Sulforaphane targets cancer stemness and tumor initiating properties in oral squamous cell carcinomas via miR-200c induction

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Abstract Background/Purpose: Cancer stem cells (CSCs) are deemed as the driving force of tumorigenesis in oral squamous cell carcinomas (OSCCs). In this study, we investigated the chemotherapeutic effect of sulforaphane, a dietary component from broccoli sprouts, on targeting OSCC-CSCs.

Methods: The effect of sulforaphane on normal oral epithelial cells (SG) and sphere-forming OSCC-CSCs isolated from SAS and GNM cells was examined. ALDH1 activity and CD44 positivity of OSCC-CSCs with sulforaphane treatment was assessed by flow cytometry analysis. In vitro and in vivo tumorigenicity assays of OSCC-CSCs with sulforaphane treatment were presented.

Results: We observed that the sulforaphane dose-dependently eliminated the proliferation rate of OSCC-CSCs, whereas the inhibition on SG cells proliferation was limited. Cancer stemness properties including self-renewal, CD44 positivity, and ALDH1 activity were also decreased in OSCC-CSCs with different doses of sulforaphane treatment. Moreover, sulforaphane treatment of OSCC-CSCs decreased the migration, invasion, clonogenicity, and in vivo tumorigenicity of xenografts. Sulforaphane treatment resulted in a dose-dependent increase in the levels of tumor suppressive miR200c.

Conclusion: These lines of evidence suggest that sulforaphane can suppress the cancer stemness and tumor-initiating properties in OSCC-CSCs both in vitro and in vivo.

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Conflicts of interest: The authors declare that they have no competing interests.

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Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer type worldwide with poor prognosis. Unfortunately, cancer therapies including extensive surgery, radiotherapy, chemotherapy, or concurrent chemo/radiotherapy are not effective for patients with advanced OSCC because of tumor recurrence, metastasis, and poor response to chemotherapy and radiotherapy. Epidemiological evidence strongly indicates that OSCC is highly associated with the areca quid chewing habit. Cancer stem cells (CSCs), also known as tumor-initiating cells, comprise a unique subpopulation of cells within a tumor. Relative to the remaining the tumor bulk, CSCs are often more tumorigenic and chemo-resistant/radioresistant than the tumor bulk. Previously, we have verified that oralspheres could be the markers to isolate and identify the CSCs from OSCC (OSCC-CSCs). Therefore, it is imperative to develop strategies on targeting CSCs.

Sulforaphane, first identified in broccoli sprouts, is well-known for pleiotropic anticarcinogenic effect on a variety of experimental cancer models including repression of cancer cell proliferation, stimulation of cancer cell apoptosis, and inhibition of tumor progression and metastasis. Sulforaphane exerted an inhibitory effect on the oncogenicity of cancer cells by modulating numerous cell signaling pathways including Keap1–Nrf2 signaling, mitogen-activated protein kinase pathway, and nuclear factor kappa B signaling. Substantial evidence has demonstrated that sulforaphane inhibited CSCs in breast cancer, prostate cancer, and pancreatic cancer. However, the efficacy of sulforaphane on the specific subset of OSCC-CSCs is still not clear.

In this study, we explore a chemotherapeutic effect of sulforaphane on targeting OSCC-CSCs by intro and in vivo xenograft mouse models. Strikingly, sulforaphane treatment diminished cancer stemness and tumorigenicity both in vitro and in vivo.

Methods

Cell culture and reagents

SAS or GNM cells were maintained in Dulbecco’s modified Eagle medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco Laboratories). Cells were grown at 37°C in a humidified incubator containing 5% CO2. Sulforaphane was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Enrichment of sphere-forming OSCC-CSCs

For enrichment of OSCC-CSCs, the two cell lines were cultured in tumor sphere medium consisting of serum-free DMEM/F12 medium (Gibco Laboratories), N2 supplement (Gibco Life Technologies), 10 ng/mL human recombinant basic fibroblast growth factor-basic (FGF) and 10 ng/mL epidermal growth factor (EGF; R&D Systems, Minneapolis, MN, USA). Cells were plated at a density of 103 live cells/low-attachment six-well plate (Corning Inc., Corning, NY, USA), and the medium was changed every other day until the tumor sphere formation was observed in about 2 weeks. For serial passage of spheroid cells, single cells will be obtained from accurtase-treated spheroids and the cell density of passage will be 1000 cells/mL in the serum-free medium as described above.

Cell proliferation determination by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Cells were plated in 96-well plates (at 1 × 104 cells/well) in 0.1% dimethyl sulfoxide (DMSO) or different concentrations of sulforaphane-containing medium and cultured at 37°C for 24 hours. Cell proliferation/survival was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The 570-nm absorbance of the DMSO-treated group was set as 100%, and data were presented as percentage of DMSO control.

CD44 staining by flow cytometry analysis

Cells were stained with anti-CD44 antibody conjugated to phycoerythrin (Milenyi Biotech., Auburn, CA, USA), with labeling according to the manufacturer’s instructions. Red fluorescence emission from 10,000 cells illuminated with blue (488 nm) excitation light was measured with a FACScalibur (Becton Dickinson, Mountain View, CA, USA) using CellQuest software.

Cell migration and cell invasion assay

Cell migration and cell invasion assays were performed as described previously.

Soft agar colony forming assay

Six-well culture dish was washed with 2 mL bottom agar (Sigma-Aldrich) mixture [DMEM, 10% (v/v) fetal calf serum, 0.6% (w/v) agar] containing 10% fetal bovine serum (Gibco Laboratories). After the bottom layer was solidified, 2 mL top agar—medium mixture [DMEM, 10% (v/v) fetal calf serum, 0.3% (w/v) agar] containing 2 × 104 cells was added, and the dishes were incubated at 37°C for 4 weeks. Plates were stained with 0.005% Crystal Violet, and the colonies were counted. The number of total colonies with a diameter ≥ 100 μm was counted over five fields per well for a total of 15 fields in triplicate experiments.

Effect of sulforaphane on tumor growth in nude mice

All procedures involving animals were in accordance with the institutional animal welfare guidelines of the Chung Shan Medical University. For the nude mice xenograft model, 5–6-week-old immunodecient nude mice (BALB/c nu/nu mice) weighing 18–22 g were used. The mice were housed with a regular 12-hour light/12-hour dark cycle and ad libitum access to standard rodent chow diet (Laboratory
Rodent Diet 5001; LabDiet, St. Louis, MO, USA) and were kept in a pathogen-free environment at the Laboratory Animal Unit. OSCC-CSCs (1 × 10^4 cells/0.2 mL/mouse) were injected subcutaneously into the right front axilla. Eight days after implantation, the mice were randomly divided into two groups (N = 6 for each group) and intaperitoneally injected with vehicle (saline) or 50 mg/kg sulforaphane. The volume was calculated according to the following formula: \[ \frac{(\text{length} \times \text{width}^2)}{2}. \] (1)

MicroRNA quantitative reverse transcription-polymerase chain reaction analysis

For miR-200c levels detection, miRNA quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using TaqMan miRNA assays with specific primer sets (Applied Biosystems, Foster City, CA, USA). All reagents and protocols were obtained from Applied Biosystems, and detection was performed using a StepOne Plus real-time PCR system (Applied Biosystems).22

Western blot assay

The extraction of proteins from cells and Western blot analysis were performed as previously described.25 Samples (15 μL) were boiled at 95°C for 5 minutes and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were wet-transferred to Hybond-ECL nitrocellulose paper (Amersham, Arlington Heights, IL, USA). The following primary antibodies were used: anti-human Bmi1 (Cell Signaling, Beverly, MA, USA) and anti-β-actin (Millipore Corp., Billerica, MA, USA). Immunoreactive protein bands were detected by the ECL detection system (Amersham Biosciences Co., Piscataway, NJ, USA).

Statistical analysis

SPSS (version 13.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The presented results are representative of three independent experiments with similar results. Statistical differences were evaluated using Student t test and were considered significant at \( p < 0.05 \).

Results

Effects of sulforaphane on the viability and self-renewal ability in OSCC-CSCs

It has been reported that OSCC-CSCs can be cultured in suspension to generate floating oralspheres under serum-free medium with bFGF and EGF.5 We further examined the cytotoxic activity of sulforaphane to sphere-forming OSCC-CSCs from SAS and GNM. With the MTT assay, sulforaphane treatment dose-dependently suppressed the proliferation rate of OSCC-CSCs, whereas the inhibition on normal oral epithelial cells (SG) proliferation was limited (Figure 1A). Secondary tumorsphere-forming ability is a common

![Figure 1](image-url)

Figure 1  Sulforaphane treatment suppresses the proliferation and self-renewal properties of OSCC-CSCs. (A) MTT assay shows that concentration-dependent inhibition of cell viability in SG and OSCC-CSCs by sulforaphane (24 hours) treatment. Cell survival is assessed and presented as percent survival relative to untreated cells. (B) Primary OSCC-CSCs treated with or without sulforaphane were subjected to a self-renewal secondary sphere-forming assay. The number of secondary spheres is calculated and presented. The experiments were repeated three times, and representative results are shown. Results are presented as mean ± standard deviation. * \( p < 0.05 \) versus Control. CSC = cancer stem cells; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OSCC = oral squamous cell carcinomas.
method to observe self-renewal capability. We then evaluated the potential role of sulforaphane in modulating the self-renewal property of OSCC-CSCs, and found that sulforaphane decreased the secondary sphere-forming ability of OSCC-CSCs (Figure 1B).

**Sulforaphane represses ALDH1 activity and CD44 positivity in OSCC-CSCs**

ALDH1 activity and CD44 expression have been demonstrated to be markers for isolating as well as identifying the putative OSCC-CSCs. Flow cytometry analysis of the ALDH1 and CD44 expression patterns indicated that sulforaphane-treated OSCC-CSCs dose-dependently induced a reduction in ALDH1 activity (Figure 2A) and CD44 positivity (Figure 2B) of OSCC-CSCs.

**Sulforaphane suppresses migration/invasion capabilities of OSCC-CSCs**

As high oncogenicity and metastasis are hallmarks of CSCs, we sought to measure the effects of sulforaphane on migration and invasion ability of OSCC-CSCs. Single-cell suspensions of control- or sulforaphane-treated OSCC-CSCs were used for analysis of their metastatic capacity in vitro as described in the Methods section. Our data indicate that sulforaphane dose-dependently inhibits the migration (Figure 3A) and invasion (Figure 3B) abilities of OSCC-CSCs.

**Effects of sulforaphane on tumor initiation activity in vitro and in vivo**

We next examined whether the anchorage-independent growth ability of OSCC-CSCs was influenced by sulforaphane treatment. We have demonstrated that sulforaphane significantly suppressed the cologenicity of OSCC-CSCs (Figure 4A). Next, we explored the therapeutic potential of sulforaphane in immunocompromised mice bearing OSCC-CSCs xenograft tumors. Eight days after OSCC-CSCs inoculation, OSCC-CSCs-transplanted nude mice were intraperitoneally injected with 50 mg/kg sulforaphane. By Day 20, sulforaphane treatment induced a reduction in tumor volume of oral CSC-transplanted nude mice (Figure 4B).

**Sulforaphane treatment induced miR-200c**

MicroRNAs (miRNAs) have been shown to be correlated with several aspects of key players of cancer-related hallmarks including cancer stemness. Previously, we have demonstrated that miR200c regulated tumorigenicity in OSCC-CSCs by directly targeting Bmi1. Elevating miR-200c expression by natural compound appears to be a promising therapeutic modality to target...
OSCC-CSCs. To investigate the molecular mechanism of sulforaphane in targeting cancer stemness, miR-200c expression in sulforaphane-treated OSCC-CSCs was determined by miRNA RT-PCR analysis. Sulforaphane treatment resulted in a dose-dependent increase in the levels of miR-200c in OSCC-CSCs (Figure 5A). Sulforaphane treatment of OSCC-CSCs cells also suppressed the protein levels of Bmi1, which were implicated as targets of miR-200c (Figure 5B).

Discussion

A large number of studies have demonstrated that tumors including OSCCs contain a crucial subset of cells, i.e., or tumor-initiating cells, which exhibit a self-renewing capacity and have the ability to reform new tumors in vivo. CSCs are key contributors to radioresistance and are responsible for tumor progression of OSCC as well as recurrence after traditional chemotherapy. Therefore, the search for approaches to target CSCs for OSCC treatment may pave a way toward a new set of clinical practices. In this study, we showed that sulforaphane, a dietary component of broccoli/broccoli sprouts, eliminated cancer stemness both in vitro and in vivo. Cancer stemness including self-renewal capability, ALDH1 activity, CD44 positivity in OSCC-CSCs was significantly inhibited by sulforaphane treatment. Moreover, sulforaphane treatment repressed migration/invasiveness/cologenicity and in vivo tumor growth in OSCC-CSCs. To our knowledge, this is the first report to demonstrate that suppression of cancer stemness could be partially augmented by the anticarcinogenic effect of sulforaphane in OSCC-CSCs.

miRNAs, a class of highly conserved small RNA molecules regulating gene expression, has emerging roles during carcinogenesis in various types of cancers. Genomic rearrangement located at locus near miRNA clusters had been reported. A pair of neighboring miRNAs is frequently deleted in human chronic lymphocytic leukemia, and miRNA genes are either lost or amplified in tumor. Conversely, the deletion and silencing of a gene encoding a miRNA that normally suppress expression of oncogenes might lead to increased protein expression and a gain in oncogenic potency. miRNAs such as miR-21, miR-31, miR-504, miR-10b, let-7, or miR-184 were revealed to be the most consistently deregulated miRNAs in OSCC. Recently, natural compounds-regulated miRNAs have been shown to be involved in the epigenetic regulation of cancer stemness in several types of malignant cancers. Our previous work has shown that miR-200c-targeting Bmi1 is involved in the regulation of cancer stemness in OSCC. The expression of miR-200c in OSCC...
tissues is significantly decreased in tumor specimens. In the study, sulforaphane treatment impaired tumor growth in OSCC-CSC tumor-bearing mice through miR-200c induction. These findings support the idea that miR-200c could be potentially used as cancer signatures and therapeutic targets.

Conclusively, the present study showed that sulforaphane inhibited cancer stemness through miR-200c induction, which resulted in the inhibition of the self-

Figure 4  Effects of sulforaphane on CD44 and ALDH1 activity expression in OSCC-CSCs. (A) Colony-forming ability was assessed in OSCC-CSCs dose-dependently treated with sulforaphane. (B) Effect of sulforaphane treatment on subcutaneous xenografts of OSCC-CSCs in vivo (N = 6 for each group). The experiments were repeated three times, and representative results are shown. Results are presented as mean ± standard deviation. *p < 0.05 versus Control. ALDH = aldehyde dehydrogenase; CSC = cancer stem cells; OSCC = oral squamous cell carcinomas.

Figure 5  Sulforaphane treatment induces miR-200c expression in OSCC-CSCs. (A) miRNA real-time RT-PCR analysis was applied to analyze the relative miR-200c expression level in sulforaphane dose-dependently treated OSCC-CSCs. (B) Western blotting was used to assess the expression level of Bmi1 in OSCC-CSCs with sulforaphane treatment. The experiments were repeated three times, and representative results are shown. Results are presented as mean ± standard deviation. *p < 0.05 versus Control. CSC = cancer stem cells; miRNA = microRNA; OSCC = oral squamous cell carcinomas; RT-PCR = reverse transcription-polymerase chain reaction.
renewal, metastasis, and tumor initiation properties of OSCC-SCs. These findings provide a strong rationale for the use of sulforaphane for OSCC chemoprevention and therapies.

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References


