

Down-regulation of cystathionine- γ -lyase/H₂S system inhibits cell growth in human breast cancer MDA-MB-231 cells

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Abstract. Hydrogen sulfide (H₂S), as the third gasotransmitter, plays important roles in cancer biological processes. Endogenous H₂S can possess pro-cancer functions. Cystathionine- γ -lyase (CSE), one of main metabolic enzymes synthesizing H₂S, possesses important roles in the development and progression of cancer. In the present study, the roles of CSE/H₂S system in human breast cancer cells were investigated. It was observed that CSE was expressed in human breast cancer MDA-MB-231 cells and inhibition of endogenous CSE/H₂S significantly reduced the cell viability and inhibited cell growth and proliferation in MDA-MB-231 cells. Meanwhile, CSE knockdown induced apoptosis of MDA-MB-231 cells and inhibited migration of MDA-MB-231. In conclusion, CSE/H₂S system possesses important roles in human breast cancer cells.

1 Introduction

Cystathionine- γ -lyase (CSE) is a key H₂S-producing enzyme in many tissues except for the central nervous system [1]. CSE catalyzes L-cystine to yield H₂S depending on pyridoxal-5'-phosphate as cofactors. Endogenous H₂S, as the third gasotransmitter signaling molecule alongside nitric oxide (NO) and carbon monoxide (CO), is found in various tissues and organs and plays important roles in many physiological processes, such as vasorelaxation, angiogenesis, cellular energy production and cytoprotection, via multiple mechanisms including activation of signal pathways and stimulation of potassium channels [2-7].

Recently the research of H₂S is also focused on the cancer field and it has been demonstrated that endogenous H₂S produced by CSE promotes proliferation of human cancer cells. Studies indicate that administer of exogenous NaHS or production of endogenous H₂S induce the proliferation of human colon cancer HCT116 cells and human hepatocellular carcinoma cells [8-10].

Currently, few of works on the roles of CSE/H₂S in breast cancer have been reported. Breast cancer is the most frequent malignancies and the sixth leading cause of cancer death for females in China. The identification of new biomarkers and new genes involved in breast cancer progress may provide novel approaches for diagnostic and prognostic evaluation. So in this study, we investigated the effect of down-regulation of CSE/H₂S system on MDA-MB-231 cell growth to explore the roles of CSE/H₂S system in human breast cancers. Our loss-of-function studies demonstrated that the

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down-regulation of CSE/H₂S system induced apoptosis accompanied by inhibiting proliferation of breast cancer cell lines in vitro.

2 Materials and methods

2.1 Reagents and antibodies

The CSE inhibitor DL -propargylglycine (PAG) was purchased from Sigma Aldrich (Saint Louis, MO, USA). Anti-CSE antibody was purchased from Abcam (Cambridge, MA, USA). Rabbit polyclonal anti- β -actin (1:2,000; cat. no. 20536-1-AP) primary antibodies and peroxidase- conjugated Affinipure goat anti- rabbit/mouse secondary antibodies (1:10,000; cat. no. SA00001 -2 and SA00001-1) were obtained from Proteintech Group, Inc. (Chicago, IL, USA).

2.2 Cell culture

Human breast cancer MDA-MB-231 cells and human normal breast MCF-10A cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a 37 °C incubator with 5% CO₂.

2.3 Western blot

Total cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) with 10 μ g/ml of the protease inhibitor PMSF and their lysates were obtained by centrifugation at 12,000 \times g for 10 min. Protein concentration of cells lysates was determined and equal amounts of protein were separated by SDS-PAGE followed by electrophoretically transferred to a PVDF membrane (Millipore, Corp.) at 70 mA for 2 h. Then the membrane was blocked in 5 % nonfat dry milk in TBST. Afterwards, the membrane was probed with specific primary antibodies at 4°C overnight. After washing in TBST, the membranes were incubated in anti-mouse IgG or anti-rabbit IgG secondary antibody for 2 h at room temperature. After the membranes were washed with TBST, the proteins were visualized using ECL detection system (Alpha, USA).

2.4 RNAi treatment

Cells were seeded in 6-well culture dish and transfected at approximately 40% confluent. Transfection was performed with LipofectamineTM2000 transfection reagent, according to the manufacturer's instructions (Invitrogen, CA, USA). Results were checked by western blot or cell viability at 48 h after transfection. si RNA was synthesized by Invitrogen (Shanghai, China) and the sequences were the following, CSE-specific si RNA (sense: 5'-GGUUUAGCAGCCACUGUAAAdTdT-3'; antisense: 5'-UUACAGUGGCUGCUAAACC dTdT-3').

2.5 H₂S detection

The production of H₂S from cancer cells was measured spectrophotometrically as described below. Briefly, MDA-MB-231 cells were transfected with CSE siRNA or exposed to DL -propargylglycine (PAG, Sigma Aldrich, Saint Louis, MO, USA) and treated with 2 mM L-cysteine and 0.5 mM pyridoxal phosphate. Meanwhile 1 % (w/v) zinc acetate (500 μ l) was added on the filter papers stick on the cover of plate to absorb H₂S. After 48 h, the filter papers were put in the tubes containing 0.2 % (w/v) N, N-dimethyl-p-phenylenediamine-dihydrochloride dye (500 μ l), 10 % (w/v) ammonium ferric sulfate (50 μ l) and 3 ml H₂O and incubated for 20 min at room temperature and absorbance was

subsequently monitored at 670 nm. The production of H₂S was determined using a standard curve of NaHS (0-1 mM ; R² = 0.9995) and presented as nmol·min⁻¹ per 1×10⁶ cells.

2.6. Cell viability, proliferation, migration and apoptosis assays

Cells were classified into CSE knockdown group and control group. In the CSE knockdown group, cells were pretreated with CSE si RNA or 2 mM PAG for 48 h. Each sample was tested at least three replications. Cell viability was performed via MTS assay (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Cell proliferation was detected with EdU assay (RUIBOBIO, Guangzhou, China). The EdU assay was performed by plating cells into 96-well dish and staining the cells according to the protocol of the EdU assay kit. The scratch wound assay was used to determine the cell migration. Cells were seeded into 6-well plate and scraped with 10 μl pipette tip at approximately 90% confluency to generate scratch wound and rinsed twice with PBS. Then cells were cultivated in the medium with 5% FBS and the distance was measured at the beginning and after 12 h, 24 h and 48 h. Meanwhile, cell cycle and cell apoptosis were investigated with flow cytometry.

2.7 Statistical analysis

Statistical analyses were performed with the SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The results are expressed as the mean±s.d. Differences between two groups were analyzed using the Paired Samples T-test. P<0.05 was considered to indicate a statistically significant difference.

3 Results

3.1 Knockdown of CSE decreases cell viability and inhibits proliferation in MDA-MB-231 cells

To explore the relationship between the CSE/H₂S system and the cell viability and proliferation of breast cancer cells, firstly, we checked the intrinsic level of CSE protein in breast cancer cell line MDA-MB-231 as well as the normal breast cell line MCF-10A. Figure 1A showed that CSE protein was slightly over-expressed in both MDA-MB-231 cells compared with MCF-10A cells. Then we knocked down CSE using siRNA transfection or inhibited the activity of CSE protein by PAG and investigated the changes of the cell proliferation using cell viability and EdU assay. Interestingly, inhibition of intrinsic CSE/H₂S with CSE siRNA or PAG significantly reduced the growth (Figure 1B-D) and proliferation (Figure 2) of MDA-MB-231 cells. The results suggested that the CSE/H₂S system could regulate the survival and proliferation of human breast cancer MDA-MB-231 cells.

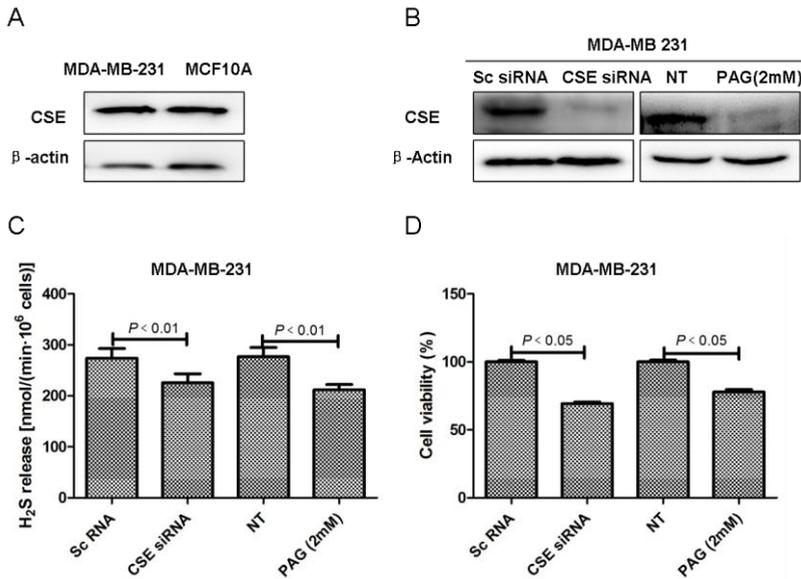


Figure 1. Expression of CSE and analyses of cell growth associated with the inhibition of CSE/ H_2S system in human breast cancer cells. (A) Expression level of CSE in human breast cancer and normal breast cells were detected by WB. (B) CSE siRNA transfection and CSE inhibitor PAG treatment in MDA-MB-231 cells were determined by WB. (C) The effects of CSE siRNA and CSE inhibitor PAG on the production of endogenous H_2S in MDA-MB-231 cells. 1×10^6 cells were seeded in 6-well plate and then treated with CSE siRNA and CSE inhibitor PAG for 48 h. The production of endogenous H_2S was assessed spectrophotometrically using N, N-dimethyl-p-phenylene diamine-dihydrochloride. (D) MTS assays were used to examine the effect of CSE siRNA and CSE inhibitor PAG on cell growth in MDA-MB-231 cells. 1×10^5 cells were seeded in 6-well plate and then treated with CSE siRNA and CSE inhibitor PAG for 48 h. Cell viability was evaluated in triplicate by Microplate Spectrofluorometer.

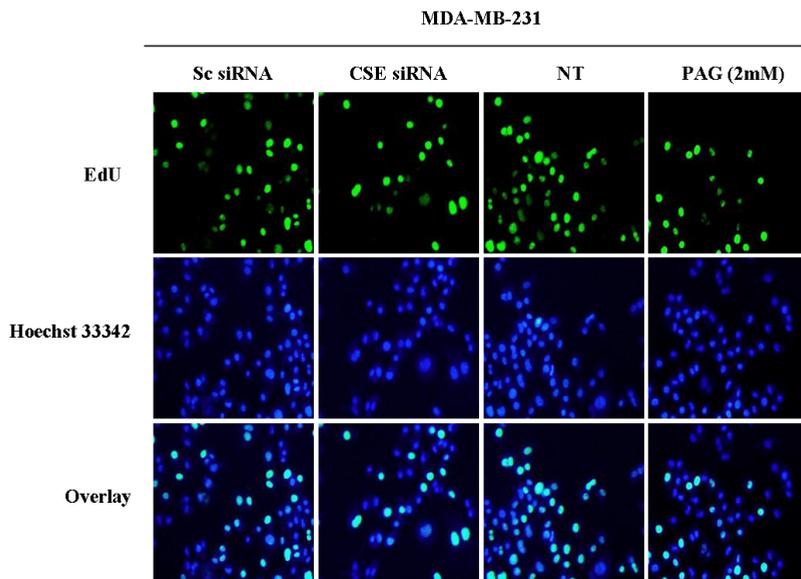


Figure 2. Analyses of cell proliferation associated with the inhibition of CSE/ H_2S system in human breast cancer cells. EdU assays were used to test the effect of CSE siRNA and CSE inhibitor PAP on cell proliferation in MDA-MB-231 cells (20 \times). EdU, 5-ethynyl-2'-deoxyuridine. NT, no treatment.

3.2 Knockdown of CSE in MDA-MB-231 cells arrests cell cycle in S phase

To further investigate the effect of knockdown of CSE on cell proliferation, we detected the changes of cell cycle in MDA-MB-231 cells treated with CSE siRNA or PAG. Flow cytometry results showed that MDA-MB-231 cells exposed to CSE siRNA or PAG were arrested in the S phase (Fig. 3). The results further proved that knockdown of CSE inhibited cell proliferation.

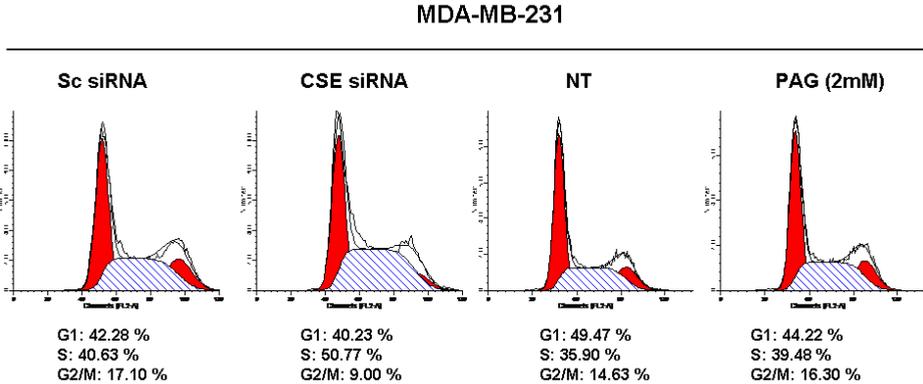


Figure 3. Analyses of cell cycle associated with the inhibition of CSE/H₂S system in human breast cancer cells. Flow cytometry was used to test the effect of CSE siRNA and CSE inhibitor PAP on cell cycle in MDA-MB-231 cells. NT, no treatment.

3.3 Knockdown of CSE in MDA-MB-231 cells induces cell apoptosis

Apoptosis is one of the major mechanisms of inhibiting cell proliferation or inducing cell death. To explore how does the CSE/H₂S system regulate the proliferation of hepatoma cells, we investigated the effect of CSE knockdown on cell apoptosis. Flow cytometry results showed that CSE siRNA transfection or CSE inhibitor PAG treatment caused the increased apoptosis rate in MDA-MB-231 cells (Figure 4).

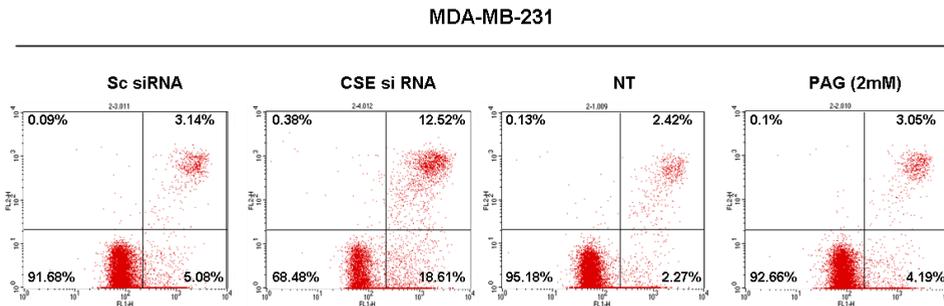


Figure 4. Analyses of cell apoptosis associated with CSE downregulation in MDA-MB-231 cells. Flow cytometry assay with Annexin V/FITC double staining. NT, no treatment.

3.4 Knockdown of CSE in MDA-MB-231 cells inhibits cell migration

Meanwhile, we performed the scratch wound assay and observed that the application of CSE siRNA or PAG inhibited the migration in MDA-MB-231 cells (Figure 5).

MDA-MB-231

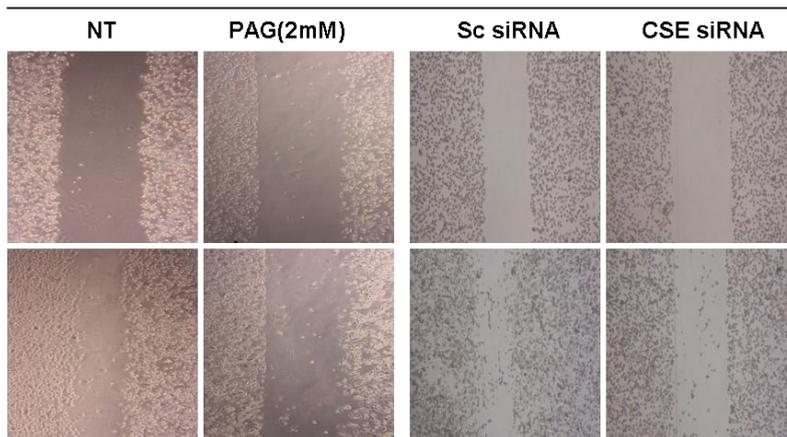


Figure 5. Analyses of cell migration associated with CSE downregulation in MDA-MB-231 cells. The scratch wound assay was performed to detect cell migration. NT, no treatment.

4 Discussion

H₂S, as the third gasotransmitter, has academically got increasing concern. Studies on H₂S are involved in many scientific fields, including many physiological and pathological processes Current studies on H₂S also mainly focus on its functions in physiological and pathophysiological processes [11]. We do not have a good understanding on the functions of H₂S in cancer field and previous studies possess a little controversy, which is exogenous H₂S could induce HCT116 cell proliferation, but inhibit WiDr cell growth [12-13]. So the functions of H₂S in cancer field need to be further investigated.

Few of works on the functions of H₂S in tumor were performed, especially breast cancer. Therefore, in this study we investigated the functions of CSE/H₂S system in breast cancer to further explore the functions of H₂S in cancer cells. We found that the knockdown of CSE/H₂S system significantly inhibited cell growth and proliferation, induced cell apoptosis and inhibited cell migration. The results showed that the CSE/H₂S system possessed vital roles in breast cancer cells.

The following things will be the researches about the mechanism of CSE/H₂S system regulating cell proliferation and apoptosis and the effect of CSE/H₂S system on tumor xenograft growth in vivo.

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