

Original Article

5-Aza-2'-deoxycytidine inhibits proliferation and induces apoptosis in oral cancer cells by enhancing glutathione peroxidase-3 expression

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Abstract: Background: Oral cancer is one of the most malignant cancers. Recent studies have suggested that glutathione peroxidase (GPX)-3 significantly affects the progression and prognosis of cancers. However, the function and mechanism of GPX3 in oral cancer are still unclear. Purpose: The aim of this study was to investigate the effects of 5-aza-2'-deoxycytidine (5-Aza-Cd) on the proliferation and apoptosis of oral cancer cells and the expression level of GPX-3. Materials and Methods: The methylation status and expression of GPX3 were analyzed using methylation-specific PCR (MSP) and qRT-PCR assays in normal oral epithelium cells (CAL-27) and oral cancer cells (HSC-3). CAL-27 and HSC-3 cells were treated with 20 $\mu\text{mol/L}$ 5-Aza-Cd, and GPX3 expression was measured by qRT-PCR assay. The methylation status of GPX3 was observed by methylation-specific PCR (MSP), and cell proliferation and apoptosis were assessed using the CCK-8 assay and flow cytometry. Results: Our results demonstrated that GPX3 expression was decreased, and methylation of the GPX3 promoter was increased in CAL-27 and HSC-3 cells compared with normal cells. The 5-Aza-Cd treatment upregulated GPX3 expression and downregulated methylation of the GPX3 promoter in CAL-27 and HSC-3 cells. In addition, 5-Aza-Cd inhibited proliferation and promoted apoptosis in oral cancer cells. Conclusions: Our results suggest that 5-Aza-Cd suppressed proliferation and accelerated apoptosis in oral cancer cells by inhibiting demethylation and thereby promoting GPX3 expression.

Keywords: 5-aza-2'-deoxycytidine, oral cancer, glutathione peroxidase-3, proliferation, apoptosis

Introduction

Oral cancer is a common malignant tumor of the head and neck, and it is ranked sixth in systemic cancers, together with oropharyngeal cancer [1]. At present, the treatment of oral cancer often results in loss of important visceral functions, including slurred speech, difficulty swallowing, eating disorders, and changes in facial appearance; thus affecting the quality of life [2, 3]. Oral cancer is a complex polygenic disease: environmental and genetic factors are involved in the occurrence and development of this disease [4-6]. Therefore, the prevention of oral cancer is particularly important, and understanding the mechanisms underlying the development and progression of oral cancer will be beneficial to the prevention, early diagnosis, and treatment of this disease.

Epigenetic regulators alter cellular activity without directly affecting and mutating DNA se-

quences [7]. DNA methylation, which serves as one of the most important factors in mammalian epigenetics, is the only known natural chemical modification of DNA in mammals [8]. A number of studies have shown that DNA methylation plays a key role in embryonic development, genetic transcription, X chromosome inactivation, and carcinogenesis [9-11]. DNA methylation patterns and levels are regulated by DNA methyltransferases and demethylases [12]. DNA demethylation includes active and passive DNA demethylation, which are controlled by DNA demethylases and methyltransferases, respectively [13]. To date, several studies have focused on DNA methyltransferases; it will be important to study the DNA demethylase genes that regulate spatial and temporal gene expression [14, 15]. In addition, although the functions of different DNA methyltransferase genes have been clarified through the study of mutants, the extent to which different DNA methyltransferase inhibitors affect methylation

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Table 1. Primer sequences for qRT-PCR analysis

Gene	Primer sequences
GAPDH	Forward: 5'-TATGATGATATCAAGAGGGTAGT-3' Reverse: 5'-TGTATCCAAACTCATTGTCATAC-3'
GPX3	Forward: 5'-CAT CCC CTT CAA GCA GTA TGC T-3' Reverse: 5'-GCC CGT CAG GCC TCA GTA G-3'

remains unclear. The demethylating reagent 5-aza-2'-deoxycytidine (5-Aza-Cd) inhibits DNA methyltransferases and induces changes in DNA methylation [16]. At present, 5-Aza-Cd has been considered as a drug with good efficacy for a variety of malignant tumors, including malignant myeloma, lung cancer, breast cancer, and liver cancer [17-20]. Not only does 5-Aza-Cd inhibit the invasion of primary lesions, but it also effectively inhibits tumor metastasis. Additionally, 5-Aza-Cd can also inhibit growth and induce apoptosis of several types of cancer cells [21-23]. In this study, oral cancer cells were treated with different concentrations of 5-Aza-Cd to explore the role of 5-Aza-Cd in the proliferation and apoptosis of oral cancer cells.

Glutathione peroxidase (GPX)-3, a member of the GPX family and a selenium-dependent enzyme, plays a vital role in restraining oxidative damage by decreasing excess reactive oxygen species [24]. GPX3 can catalyze a decrease in catalase, lipid peroxidase, and organic peroxidase levels by reducing glutathione. Several studies have shown that GPX3 acts as a tumor suppressor in multiple tumor types. In addition, it has been confirmed that downregulation of GPX3 expression is mediated by hypermethylation of the GPX3 promoter in a variety of solid tumors [25-28]. However, the exact mechanism and function of GPX3 in oral cancer remain unclear.

In the present study, we investigated the influence of 5-Aza-Cd on the expression level and methylation status of the GPX3 promoter in oral cancer. In addition, we explored the role of 5-Aza-Cd in the proliferation and apoptosis of oral cancer cells by GPX3.

Materials and methods

Cell culture and transfection

Human oral mucosal epithelial cells (OMECS) and oral cancer cells (CAL-27 and HSC-3) were provided by the Chinese Academy of Sciences

(Shanghai, China). OMECS were maintained in defined keratinocyte serum free medium supplemented with 6% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA). CAL-27 and HSC-3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% FBS. Cells were cultivated at 37°C with 5% CO₂ and 95% air. CAL-27 and HSC-3 cells were treated with 20 µmol/L 5-Aza-Cd for 72 h.

RNA extraction and quantitative real-time PCR (qRT-PCR) assay

Total RNA was extracted with TRIzol reagent (#9109; Takara, Tokyo, Japan) from OMECS and CAL-27 and HSC-3 cells according to the manufacturer's instructions. RNA (5 µg) was transcribed to cDNA using a Reverse Transcription kit (Takara) according to the manufacturer's instructions. The expression level of GPX3 was analyzed using the Bestar™ qPCR MasterMix (#2043, DBI Bioscience, China) on the ABI PRISM 7500 Sequence Detection System (Life Technologies, Carlsbad, CA, USA). The primers are shown in **Table 1**. The relative mRNA expression level was analyzed using the 2^{-ΔΔCt} method [29].

Methylation-specific PCR (MSP)

High-purity DNA was extracted using the TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China) from the treated CAL-27 and HSC-3 cells according to the manufacturer's instructions. The concentration of the extracted DNA was measured by UV absorbance at 260 and 280 nm. DNA (1 g/100 L) was treated with bisulfite using the Methylamp™ One-Step DNA Modification kit. The DNA modified by sodium bisulfite was analyzed by MSP using an EpiTect bisulfite reagent kit (Qiagen, Venlo, The Netherlands). The DNA was then amplified using the S1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The PCR reactions were prepared as follows: Ex Taq DNA Polymerase (1.25 U), 10× Ex Taq buffer (5 µL), dNTPs (4 µL, 1 mM each), primers (0.2 µM each), and cDNA template (5 ng). The reaction was as follows: 94°C for 4 min, followed by 94°C for 40 s, 58°C for 30 s, 72°C for 1 min for 30 cycles, with a final step for 5 min at 72°C. PCR products were detected by electrophoresis in 1× TAE buffer on a 1.0% agarose gel. Primer sequences are provided in **Table 2**.

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Table 2. Primer sequences for MSP analysis

Gene	Primer sequences
Methylated	Sense: 5'-TTA CGA GGG GCG GTC GTA CGA GGG-3'
	Antisense: 5'-AAA ACG ACC GAC GCG AAC GCC TGC-3'
Unmethylated	Sense: 5'-TTA TGA GGG GTG GTT GCA TGT GGG-3'
	Antisense: 5'-AAA ACA ATC AAC ACA AAC ACC TCC-3'

Cell proliferation assay

The treated CAL-27 and HSC-3 cells in logarithmic growth phase were re-suspended in DMEM medium with 10% phosphate-buffered saline (PBS). The suspended cells were seeded into 96-well plates at a concentration of 5×10^3 cells/100 μ L per well and cultivated at 37°C and 5% CO₂ for 48 h. Cell counting kit-8 (CCK-8) solution (10 μ L; Dojindo, Japan) was added to each well and the plate was incubated at 37°C and 5% CO₂ in the dark for 2 h. The absorbance (OD) was measured at 450 nm using a microplate reader (SpectraMax, Molecular Devices, San Jose, CA, USA).

Cell apoptosis analysis

The number of apoptotic cells was assessed using an annexin V-FITC/propidium iodide (PI) double-staining detection kit (Sigma-Aldrich). The treated CAL-27 and HSC-3 cells (5×10^5 /well) in logarithmic growth phase were digested with trypsin, harvested, and washed with PBS. According to the manufacturer's instructions, the collected cells were incubated with 5 μ L annexin V-FITC and 10 μ L PI for 15 min in the dark. The apoptotic cells in each group were detected by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

Experimental data are presented as mean \pm standard deviation (SD) of at least three independent experiments. The data was analyzed using IBM SPSS Version 20 (SPSS, Inc., Chicago, IL, USA) with Student's *t*-test. The statistical significance was defined as $P < 0.05$.

Results

Downregulation of GPX3 expression and hypermethylation of the GPX3 promoter in oral cancer cells

In this study, we observed that the mRNA expression level of GPX3 was significantly

downregulated in the oral cancer cells CAL-27 and HSC-3, relative to normal OMECs ($P < 0.05$, **Figure 1A**). Methylation-specific PCR (MSP) was performed to detect the methylation status of the GPX3 promoter in oral cancer cells. The results indicated that the GPX3 promoter was not methylated in OMECs, and that the GPX3 promoter was hypermethylated in the oral cancer cell lines CAL-27 and HSC-3 (**Figure 1B**). Therefore, CAL-27 and HSC-3 cells displayed decreased GPX3 expression and hypermethylation of the GPX3 promoter.

5-Aza-Cd-induced demethylation increases GPX3 expression in oral cancer cells

Several studies have confirmed that the demethylating reagent 5-Aza-Cd can inhibit DNA methyltransferases, resulting in altered methylation status [16, 30, 31]. It has been shown that 5-Aza-Cd can inhibit the development and progression of multiple types of cancers [32-34]. To further verify the relationship between GPX3 expression and methylation of the GPX3 promoter, CAL-27 and HSC-3 cells were treated with 20 μ mol/L 5-Aza-Cd for 72 h and qRT-PCR and MSP were performed to assess GPX3 expression and the methylation status of the GPX3 promoter, respectively. As shown in **Figure 2A**, the mRNA expression level of GPX3 was significantly increased following demethylation with 5-Aza-Cd ($P < 0.05$). Methylation of the GPX3 promoter was also reduced following 5-Aza-Cd treatment compared with the control (**Figure 2B**). Therefore, our results suggest that 5-Aza-Cd treatment increased the expression of GPX3 and decreased the methylation of the GPX3 promoter in oral cancer cells.

5-Aza-Cd inhibits proliferation and promotes apoptosis in oral cancer cells.

To further examine the roles of GPX3 promoter demethylation following treatment with 5-Aza-Cd on oral cancer cell proliferation and apoptosis, CAL-27 and HSC-3 cells were treated with 20 μ mol/L 5-Aza-Cd for 72 h. The CCK-8 assay and flow cytometry analyses were performed to determine the influence of 5-Aza-Cd on oral cancer cell proliferation and apoptosis. The CCK-8 assay revealed that 5-Aza-Cd treatment resulted in a significantly lower proliferative

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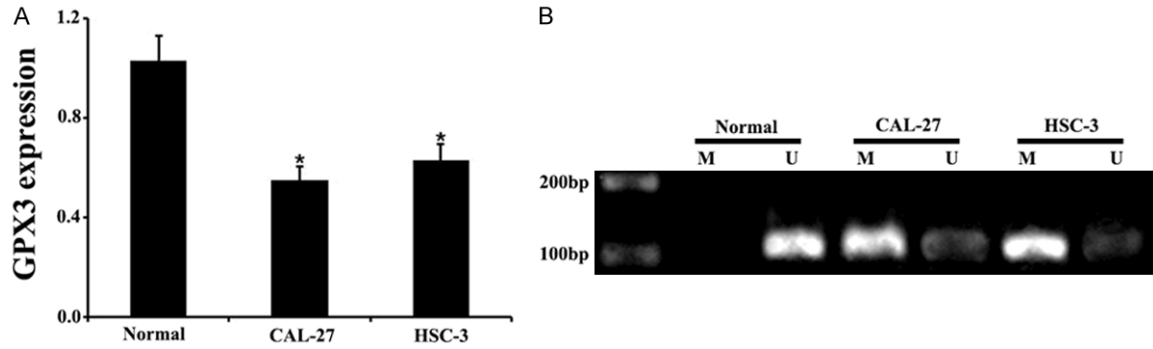


Figure 1. Expression level of GPX3 mRNA and methylation status of the GPX3 promoter in oral cancer cells. A. Relative expression level of GPX3 was assessed by qRT-PCR in oral mucosal epithelial cells (OMECS, normal) and oral cancer cells (CAL-27 and HSC-3) (* $P < 0.05$ vs. normal group). B. The methylation status of GPX3 promoter was detected by methylation-specific PCR (MSP). M, methylated; U, unmethylated.

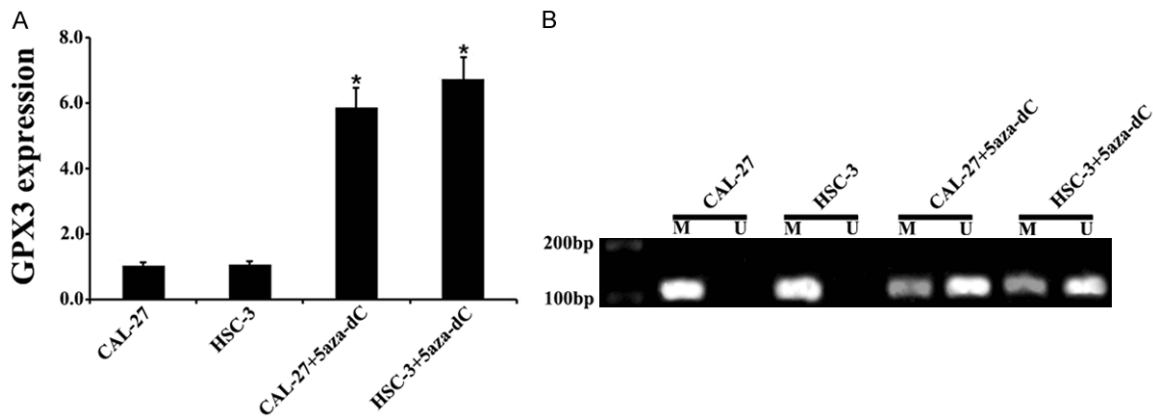


Figure 2. Restoration of GPX3 expression in CAL-27 and HSC-3 cells by demethylation. CAL-27 and HSC-3 cells were treated with 20 $\mu\text{mol/L}$ 5-Aza-Cd for 72 h. A. qRT-PCR was performed to analyze GPX3 expression (* $P < 0.05$). B. Methylation-specific PCR (MSP) was performed to measure methylation of the GPX3 promoter. M, methylated; U, unmethylated.

capacity of CAL-27 and HSC-3 cells ($P < 0.05$, **Figure 3A**). Flow cytometry analyses indicated that the number of early and late apoptotic cells was significantly increased in CAL-27 and HSC-3 cells treated with 5-Aza-Cd compared with control groups ($P < 0.05$, **Figure 3B**). Therefore, our results suggested that 5-Aza-Cd inhibited oral cancer cell proliferation and induced apoptosis.

Discussion

Tumor formation is a complex process related to multiple genes, multiple factors, and multiple stages [35]. In recent years, DNA methylation has attracted much attention in epigenetics research. DNA methylation is the process by which methyl groups are transferred to

a specified DNA base site in a methyltransferase-dependent manner [10]. DNA methylation is abnormally expressed in tumors, i.e., tumor suppressor genes are frequently unmethylated in normal tissues or cells, and methylated in malignant tissues or cells [36]. Most DNA methylation occurs in the CpG islands of the promoter region, and there are approximately 29,000-45,000 CpG islands in the human genome [37]. GC base pairs account for about 60-70% of the CpG islands, and approximately 50% of the genome contains CpG islands. These islands are mainly located in the promoter region or in the first exon region of a gene, which is rich in the CpG sequence. The cytosine in the CpG sequence can be easily methylated to form 5-methylcytosine. Abnormal hypermethylation of DNA in CpG islands in a promot-

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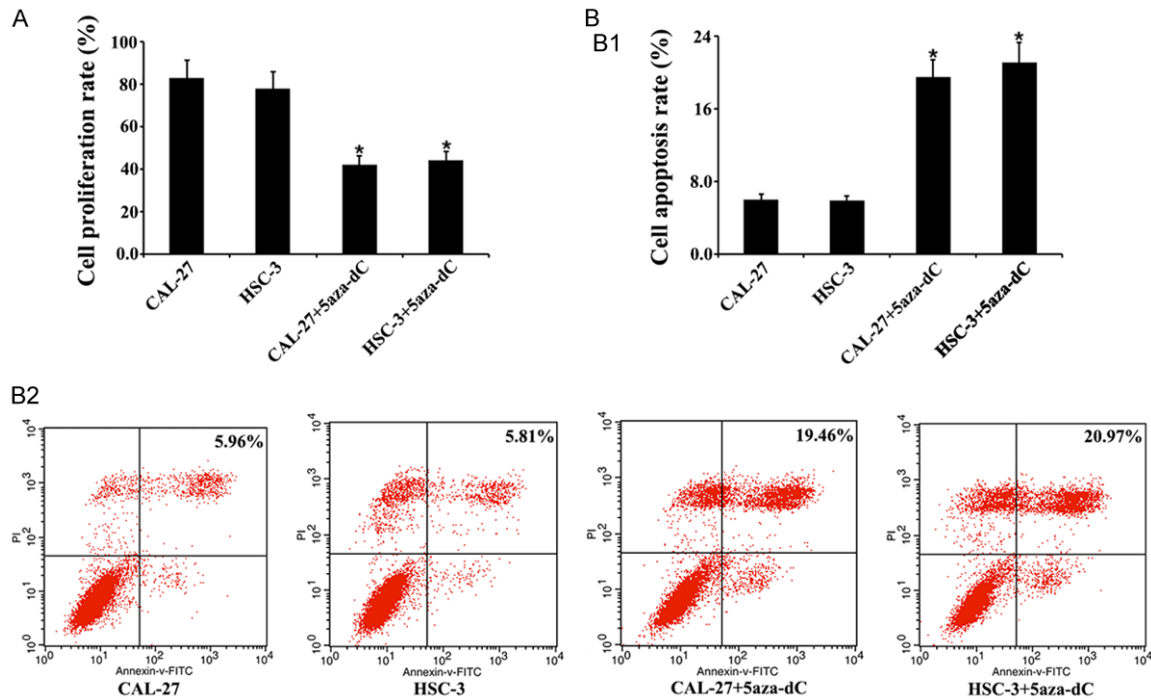


Figure 3. The effects of 5-Aza-Cd on cell proliferation and apoptosis in oral cancer. A. CAL-27 and HSC-3 cells were treated with 20 $\mu\text{mol/L}$ 5-Aza-Cd for 72 h. Proliferation was examined with the CCK-8 assay ($*P < 0.05$). B. CAL-27 and HSC-3 cells treated with 20 $\mu\text{mol/L}$ 5-Aza-Cd were stained with annexin V-FITC and propidium iodide and analyzed by flow cytometry ($*P < 0.05$).

er can lead to gene deletion or low expression, which is an important marker of tumor formation and tumor development in humans [37].

GPX is a widely distributed peroxidase in the body. GPX3, a member of the GPX protein family, is mainly distributed in the fluids outside cells, such as plasma, eye chambers, and amniotic fluid [38, 39]. GPX3 functions as a tumor suppressor gene and is downregulated in a variety of cancers because of genomic deletion or epigenetic alteration. For example, GPX3 inhibits esophageal squamous cell carcinoma migration and invasion by regulating the FAK/AKT pathway [40]; GPX3 has been shown to exhibit clinical significance and therapeutic value in hepatocellular carcinoma [25]; decreased expression of GPX3 has been linked to favorable/intermediate karyotypes in patients with *de novo* acute myeloid leukemia [41]; silencing of GPX3 accelerated human thyroid cancer metastasis [42]; downregulation of GPX3 was linked to the prognosis and lymph node metastasis of cervical cancer [26]; low expression of GPX3 has been shown to be a biomarker for poor prognosis in gallbladder

cancer [43]; and GPX3 has been shown to act as a tumor suppressor in colitis-associated carcinoma [44]. In addition, GPX3 has been found to be methylated in a variety of tumors, resulting in downregulation or complete loss of expression. By screening the genes silenced by epigenetic modifications a previous study found that GPX3 was heavily methylated in prostate cancer [45]; methylation or deletion of the GPX3 gene promoted prostate cancer growth and metastasis [46]; GPX3 has been shown to be methylated and deleted in Barrett's esophageal carcinoma [47]; DNA hypermethylation affects the expression of glutathione peroxidases in Barrett's-related adenocarcinomas [48]; GPX3 hypermethylation acts as a prognostic biomarker of non-M3 acute myeloid leukemia [49]; hypermethylation of the GPX3 promoter has been observed in chronic myeloid leukemia [50]; the methylation status of GPX3 can be used as a predictive biomarker for platinum sensitivity in colorectal cancer [51]; and GPX3 expression was shown to be decreased and the methylation of GPX3 increased in hepatocellular carcinoma tissue [52]. In this study, we further explored the expression level and

methylation of the GPX3 gene in oral cancer cells, and observed downregulation of GPX3 expression and hypermethylation of the GPX3 promoter in oral cancer cells. Therefore, we showed that hypermethylation of GPX3 might also be useful as a prognostic biomarker for the treatment of oral cancer. 5-Aza-Cd, a DNA methyltransferase inhibitor, can reverse the malignant phenotype of tumor cells by demethylation [31]. Demethylation can lead to the expression of many CpG over-methylated tumor suppressor genes and restore their anti-cancer function [53, 54]. In our study, 5-Aza-Cd was adopted to treat oral cancer cells and induce demethylation. We found that 5-Aza-Cd resulted in increased GPX3 expression and decreased methylation of GPX3. In addition, 5-Aza-Cd treatment inhibited oral cancer cell proliferation and promoted apoptosis. The demethylation of 5-Aza-Cd in our study is consistent with previous reports.

Conclusions

We demonstrated that methylation of the GPX3 promoter was increased and GPX3 was down-regulated in oral cancer. In addition, we showed that 5-Aza-Cd inhibited oral cancer cell proliferation and accelerated apoptosis by regulating GPX3 methylation. However, our study still has some limitations. For example, the level and methylation status of GPX3 should be verified in a mass of oral cancer tissues. Further experiments should also be carried out to assess the influence of GPX3 methylation and demethylation on oral cancer proliferation, migration, and invasion; other oral cancer cells should also be studied. Finally, *in vivo* experiments should be conducted to verify the role of GPX3 methylation. The exact mechanism of GPX3 methylation on oral cancer progression should be further explored.

Disclosure of conflict of interest

None.

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