer is the leading cause of cancer mortality worldwide, accounting for about 13% of total cancer deaths [1,2]. Despite significant improvement being made in cancer therapy, the 5-year relative survival rate of lung cancer still remains low [1]. Accumulating evidence has shown that cancer stem cells (CSCs), a rare subpopulation of cancer cells, possess the capacity of self-renewal and multipotent differentiation [3,4]. CSCs express distinct and specific cell markers. For example, CD133, CD44 and aldehyde dehydrogenase (ALDH) are widely used to identify lung CSCs [5,6]. It is well acknowledged that CSCs are responsible for the heterogeneity, metastasis and relapse of tumors [6]. Thus, the CSCs concept provides more effective intervention strategies in the prevention and treatment of cancers.

Several signal pathways are crucial in orchestrating CSCs activity. Dysregulation of these pathways has been implicated in the maintenance and function of CSCs. Aberrant Wnt/β-catenin signal pathway has been reported in several types of CSCs [7–10]. The activation of Wnt/β-catenin pathway depends on the key regulator β-catenin, the intracellular level of whose is mainly modulated by glycogen synthase kinase-3β (GSK3β). In the absence of Wnt, the cytoplasmic β-catenin is phosphorylated by the destruction complex consisting of Axin, adenomatous polyposis coli (APC), casein kinase 1α (CK1α) and GSK3β and subsequently undergoes ubiquitin-proteasome degradation. Upon the Wnt ligand binding to the Frizzled receptor, β-catenin is dissociated from the destruction complex and accumulates in cytoplasm. The stabilized β-catenin then translocates into the nucleus where it forms a transcriptional complex with the T-cell factor (TCF)/lymphocyte enhancer factor (LEF), leading to the activation of downstream genes such as c-Myc, Cyclin D1, CD44, ALDH and others [11–13].

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miRNAs are 21–25 nucleotides in length, small noncoding RNAs that regulate target genes by binding to the 3′-untranslated regions (3'UTRs), which leads to either degradation of mRNA or inhibition of protein translation [14]. Mounting evidence has indicated the pivotal role of miRNA in the development and progression of cancers by regulating CSCs activity [15,16]. Some oncogenic miRNAs have been characterized, for example, the miR-17-92 cluster [17–19]. Among the miR-17-92 cluster, miR-19 is the key miRNA which exerts oncogenic function by targeting several critical tumor suppressor genes such as PTEN [20]. Previous studies revealed that miR-19 triggers the epithelial–mesenchymal transition (EMT) and is correlated with metastasis of lung cancer [21]. In gastric cancer, miR-19 promotes multidrug resistance (MDR) and regulates the self-renewal and proliferation of gastric CSCs [22,23]. Moreover, a recent study has elucidated the role of miR-17-92 in lung cancer stem cells through regulation of Wnt signaling [24].

Numerous epidemiological studies have substantiated the efficient anticancer properties of dietary components in vegetables and fruits [25]. These bioactive and non/low-toxic phytochemicals have been proved to be promising candidates for cancer intervention [25,26]. Sulforaphane (SFN), a major isothiocynate (ITC) abundant in broccoli or broccoli sprouts, has been shown to possess anticarcinogenic potential *in vitro* and *in vivo* [27]. Recently, it has been documented that SFN exhibits its anticancer property by targeting CSCs in various cancer types [28–31]. In addition, by combining with other chemotherapeutic agents, SFN effectively abolishes CSCs characteristics [32]. To date, however, no studies have been conducted yet to examine the inhibitory effect of SFN on lung CSCs. Therefore, the present study aimed to investigate the regulatory role of the oncogenic miR-19 in lung CSCs and SFN modulation of lung CSCs.

2. Materials and methods

2.1. Cell culture

Human lung cancer cell lines A549 and H1299 were purchased from Chinese Academy of Typical Culture Collection Cell Bank. Both cell lines were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum and antibiotics (100-units/mL penicillin and 100-μg/mL streptomycin) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Tumorsphere formation assay

One major feature of CSCs is their ability to form three-dimensional spheres in serum-free medium (SFM) culture condition. In SFM with some specific growth factors, a small portion of tumor cells can form tumorspheres, while most of other cells undergo apoptosis. These sphere-forming cells possess CSCs characteristics. Thus, tumorsphere formation in SFM provides a convenient tool for the investigation of CSCs biology [33–37]. In our study, SFM culturing was used to isolate and enrich lung CSCs from A549 and H1299 lung cancer cell lines. In brief, A549 and H1299 cells were seeded into ultra-low-attachment six-well plates at a density of 20,000 cells/well in serum-free medium (SFM) [Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12) (Gibco) supplemented with 2% B27 Supplement (Gibco), 20-ng/ml human basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, NJ, USA) and 20-ng/ml epidermal growth factor (EGF) (Peprotech)] at 37 °C. Formation of tumorspheres was observed, and images were acquired under a light microscope (Nikon, Japan). To analyze the inhibitory effect of SFN (Sigma, St. Louis, MO, USA) on sphere formation of these cells, different concentrations of SFN (0, 1, 5, 15 μM) were added to SFM, using 0.1% Dimethyl Sulfoxide (DMSO) (Sigma) as the vehicle control. The size and number of the tumorspheres were obtained on day 7 (only when spheres>50 μm in diameter were counted).

2.3. Detection of CD133-positive cells by flow cytometry

Tumorsphere-forming cells were collected and washed twice with ice-cold PBS. A 1×10^6 single-cell suspension was stained with 1-μL APC-conjugated human monoclonal CD133/1 (AC133) (Miltenyi Biotech, Teterow, Germany) antibody or corresponding isotype control antibody (Mouse IgG1) (Miltenyi Biotech) at 4 °C in dark for 10 min, followed by flow cytometic analysis.

2.4. Detection of apoptotic cells by flow cytometry

The apoptosis assay was performed using the Annexin V/propidium iodide (PI) apoptosis kit (BD Bioscien, Frankin Lake, NJ, USA) according to the manufacturer's instructions. Briefly, A549 and H1299 sphere-forming cells were exposed to different concentrations of SFN for 7 days. The cells were harvested, washed twice with ice-cold PBS and resuspended in 400-μL binding buffer. The cells were then incubated with 5-μL Annexin V-FITC at 4 °C in the dark for 15 min, followed by incubating with 10-μL propidium iodide (PI) at room temperature in the dark for 5 min. Stained cells were subjected to flow cytometry analysis and a total of 10,000 events were acquired. Both PI and Annexin V negative cells were defined as normal cells, PI negative and Annexin V positive cells were defined as early apoptotic and cells that were both PI and Annexin V positive were defined as late apoptotic. Therefore, apoptotic cells contained both PI negative/Annexin V positive and PI positive/Annexin V positive cells.

2.5. Western blot analysis

Tumorsphere-forming A549 and H1299 cells were collected by centrifugation and washed twice with ice-cold PBS, then lysed in RIPA buffer [20-mM HEPES (pH 6.8), 5-mM EDTA, 10-mM EGTA, 5-mM NaF, 0.1-μg/mL okadaic acid, 1-mM dithiothreitol, 0.4-M KCl, 0.4% Triton X-100, 10% glycerol, 5-μg/mL leupeptin, 50 μg/mL of phenylmethanesulphonylfluoride, 1-mM benzamidine, 5-mg/mL aprotinin and 1-mM sodium orthovanadate]. Concentrations of the precipitated proteins were determined by the BCA Protein Assay (Pierce, Rockforsd, WI, USA). Proteins were separated on 10% SDS-polyacrylamide gels and were then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% milk, membranes were incubated with primary antibodies and HRP-conjugated secondary antibodies. GAPDH was used as a loading control. The primary antibodies against CD133, CD44, ALDH1A1, Nanog, Oct4, β-catenin, GSK3β, phosphorylated-GSK3β (Ser9), c-Myc, Cyclin D1, Cleaved-Caspase 3, Cleaved-Caspase 9, Caspase 8, Bax, Bcl2, PCNA and GAPDH were obtained from Proteintech (Rosemont, IL, USA).

2.6. RNA extraction and quantitative real-time PCR

Total cellular RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. One μg of RNA was converted into cDNA according to the manufactures' instructions (abm, Canada). qRT-PCR was performed *via* the Power SYBR Green Master Mix (Applied Biosystems, Foster city, CA, USA) using ABI 7300 real-time PCR detection system (Applied Biosystems). For detection of miR-19a and miR-19b, the forward (F) and reverse (R) primers were as follows:

miR-19a-F, 5′-CCTCTGTTAGTTTTGCATAGTTGC-3′;
miR-19a-R, 5′-CAGGCCACCATCAGTTTTG-3′;
miR-19b-F, 5′-CACCATGGCATGCTTTAGATTATATATTCCGC-3′;
miR-19b-R, 5′-GCGGAATATATAATCTAAAGCATGGGTGCCATGGTG-3′;
U6-F, 5′-CGCTTCGGCAGCACATATACTAAAATTGGAAC-3′;
U6-R, 5′-GCTTCACGAATTTGCGTGTCATCCTTGC-3′; all of these primers were synthesized by RIBOBIO (Guangzhou, China). The U6 snRNA was used as an internal control. For the mRNA measurements of CD133, CD44, ALDH1A1, Oct4, Nanog, Cyclin D1, GSK3β and c-Myc, the primers were as follows:

CD133-F, 5′-GCACTCTATACCAAAGCGTCAA-3′;
CD133-R, 5′-CTCCCATACTTCTTAGTTTCCTCA-3′;
CD44-F, 5′-AGGATTTCCCCAGAACTTAG-3′;
CD44-R, 5′-ACAGGTCAAGATGGAAGATG-3′;
ALDH1A1-F, 5′-CCGCAAGACAGGCTTTTCAG-3′;
ALDH1A1-R, 5′-CATTGTCGCCAGCAGCAGA-3′;
Oct4-F, 5′-ACATCAAAGCTCTGCAGAAAGAACT-3′;
Oct4-R, 5′-CTGAATACCTTCCCAAATAGAACCC-3′;
Nanog-F, 5′-AGAAGGCCTCAGCACCTA-3′;
Nanog-R, 5′-GGCCTGATTGTTCCAGGATT-3′;
Cyclin D1-F, 5′-TGGAGCCCCTGAAGAAGAG-3′;
Cyclin D1-R, 5′-AAGTGCGTTGTGCGGTAGC-3′;
GSK3β-R, 5′-GCTTTGAAAGTAATCCCTGGGGTTTGG-3′;
GSK3β-R, 5′-TGCAGAGGTGCAAAACGGAGCA-3′;
c-Myc-F, 5′-TCAAGAGGCGAACACACAAC-3′;
c-Myc-R, 5′-GGCCTTTTCATTGTTTTCCA-3′;
GAPDH-F, 5′-CAAGGTCACCATGACAACTTTG-3′;
GAPDH-R, 5′-GTCCACCACCCTGTTGCTGTAG-3′; all of these primers were synthesized by Invitrogen. Data were normalized using GAPDH as an internal control and fold changes in expression of each gene were calculated by $2^{-(\Delta\Delta Ct)}$ method.

2.7. Transient transfection of miR-19a/b mimics/inhibitors

A549 and H1299 cells were plated onto six-well plates at a density of approximately 2×10^5 cells in the RPMI 1640 medium containing 10% FBS without antibiotics. Following 12 h of incubation, cells were then transiently transfected with miR-19a/miR-19b mimics (50 nM), miR-19a/miR-19b inhibitors (50 nM), and their corresponding control mimic and control inhibitor (50 nM) with lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's procedure. For total RNA isolation, cells were harvested 48 h after oligonucletide treatment. For turmorsphere assay, cells were trypsinized 24 h post transfection and then cultured in SFM for another 72 h. miR-19a/miR-19b mimics, miR-19a/miR-19b inhibitors, control mimic and control inhibitor were purchased from RIBOBIO.