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Promoter methylation and clinical significance of GPX3 in esophageal squamous cell carcinoma

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ABSTRACT

Background: Glutathione peroxidase 3 (GPX3) provides critical protection against reactive oxygen species (ROS) in cells. Downregulation of GPX3 may contribute to carcinogenesis of esophageal squamous cell carcinoma (ESCC), but the mechanisms are not clear.
Materials and methods: We examined the differences in gene expression between ESCC and normal esophageal epithelial, by using GEO datasets, and collected 136 ESCC tumors and adjacent normal tissues to confirm our findings. GPX3 expression was measured, at the mRNA and protein levels, by qRT-PCR, Western Blot and immunohistochemistry (IHC).
Results: GPX3 mRNA and protein levels were 3.3-fold higher in normal epithelium compared with case-matched ESCC tissues. There was no significant correlation between clinical parameters and expression levels of GPX3 in ESCC. Promoter methylation of the GPX3 gene correlated with decreased expression.
Conclusion: Downregulation of GPX3 might be a key factor in the process of ESCC carcinogenesis.

1. Introduction

ESCC is a multiple stage process involved genetic and epigenetic changes, including DNA methylation, which inactivates tumor suppressor genes to promote carcinogenesis. Reactive oxygen species (ROS), such superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^·$), are generated under normal metabolic conditions through cellular respiration, and participate in a wide range of human diseases, including cancer [10]. Tumor cells generate but have decreased capacity to eliminate ROS. Antioxidant enzymes, such as glutathione peroxidase (GPX), play important roles in eliminating ROS [2]. In animals, the glutathione peroxidases has eight members (GPX1-8), five of which are in humans(GPX1-4, 6) [7]. Previous, the roles of GPX in carcinogenesis has been uncovered [1]. Glutathione peroxidase 3 (GPX3) is a selenium-dependent enzyme that detoxifies ROS to protect organs from overwhelming oxidative stress [21]. GPX3 catalyzes the reduction of hydrogen peroxidase, organic peroxidase and lipid peroxidase by reducing glutathione. GPX3 is reported to be down-regulated in several types of cancer, such as hepatocellular carcinoma and esophageal cancer [8,11]. Moreover, circulating GPX3 was identified to be significantly lower in patients with glioblastoma compared with non-tumor patients [12]. It has been demonstrated that GPX3 DNA methylation will reduce the expression of GPX3 protein in gastric cancer, prostate cancer and so on [6,19,20]. However, the expression of GPX3 and the mechanism of its downregulation in ESCC was not identified.

To further elucidate the role(s) of GPX3 in human ESCC carcinogenesis, we analyzed GPX3 expression and DNA methylation in a panel of cell lines representing human ESCC malignancies. In addition, associations of GPX3 expression with clinical parameters were analyzed in a large cohort of ESCC patients.

2. Material and methods

2.1. Patient samples and cell lines

We collected 136 consecutive ESCC samples and case-matched normal esophageal epithelium at the Tumor Hospital of Shantou University between 2008 and 2012. All patients underwent potentially curative surgery without preoperative chemotherapy or radiotherapy. In the ESCC cohort, 98 were men and 38 were women, and the age range was 37–80 years, with a median of 57.9 years. This study was approved by the ethical review committees of the Medical College of Shantou University. All participants involved in our study gave

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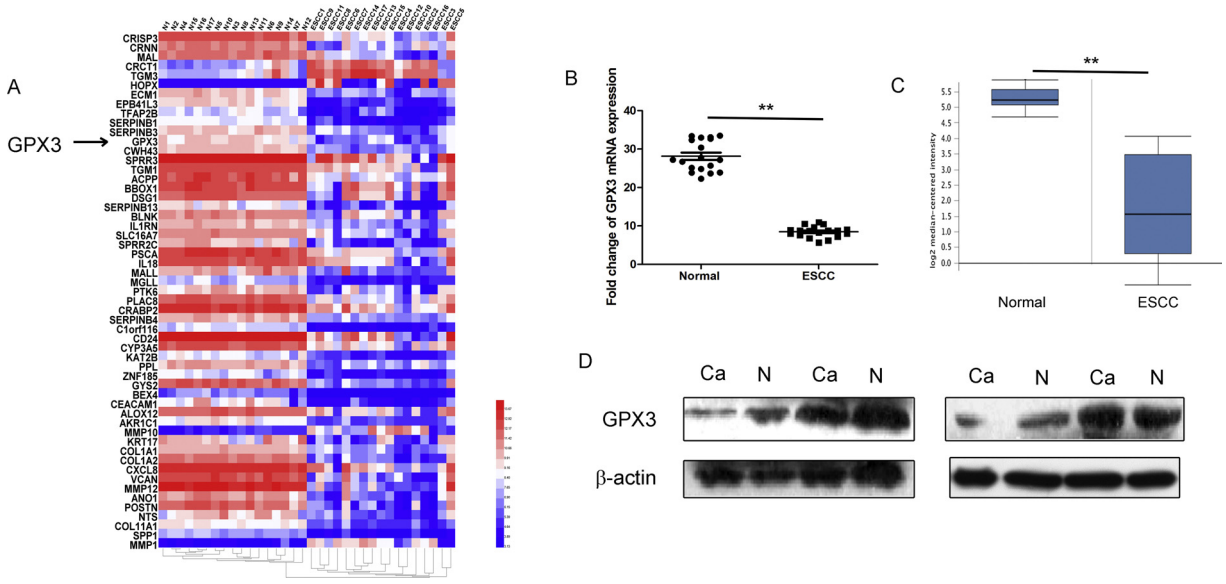

Fig. 1. GPX3 mRNA and protein expression in ESCC tumors.
A. Heatmap clustering of expression array data obtained from the GSE20347 dataset. B. Quantitative RT-PCR showing GPX3 mRNA expression level was 3.07 times higher than the case-matched normal tissues. C. Comparison of the expression of GPX3 between esophageal squamous epithelium and squamous cell carcinoma samples in the Su esophagus database, using Oncomine. D. Western blot for GPX3 in ESCC tumors and case-matched normal esophageal epithelium was examined. Compared to benign esophageal tissue harvested at the surgical margins in the same specimens (labeled as N), cancerous tissues (labeled as Ca) expressed a significantly lower level of GPX3 (** $p < 0.01$).

informed consent.

Four ESCC cell lines (TE1, TE13, KYSE150 and KYSE520) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1× antibiotic mixture (Invitrogen, Carlsbad, CA, USA).

2.2. Microarray data analyses for obtaining the differential gene signature in ESCC vs. normal esophageal epithelium

To identify poor outcome gene signatures, we used the GSE20347 dataset, which included 17 ESCC samples and 17 normal esophageal tissues (N = 34) [5]. Expression data for GSE20347 was downloaded from the Gene Expression Omnibus (GEO) website (http://www.ncbi.nlm.nih.gov/gds/). Background correction, normalization, and summarization were performed and a baseline transformation to the median of all samples was applied. A gene list was made by selecting genes with a fold change > 3 or < -3 and *p*-value < 0.05 between ESCC and normal tissues.

2.3. Statistical analysis of GPX3 expression in ESCC

To determine the expression pattern of GPX3 in ESCC, we used the datasets in the Oncomine database (https://www.oncomine.org). Oncomine is an online database consisting of previously published and publicly available microarray data. The analysis enables multiple comparisons of gene expression (DNA or RNA) between different studies. The significance of the gene expression across the available studies was also considered. The GPX3 gene was queried in the database and the results were filtered by selecting ESCC and Cancer vs. Normal Analysis. Comparison statistical analysis was conducted using Oncomine algorithms. Details of standardized normalization techniques and statistical calculations are provided on the Oncomine platform. The correlation between GPX3 expression and DNA methylation was detected in the cBioPortal based on the TCGA data portal (https://tcgadata.nci.nih.gov/tcga/tcgaHome2.jsp). The survival of ESCC patients with different GPX3 expression levels was analyzed in the Gene expression Profiling Interactive Analysis based on the TCGA data. (http://gepia.cancer-pku.cn/index.html).

2.4. DNA demethylation and western blot analysis

To demethylate DNA, all ESCC cell lines were treated with 10 μM 5-aza-deoxycytidine(5-Aza-dC) (Sigma–Aldrich, St. Louis, MO, USA), for 96 h. Control cells received DMSO treated. Western blotting was performed as described previously [16]. Antibodies against human β-actin (1:1000) and GPX3 (1:1000) were purchased from Abcam (Cambridge, USA).

2.5. Immunohistochemistry (IHC)

IHC staining was performed using the Envision Labeled Peroxidase System (Dako, Carpinteria, CA) as described previously [17]. Immunostaining of GPX3 (Abcam, Cambridge, USA; 1:200) was evaluated by two independent pathologists, and a high degree of concordance between the two pathologists was indicated by an inter-rater agreement kappa value of 0.93. The immunostaining of GPX3 was evaluated by optical density using Image-Pro Plus 6.0 software.

2.6. Quantitative RT-PCR

Expression of GPX3 in ESCC cells was determined by real time PCR. Using the RNeasy Mini kit (Qiagen), total cellular RNA was extracted from cells. Quantitative RT-PCR was performed according to the manufacturer's instructions. Primers for GPX3 were: forward: 5′-CTTCCTA CCCTCAAGTATGTCCG-3′, reverse: 5′-GAGGTGGGAGGACAGGAGTT CTT-3′, GAPDH, forward, 5′-AGAAGGCTGGGCTCATTTG-3′, reverse, 5′-AGGGGCCATCCA CAGTCTTC-3′.

2.7. Statistical analysis

Data was expressed as mean ± SD of at least three separate experiments. A $p < 0.05$ was considered as statistically significant. Differences among the treatment groups were assessed via ANOVA using statistical software (SPSS, IBM, USA). A *p*-value of ≤0.05 was considered significant.

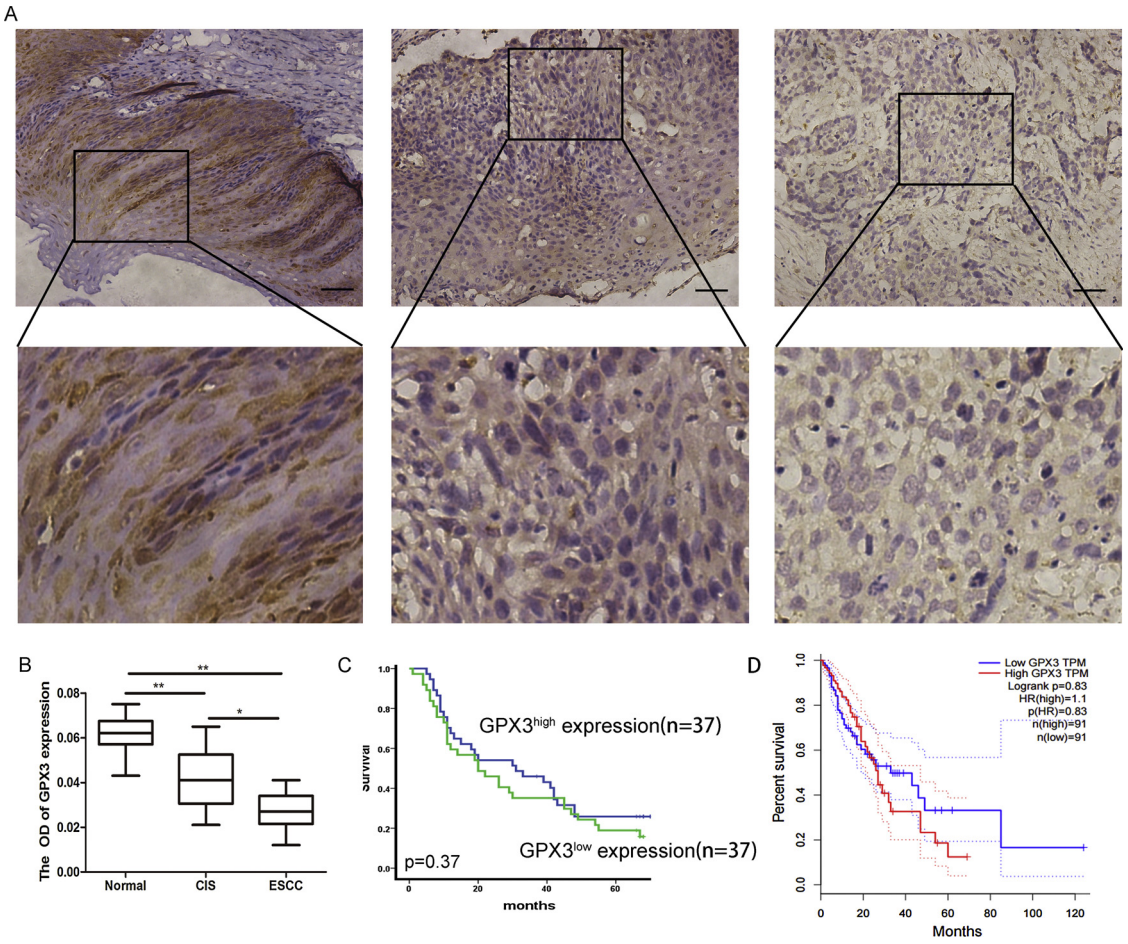

Fig. 2. Immunostaining of GPX3 in normal esophageal epithelium and ESCC.
Immunostaining for GPX3 was strong in normal epithelium, moderate in carcinoma in-situ and weak in ESCC tissues (IHC staining, scar bar: 50 μm). B. Expression of GPX3 was significantly higher in normal esophageal epithelium compared with case-matched ESCC tissues (*$p < 0.05$). C. Kaplan-Meier test shows no significant association between overall survival and the expression level of GPX3 ($P = 0.37$), when the two groups were defined as low and high. D. Survival data from the TCGA database shows that there is no significant difference between GPX3 high and low patient groups (*$p < 0.05$).

Table 1
Correlation of GPX3 and clinical parameters in 136 ESCC cases.

Parameter		Case NO. 136	OD of GPX3 *p*	
Age	< 58	67	0.031 ± 0.006	0.872
	≥58	69	0.033 ± 0.011	
Gender	Male	98	0.032 ± 0.013	0.458
	Female	38	0.026 ± 0.006	
Differentiation	Poor	14	0.026 ± 0.015	0.894
	Intermediate	76	0.027 ± 0.012	
	Well	46	0.027 ± 0.009	
Tumor size	≥5 cm	48	0.037 ± 0.005	0.397
	< 5 cm	88	0.028 ± 0.006	
Depth of invasion	T1-T2	32	0.037 ± 0.008	0.562
	T3-T4	104	0.032 ± 0.012	
Lymph node metastasis	Yes	59	0.033 ± 0.011	0.581
	No	77	0.031 ± 0.009	

3. Results

3.1. Microarray screen for differentially-expressed genes in ESCC

To explore the differences in gene expression profiles between ESCC and normal esophageal epithelium, we analyzed a microarray dataset (GSE20347) that included 17 primary ESCC samples and 17 normal esophageal [5]. The gene expression profile was downloaded from the GEO database, and the GEO2R method was used to identify differentially-expressed genes in ESCC samples compared with normal epithelial. A $P < 0.05$, $\log_2$FC (fold control) > 3.0 or $\log_2$FC < -3.0 was used as the criteria for significance, leading to 78 genes being identified as DEGs. Among these, 14 genes (17.95%) were upregulated, and the remaining 64 genes (82.05%) were downregulated. Some of the genes are listed in Fig. 1A. Some of these downregulated genes, including CRNN and TGM3, have been previously reported to be involved in esophageal cancer [4]. The identification of these known differentially-expressed genes suggested that our platform was accurate for the discovery of genes showing different expressions between ESCC and normal esophageal epithelium.

3.2. GPX3 mRNA and protein levels in ESCC and non-cancerous tissues

Among these differentially-expressed genes, we focused on GPX3, a ROS scavenger in this study. Based on our qRT-PCR results, there was a greater than 3-fold increase in GPX3 expression in normal esophageal epithelium compared with that detected in 32 case-matched ESCC tissues (Fig. 1B). To confirm our results, we compared GPX3 gene expression in normal human esophageal epithelium and ESCC tissues using the Oncomine database (http://www.oncomine.org). Analysis of a representative data set (Su esophagus) revealed that GPX3 expression levels were also 3-fold higher in esophageal squamous epithelium than in ESCC (Fig. 1C), which is consistent with our results.

We next examined GPX3 protein expression levels in same cohort of 32 case-matched ESCC samples and found that 26 cases (81.2%)

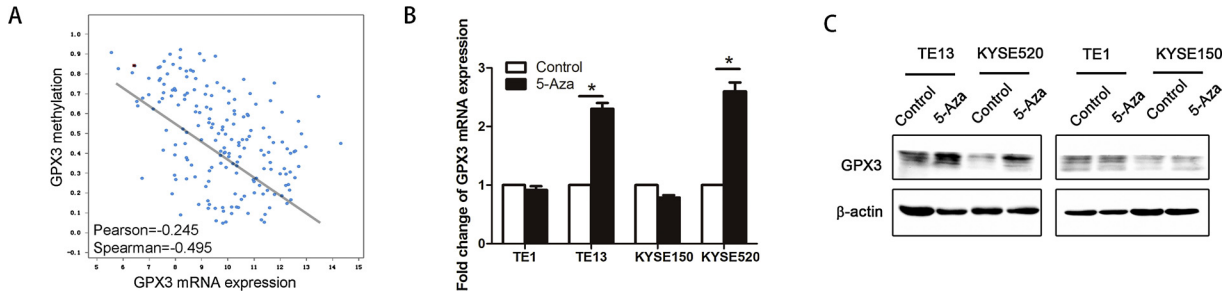

Fig. 3. GPX3 expression is associated with methylation of the GPX3 promoter.
A. cBioPortal data showing that the downregulation of GPX3 was correlated with its methylation status. B. The mRNA level of GPX3 was significantly elevated after 96 h of 5-Aza-dC treatment at 10 μM in four ESCC cell lines, TE1, TE13, KYSE150 and KYSE520 cells. C. Protein level of GPX3 was increased after 96 h 5-Aza treatment at 10 μM (*$p < 0.05$).

showed higher GPX3 expression levels in normal epithelium than ESCC tissues, 3 (9.4%) cases showed equal expression and 3 (9.4%) showed lower expression in normal epithelium than ESCC tissues (Fig. 1D). Data generated from the qRT-PCR and western blot studies strongly correlated with each other ($p < 0.001$, Fisher's exact test).

3.3. Expression of GPX3 and its clinical significance in ESCC

To further evaluate the clinical relevance of GPX3 in ESCC, we collected 136 pairs of samples of normal squamous epithelial and their corresponding primary ESCC tissues. Immunoreactivity of GPX3 was determined based on the presence of cytoplasmic staining. GPX3 staining was mainly observed in the cytoplasm of tumor cells (Fig. 2A), Among 136 cases, there were 12 carcinoma in situ (CIS) tissues. The expression of GPX3 was significantly higher in benign epithelial tissues (n = 136) (IS: 0.064 ± 0.18), as compared to CIS tissues (n = 12) (IS: 0.041 ± 0.19, $p < 0.01$) (Fig. 2B). The expression of GPX3 in ESCC (n = 136) was lower than that of CIS tissues (IS: 0.023 ± 0.04, $p < 0.05$).

We then analyzed the correlation between GPX3 expression and clinical parameters. No statistically significant correlations were observed between GPX3 expression and gender, age, tumor size, tumor differentiation or lymph node metastasis (Table 1). Clinical follow-up data was available for 74 of the 136 patients included in this study. Survival was analyzed using the Kaplan-Meier method. Based on the intensity and percentage of immunostained cells described above, half of these tumors (37 of 74) were assessed to have $GPX3^{high}$ expression, and 37 (50.0%) were assessed as $GPX3^{low}$ expression in ESCC. Patients with $GPX3^{low}$ expression levels tended to have worse overall survival than $GPX3^{high}$ expression levels (29.4 ± 3.9 months vs. 34.4 ± 4.0months, $p = 0.37$), but was not significant (Fig. 2C). Survival data from the TCGA database also supported our result that there was no significant difference in survival outcome between $GPX3^{low}$ expression and $GPX3^{high}$ expression patients.

3.4. Decreased GPX3 expression levels are linked to methylation of the GPX3 gene promoter in ESCC

The previous research indicates that methylation is associated with the transcriptional silencing of GPX3 in ESCC cells [13]. The expression of GPX3 mRNA levels in the cBioPortal dataset revealed that there was a significant correlation between decreased GPX3 expression and its promoter methylation (Fig. 3A). To determine whether GPX3 expression could be reactivated by pharmacological demethylation of genomic DNA, four ESCC cell lines (TE-1, TE13, KYSE-150 and KYSE-520) were treated with the demethylating agent 5-Aza-dC at 10 μM for 96 h. GPX3 protein and mRNA expression were elevated 2.3 ~ 2.5 times in TE13 and KYSE520 ESCC cell lines. These results further support the role of methylation as a mechanism of GPX3 downregulation in ESCC.

4. Discussion

In this study, we report that, in a large cohort of ESCC tissue and case-matched normal esophageal epithelium, loss of GPX3 expression in ESCC is due in part to methylation of the GPX3 promoter. However, we did not find significant correlation between GPX3 and pathological parameters nor prognostic value.

To identify novel genes that are differentially-expressed in ESCC, we downloaded oligonucleotide microarray data (GSE20347) from the GEO database. Simultaneously, we also investigated the gene expression profiles of 34 paired tumor/non-tumor tissues to select genes whose expression are indeed increased or decreased in ESCC, compared with paired non-tumor tissue. Several genes have been reported as functional genes in ESCC tumorigenesis, such as CRNN [4]. In this gene dataset, GPX3 was selected as a gene of interest because our previous research showed that GPX3 expression is mediated by the hypermethylation of its promoter (data not published).

GPX3, a secreted form of GPX that is readily detectable in plasma and mucosal surfaces, detoxifies ROS before it enters cells [2]. The functions and expression of GPX3 in carcinogenesis has been reported in several types of cancer, such as myeloid leukemia, colorectal cancer and thyroid cancer [9,18,21]. Reduced GPX3 expression is closely associated with clinical pathological behaviors and poor prognosis of gallbladder cancer [14]. Silencing GPX3 is related to tumor size and lymph node metastasis in thyroid cancer [18]. Moreover, several reports demonstrate that the tumor suppression function of GPX3 is mediated through induction of apoptosis in prostate and colon cancer [9,15]. In hepatocellular carcinoma cells, tumor suppressive activity of GPX3 was mediated through ERK-NFkB-SIP1 pathway [3].

Epigenetic hypermethylation and genome deletion are reported as two reasons for the down-regulation of GPX3 during cancer development [21]. Since methylation is a reversible process, this suggests that removal of methylation could restore GPX3 gene function. Previous studies showed that treatment of cell lines cultured with 5-aza deoxycytidine results in demethylation and restores expression of GPX3 in several cancer cell lines [8,15]. Methylation of GPX3 promotes thyroid cancer cell metastasis through Wnt/β-catenin signaling [18]. In acute myeloid leukemia, GPX3 methylation predicts adverse clinical outcome, and GPX3 expression is regulated by its promoter methylation [21]. GPX3 methylation has been found in Barrett's adenocarcinoma [8], while corresponding adjacent noncancerous liver tissues showed no methylation, which is consistent with our results.

In conclusion, we show that down-regulation of GPX3 can occur in ESCC tissues compared with normal tissues. Although GPX3 expression has no significant correlation with any pathological parameters in ESCC. Moreover, silencing of GPX3 can occur because of promoter methylation in ESCC.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The Authors declare that there is no conflict of interest.

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