Gene 678 (2018) 115–123

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Purified sulforaphane from broccoli (*Brassica oleracea* var. *italica*) leads to alterations of *CDX1* and *CDX2* expression and changes in miR-9 and miR-326 levels in human gastric cancer cells

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ARTICLE INFO

Keywords:
Gastric cancer
Sulforaphane
Epigenetic alteration
CDX1
CDX2
miR-9
miR-326

ABSTRACT

Background: Genetic alterations and epigenetic modifications are two main factors involved in gastric carcinogenesis, progression, and metastasis. Several miRNAs such as miRNA-9 and miRNA-326 may play important role in gastric cancer by targeting the 3′UTR of the caudal type homeobox (*CDX*) *1* and 2 mRNA respectively. The use of herbal medicines has been widely considered in the treatment of cancers such as gastric cancer. Sulforaphane extracted from broccoli may indirectly prevent cancer through affecting different signaling pathways. The aim of this study was to evaluate the effect of different concentrations of sulforaphane extracted from broccoli sprout (SEBS) on viability, death pattern, and expression alterations of *CDX1/2* as well as miRNA-9 and miRNA-326 in normal (HF2FF) and gastric cancer cell lines.

Methods: Two gastric cancer cell lines (AGS and MKN45) and HF2FF normal cell line were cultured and treated with different concentrations (31.25, 62.5, 125, and 250 μg/ml) of the purified sulforaphane. Expression levels of *CDX1* and *CDX2* as well as miRNA-9 and miRNA-326, and mechanisms leading to cell death were assessed by Taqman real time PCR assay and flow cytometry, respectively.

Results: Significant dose-dependent and anti-proliferative effects of the SEBS were observed on AGS and MKN45 cells after 48 h with an IC50 value of about 112 and 125 μg/ml, respectively ($P < 0.001$). Apoptotic cells were observed in AGS and MKN45 cells but not HF2FF after 48 h of treatment with SEBS. Furthermore, significant changes in expression of *CDX1*, *CDX2*, miR-9 and miR-326 in the gastric cancer lines (AGS and MKN45), were observed under different concentrations of SEBS.

Conclusion: Our present study suggests that the SEBS may influence gastric cancer cell lines at specific doses and change their proliferation rate by altering the expression of *CDX1*, *CDX2*, miR-9, and miR-326.

1. Introduction

Gastric cancer is the fourth prevalent cancer type and the second cancer-related death cause throughout the world (Malekzadeh et al., 2009). Although common cancer therapies such as chemotherapy, radiotherapy, and surgery are widely used to treat gastric cancer, but these methods have serious side effects, and damage natural cells. Plant extracts have many chemical compounds with a high potential for cancer treatment and low side effects, and high efficacy (Amini Navaie et al., 2015; Fattahi et al., 2013; Fattahi et al., 2018). Cruciferous vegetables are a diverse group of vegetables that belong to the Brassicaceae family, and have anti-cancer properties (Higdon et al., 2007). Broccoli (*Brassica olerasea* var. *italica*) is an eminent cruciferous vegetable that has a high content of sulforaphane alkaloids. Sulforaphane is a type of organosulfur compound that is secreted under some tensions such as drought and oxidative stress, and helps therefore the plant to survive (Mittler, 2002). Previous studies on different species of broccoli indicated an increase in sulforaphane level and improved antioxidant

Abbreviations: CDX1, *caudal type homeobox 1*; *CDX2*, *caudal type homeobox 2*; *GAPDH*, *glyceraldehyde-3-phosphate dehydrogenase*; miR, MicroRNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HPLC, high-performance liquid chromatography; FBS, fetal bovine serum; PBS, phosphate buffered saline; IC50, the half maximal inhibitory concentration; SEBS, sulforaphane extracted from broccoli sprout

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https://doi.org/10.1016/j.gene.2018.08.026
Received 2 August 2018; Accepted 6 August 2018
Available online 07 August 2018
0378-1119/

activity of broccoli sprout extract under drought condition (Kiani et al., 2017). Sulforaphane has been shown to be an effective antimicrobial, antioxidant, anti-aging, anticancer, anti-diabetic, and antifibrotic compound (Kim and Park, 2016; Sun et al., 2016a, 2015). Sulforaphane exerts its anticancer effects by triggering a variety of tumor suppressive and anti-proliferative cellular programs such as cell cycle arrest, apoptosis, and angiogenesis prevention through activating or repressing key effector molecules (Clarke et al., 2008). Sulforaphane was shown to induce G2/M phase transition arrest by downregulating cyclin D1, cyclin B1, and *c-MYC* genes, as well as upregulating *p21* (Oh et al., 2017; Li and Zhang, 2013; Myzak et al., 2007). In addition, sulforaphane triggers apoptosis through upregulating BAX (BCL2 associated X)/BCL-2 (B-cell lymphoma 2) ratio, caspase 7, caspase 9, and increasing PARP (poly ADP ribose polymerase) cleavage in cancer cells (Clarke et al., 2008; Su et al., 2018; Shen et al., 2006). Sulforaphane can prevent further formation of reactive oxygen species (ROS) under stress conditions by activating the nuclear factor erythroid 2–related factor 2 (Nrf2) signaling pathway (Oh et al., 2017). Moreover, sulforaphane is capable of inhibiting Wnt/β-catenin pathway, and hence, preventing tumor progression (Li and Zhang, 2013). Sulforaphane was shown to target cancer stem-cells through altering different signaling pathways (Li and Zhang, 2013). Sulforaphane reduces NF-κB's DNA binding capacity, and inhibits sonic hedgehog (SHH) and PI3/AKT pathways in pancreatic cancer cells. Sulforaphane prevents cancerigenesis through distinct epigenetic mechanisms such as histone acetylation, DNA metylation, and alteration of noncoding RNAs (ncRNAs) expression. Relatively, histone deacetylase activity was significantly decreased in peripheral blood mononuclear cells (PBMC) following sulforaphane consumption in human subjects (Myzak et al., 2007). Also, sulforaphane reduced the expression of DNA methyl transferases in cancer cells. Promoter demethylation in several tumor suppressor genes such as cyclin D2, phosphatase and tensin homolog (*PTEN*), Cadherin 1 (*CDH1*), death-associated protein kinase 1 (*DAPK1*), glutathione S-transferase P (*GSTP1*), and human telomerase reverse transcriptase (*hTERT*) have been reported in cancer cells treated with sulforaphane (Hsu et al., 2011; Ali Khan et al., 2015; Lubecka-Pietruszewska et al., 2015). Additionally, sulforaphane was shown to suppress invasion, and induce apoptosis by down-regulating oncomiRs (miR-616-5p and miR-21) as well as up-regulating tumor suppressor miRNAs (miR-9-3, miR200c) (Su et al., 2018; Gao et al., 2018). Caudal type homeobox 1 (*CDX1*) and *CDX2* are the most important genes belonging to homeobox family, and are intestine-specific transcription factors that play an important role in the final intestinal differentiation and stem cell's growth regulation (Samadani and Akhavan-Niaki, 2015; Silberg et al., 2000). *CDX1* and *CDX2* have been reported as tumor suppressors in more than 85% of patients with leukemia. Aberrant methylation of the CpG islands present in *CDX1* and *CDX2* promoter was reported in human malignancies (Wang et al., 2016; Wong et al., 2004). Another epigenetic issue that was focused by many researchers was post transcriptional modifications, and specifically the role of miRNAs in diagnosing and treating gastric cancer. Correspondingly, several miRNAs were reported to contribute to gastric cancer (Pan et al., 2013). MiRNA-9 and miRNA-326 play a role in gastric cancer, as miRNA-9 was reported to reduce *CDX2* and *NF-KB* expression, and therefore modulate gastric cancer progression (Rotkrua et al., 2011; Wan et al., 2010). Some studies also showed that increased expression of miRNA-9 stimulates metastasis in gastric cancer by changing the expression pattern of E-cadherin (Ma et al., 2010). MiRNA-326 is known to act as a tumor suppressor in several cancer types (Sun et al., 2016b; Zhang et al., 2017). In colorectal carcinoma, miRNA-326 targets one binding protein 1 (*NOB1*) and inhibits the regeneration, migration, and aggression, and also induces apoptosis (Wu et al., 2015). Based on the availability of broccoli and its main ingredient, sulforaphane, the present study aimed to examine the effect of sulforaphane extracted from broccoli sprout (SEBS) on two gastric cancer cell lines AGS and MKN45, and its effect on the expression of *CDX1*, *CDX2*, and miRNA-9 and miRNA-326 as their epigenetic regulators.

2. Materials and methods

2.1. Seeds germination

Seeds of the F1 Marathon broccoli were purchased from PS America company. Seeds germination was performed according to Kiani et al. (2017). Briefly, after sterilization with 70% ethanol and 20% sodium hypochlorite, seeds were washed 4 times with deionized water. Then seeds were cultured in a wet sterile petri dish under standard conditions with 16 h light and 8 h darkness at 23 °C (day)/20 °C (night), and 70% humidity. In order to create drought stress, sprouts of broccoli were treated on the fifth day with 88 mM mannitol for two days. The seven days old sprout was collected for further experiments.

2.2. Purification of sulforaphane from broccoli extract

The amount of sulforaphane that was purified from broccoli sprouts in seven-day buds of the post-stress broccoli cultivars was evaluated by HPLC according to the method of Gu et al. with minor modifications (Gu et al., 2012). Each tube contained 3 g of broccoli sprouts powdered with liquid nitrogen, with 4 ml of HPLC grade hexane (Merck, Germany). Then, 2 ml deionized water was added to the dried and depleted broccoli sprouts powder. After complete mixing in hot water bath, tubes were placed at 35 °C for 2 h. The extracts obtained after this step were combined, and further purified with 5 ml HPLC grade ethyl acetate (Merck, Germany) and 1 g HPLC grade sodium chloride (Merck, Germany), and then ethyl acetate was dried by rotary device at 35 °C, and the residue was dissolved in 10% acetonitrile solution. Finally, the extracted solution was filtered by Whatman 0.45 μM membrane filters (GE Healthcare, United Kingdom), and injected into HPLC instrument (Waters1525, USA), using a HPLC Spherisorb C8 column (silica-based reversed-phase column, Waters, USA), with a mobile phase containing acetonitrile/water (40/60) at a flow rate of 0.6 ml/min, and the sulforaphane content was recorded at 205 nm using a sulforaphane standard (Sigma, USA).

2.3. Cell culture

Two human gastric cancer cell lines (AGS and MKN45) and human skin fibroblast cell line (HF2FF) as a normal cell, were obtained from the Pasteur Institute, Tehran, Iran. AGS and MKN45 cells were grown in RPMI supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin. HF2FF cells were grown in DMEM, supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin in a humidified incubator under 95% air and 5% CO_2. The culture medium was renewed every 2 days.

2.4. Cell viability assay

Anti-proliferative activities of the SEBS against AGS and MKN45 human gastric cancer cell lines, and HF2FF human skin fibroblast were determined by the MTT assay. 7×10^3 cells/well for AGS and MKN45, and 1×10^4 cells/well for HF2FF were seeded on 96-well and 24-well plates, respectively. After 24 h incubation in the CO_2 incubator, culture media were removed and replaced by culture media containing different concentrations (31.25, 62.5, 125, and 250 μg/ml) of the SEBS, and cells were incubated for 48 h. After 48 h incubation, culture media were removed, washed with PBS, and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, USA) solution (5 mg/ml) was added to each well, and incubated for an additional 4 h at 37 °C. Then, DMSO (Merck, Germany) was added, and after complete dissolution, the absorbance at 570 nm (formation of formazan) was recorded with a BioTekmicroplate reader (BioTek, USA).