

## Original Research Article

# Clinical significance of peroxiredoxin 2 in esophageal squamous cell carcinoma and its role in cell proliferation and apoptosis *in vitro*

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### Abstract

**Purpose:** To evaluate the expression of peroxiredoxin 2 (PRDX2) and its clinical significance in esophageal squamous cell carcinoma (ESCC) and its effect on the proliferation and apoptosis of ESCC cells.

**Methods:** The expression of PRDX2 in clinical tissues (120 samples of tumor tissues compared to 20 samples of non-tumor tissues) was determined. The tissues were divided into high-expression group ( $n = 56$ ) or low-expression group ( $n = 64$ ) based on the level of PRDX2. Association between PRDX2 expression and clinicopathological features of patients was further analyzed. *In vitro*, si-NC and si-PRDX2 were transfected into ECA-109 cells, respectively, to determine the influences of PRDX2 on the function of ESCC cells. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the different expressions of PRDX2 in the clinical tissues.

**Results:** The results from qRT-PCR indicated that PRDX2 was upregulated in ESCC tissues compared to normal control. Correlation between the expression of PRDX2 and clinical pathological data revealed that the expression of PRDX2 showed correlation with tumor differentiation, size, and clinical tumor node metastasis (TNM) stage. Modulation of PRDX2 expression by transfecting si-PRDX2 in ESCC cell lines demonstrated effective regulation of cell proliferation and apoptosis. Specifically, it was observed that cell proliferation decreased, while cell apoptosis was increased.

**Conclusion:** Peroxiredoxin 2 is a novel factor that plays a significant role in ESCC progression, and constitutes a potential biomarker and therapeutic target for ESCC.

**Keywords:** Esophageal squamous cell carcinoma (ESCC), Peroxiredoxin 2 (PRDX2), Proliferation, Apoptosis

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## INTRODUCTION

Esophageal carcinoma (EC) is a common malignant tumor of the digestive tract. Statistics have revealed that more than 300,000 people die from EC every year globally [1,2] and its 5-year

survival rate was reported to range from 10 - 40 % [3,4]. Esophageal squamous cell carcinoma (ESCC) is the main pathological type of EC [5]. Research indicates that long-term smoking and heavy drinking are the major triggering factors for ESCC [6,7]. In an attempt at reducing the high

morbidity and mortality rates, current treatment methods have been far from meeting the needs of clinical patients. Furthermore, due to the lack of effective early diagnosis for ESCC in clinics, most patients are in the advanced stage when definitive diagnoses are achieved. Therefore, it is of great significance to elucidate the molecular mechanism of ESCC cells' chemosensitivity and find treatment targets for clinical treatment. Peroxiredoxins (PRDXs) are small-molecule proteases with a molecular weight ranging from 22 to 27 kDa. They are widely expressed in organisms and play a crucial role in catalyzing various peroxides while counteracting the toxic effects caused by peroxidation [8,9]. Besides, PRDXs function in regulating multiple physiological processes, including cell growth, differentiation, apoptosis, embryonic development, lipid metabolism, immune response, and intracellular homeostasis [10,11]. New evidence indicates that PRDXs influence the occurrence of malignant tumors and have a correlation with the formation of drug resistance of tumors [12-14].

Primarily located in the cytoplasm of cells, Peroxiredoxin 2 (PRDX2) has a higher reactivity to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) than other members of the family, so it could rapidly lower the level of intracellular redox metabolites, thus reducing the degree of oxidative stress [15]. In the meantime, it affects the expression level of signaling pathway factors related to cell proliferation and apoptosis [16,17]. The importance of PRDX2 in tumor progression has begun to receive the attention of scholars. From clinical evidence, the role of PRDX2 as a cancer-promoting factor has

been reported [18,19]. However, its role in ESCC remains unclear. Therefore, the present study aims to study the expression of PRDX2 in ESCC tissues, and its clinical significance and role in the proliferation and apoptosis of ESCC cells.

## EXPERIMENTAL

### Tissue specimens

Tissue specimens were harvested from 120 ESCC patients treated from April 2016 to December 2018. At the same time, 20 samples of esophageal epithelium tissues were collected from non-tumor patients as controls. The cancer tissues and normal tissues were immediately placed into liquid nitrogen for RNA extraction using the TRIzol method. The profile of the patients, including age, gender, tumor size, tumor differentiation, and tumor node metastasis (TNM) stage are shown in Table 1. All patients had complete data and received no chemoradiotherapy before the operation. This study was approved by the Ethics Committee of The First People's Hospital of Fuyang District (approval no. CN-ZJ-IRB-2018-367896). All patients signed the informed consent document and all procedures followed the guidelines stated in the Declaration of Helsinki [20].

### Cell culture and transfection

Human ESCC cell lines ECA-109 were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and were routinely cultured in the Roswell Park Memorial Institute 1640 (RPMI 1640)

**Table 1:** PRDX2 expression and clinical features of patients with ESCC cancer

Variable	N	PRDX2 level		P-value
		Low	High	
<b>No. of samples</b>	120	56	64	
<b>Gender</b>				0.8527
<b>Male</b>	71	34	37	
<b>Female</b>	49	22	27	
<b>Age (years)</b>				0.1416
<b>&lt; 60</b>	53	29	24	
<b>≥ 60</b>	67	27	40	
<b>Differentiation</b>				0.0075
<b>Well and moderate</b>	44	28	16	
<b>Poor</b>	76	28	48	
<b>Tumor size (cm)</b>				0.0055
<b>&lt; 5</b>	50	31	19	
<b>≥ 5</b>	70	25	45	
<b>Lymph nodes metastasis</b>				0.5504
<b>Negative</b>	84	40	44	
<b>Positive</b>	36	16	20	
<b>TNM stage</b>				0.0089
<b>I-II</b>	75	42	33	
<b>III-IV</b>	45	14	31	

medium (HyClone, South Logan, UT, USA) containing 10 % fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) in an incubator with 5 % CO<sub>2</sub> at 37 °C. Cells in the logarithmic growth phase were used for the experiments. Transfection was performed when 60 % of cells were fused, si-PRDX2 and si-NC (negative control) were transfected in cell lines with the instruction of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). *In vitro*, experiments were performed 48 h after transfection.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues and cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into complementary deoxyribose nucleic acid (cDNA) according to the AMV reverse transcription kit instruction (2 µg total RNA added to 20 µL system), followed by real time-PCR in a PCR instrument using the 2xSYBR Green PCR Mastermix.

Then, an appropriate amount of cDNA was taken as the template, and the corresponding forward and reverse primers were designed and synthesized according to the target genes. This was followed by amplification with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, the data obtained were analyzed using relative quantification (RQ) shown in Eq 1.

$$RQ = 2^{-\Delta\Delta Ct} \dots\dots\dots (1)$$

### Cell proliferation

The ESCC cells were inoculated into a 96-well plate at a density of 3 × 10<sup>3</sup> cells/100 µL, and 8 replicate wells were set in each group. The cell proliferation was determined by MTT assay (Sigma-Aldrich, St. Louis, MO, USA). Absorbance at 450 nm (A-450) value of each group was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) for 3 consecutive days.

### Clone formation

The transfected ESCC cells in the logarithmic growth phase were digested with trypsin to prepare cell suspension and cell density was adjusted. Then, the cells were inoculated into a 6-well plate, with each group of cells in 3 wells (500 cells/well), and the 6-well plate was gently shaken to disperse the cells evenly. Subsequently, the cells were incubated for 10 days with 50 mg/L CO<sub>2</sub> at 37 °C, and the culture

solution was discarded after visible clones appeared. Thereafter, the cells were carefully washed with phosphate-buffered saline (PBS) twice, fixed by formaldehyde for 15 min, and stained with 0.4 % crystal violet for 10 min.

Next, the staining solution was slowly rinsed with running water, followed by air drying, and the number of clones (NC) of more than 50 cells was calculated under a microscope. Finally, the clone formation was calculated based on Eq 2.

$$CF = (NC/ICN)100 \dots\dots\dots (2)$$

where CF is the Clone formation rate, and ICN is the inoculated cell number

### Flow cytometry

#### Cell cycle

After 48 h of transfection, ECA-109 cells were determined according to cell cycle test kit instructions. In brief, ECA-109 cells were digested with Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin, centrifuged, and suspended, after incubation at 37 °C for 1 h, 5 µL Rnase was added at a final concentration of 10 mg/mL. Subsequently, the propidium iodide staining solution was added, and the cell cycle was determined using flow cytometry after staining for 30 min at room temperature.

#### Cell apoptosis

Cells after transfection were extracted, and suspended with 300 µL of binding buffer. The cell concentration was adjusted to 1 × 10<sup>6</sup> cells/mL. A volume of 100 µL of cell suspension was introduced into the flow tube. Subsequently, 5 µL of Annexin V-fluorescein isothiocyanate 1 (FITC) and 5 µL of propidium iodide (PI) were added to the mixture. The resulting solution was then incubated at room temperature for 15 min in a dark environment after thorough mixing. A volume of 400 µL of PBS was added to the reaction tube and subsequently analyzed using flow cytometry.

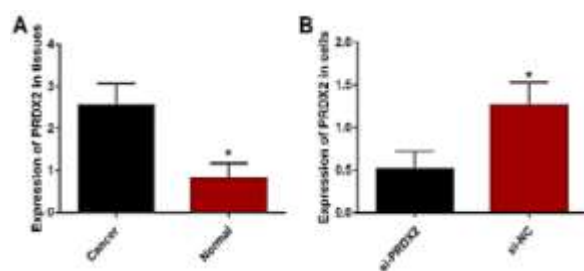
### Statistical analysis

Statistical Package for the Social Sciences (SPSS) 13.0 software (SPSS Inc, Chicago, IL, USA) was used for data analysis. Measurement data were analyzed *via* *t*-test or one-way analysis of variance (ANOVA). Chi-square test was performed for the association between PRDX2 and clinicopathological parameters of patients. The level of significance was set at *p* < 0.05.

## RESULTS

### PRDX2 was up-regulated in ESCC

Expression of PRDX2 in clinical tissues (120 cases of tumor tissues compared to 20 cases of non-tumor tissues) was determined. The results from PCR showed that the expression of PRDX2 in ESCC patients was significantly higher than that in non-tumor patients. The expression level of PRDX2 in 120 ESCC tissues was  $2.599 \pm 0.537$  which was statistically significant compared with that in 20 non-tumor tissues ( $0.645 \pm 0.420$ ) (Figure 1).



**Figure 1:** The expression of PRDX2 in cancer and normal tissues. (A) The expression of PRDX2 in ESCC is higher than that in normal tissues. (B) Transfection efficiency was determined using qRT-PCR. \* $P < 0.05$

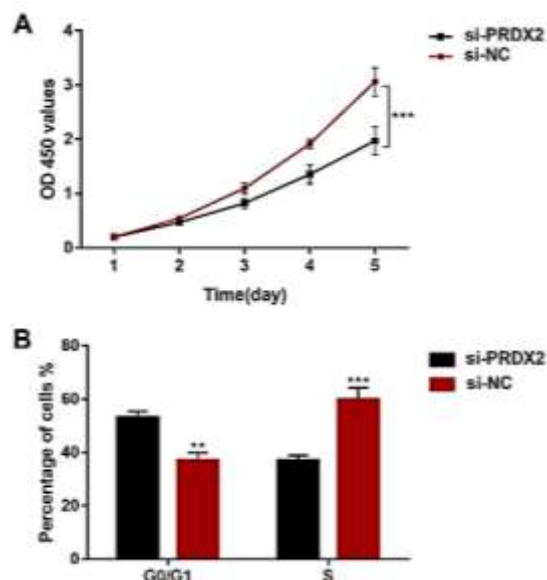
### Relationship of PRDX2 with clinicopathological features

The tissues were divided into high-expression group ( $n = 56$ ) and a low-expression group ( $n = 64$ ) based on the mean expression level of PRDX2 (2.599). Then, the association between PRDX2 expression and clinicopathological features of patients was further analyzed as shown in Table 1, significant correlation was found between the expression of PRDX2 with the tumor differentiation, tumor size, and clinical TNM stage ( $p < 0.05$ ).

### Downregulation of PRDX2 expression accelerated cell proliferation and invasion

The results revealed the regulatory effect of si-PRDX2 on the intracellular PRDX2. The proliferative ability of ECA-109 cells was significantly enhanced following transfection with si-PRDX2. MTT results depicted a more pronounced proliferation curve in si-NC cells (Figure 2 A). One potential explanation for this outcome was that si-PRDX2 might impede the transition of cells from the G0/G1 phase to the S phase, thereby preventing most cells from entering the division phase (Figure 2 B). Moreover, the colonies formed by ECA-109 cells that were transfected with si-PRDX2 were

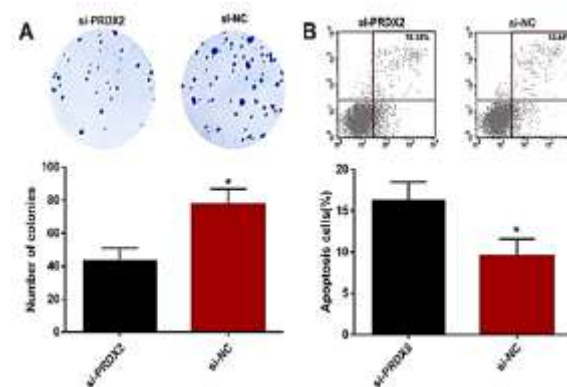
obviously less in number and smaller in size than those formed by cells in the other group (Figure 3 A).



**Figure 2:** Effects of PRDX2 on cell proliferation and invasion. (A) si-PRDX2 limited the proliferation ability of ESCC cells. (B) si-PRDX2 blocked cell transition from G0/G1 phase to S phase. \*\* $P < 0.01$  vs. si-NC, \*\*\* $p < 0.001$  vs. si-NC

### Inhibitory effects of PRDX2 on cell apoptosis

After transfecting ECA-109 cells with si-PRDX2, flow cytometry was employed to assess cellular apoptosis. As depicted in Figure 3B, the downregulation of PRDX2 in ECA-109 cells resulted in a significant increase in cell apoptosis. The apoptosis rates were  $11.41 \pm 2.05\%$  and  $20.29 \pm 2.73\%$  in si-NC and si-PRDX2 groups, respectively.



**Figure 3:** Effects of PRDX2 on cell apoptosis. (A) si-PRDX2 decreased the colony formation potential of ESCC cells (Magnification:  $\times 40$ ), (B) si-PRDX2 promoted the cell apoptosis of ESCC cells. \* $P < 0.05$  vs. si-NC

## DISCUSSION

Esophageal cancer (EC) is a significant global health issue. Despite considerable advancements in the medical field in recent decades, the outlook for improving survival rates and prognosis among EC patients remains challenging. Most patients are found to be in the advanced stage when diagnosed. Therefore, exploring effective biomarkers for EC was helpful for the early diagnosis and treatment of EC, which prolonged the survival time of patients. The stability of oxygen metabolism is very important for the maintenance of normal functions of cells.

Oxidative stress refers to the condition where various factors cause an abnormal increase in peroxides within the body, along with an abnormal decrease in the function and quantity of antioxidant substances. This condition results in oxidative damage to cells and tissues. Oxidative stress is a fundamental driving factor in the development and advancement of malignant tumors. In particular, reactive oxygen species (ROS) promote tumor progression in multiple regulatory processes. This function was first discovered by Loven *et al* [21] in 1982. They reported that insulin increases the level of hydrogen peroxide in tumor cells, accompanied by obvious cell proliferation. Therefore, the up-regulated ROS level resulting from internal and external factors facilitated the proliferation and survival of tumor cells through a series of complex signal interactions.

Peroxiredoxin 2 participates in the regulation of different signal pathways that influence the progression of tumors. For example, the overexpression of PRDX2 suppresses the production of H<sub>2</sub>O<sub>2</sub> that depends on platelet-derived growth factor (PDGF) and EGF pathways as well as NF- $\kappa$ B transcription activity induced by TNF- $\alpha$ . Moreover, PRDX2 was involved in the regulation of MAPK signaling pathway and selectively phosphorylates specific tyrosine sites on PDGF receptors by controlling the activity of membrane-associated protein tyrosine phosphatases (PTPs) [17].

In the past few years, several studies have shown that expression of PRDX2 was up-regulated in various human malignant tumors, suggesting that PRDX2 might be a tumor-promoting factor. Besides, according to some studies, PRDX2 exhibited a higher expression level in some borderline ovarian tumors than in some benign ovarian tumors so it could be used as a marker in the differential diagnosis of benign and borderline epithelial ovarian tumors [12]. In

breast cancer, PRDX2 acts like a "metabolic adapter" kinesin, by specifically inducing the growth of pulmonary metastases by antagonizing oxidant stress [19]. The bidirectional regulatory effect of PRDX2 in tumor occurrence and progression was also demonstrated in colorectal cancer (CRC) model.

Other studies have reported that overexpression of PRDX2 in CRC tissues had positive correlation with the invasive biological behavior, distant metastasis ability, and TNM stage of cancer cells, suggesting that PRDX2 plays a promoting role in CRC progression [18,22]. However, silencing of CRC cell line PRDX2 has also been reported to increase apoptosis and further facilitated endogenous ROS production, and resulted in changes in the expression level of the Wnt signaling pathway-related regulatory proteins [18].

The findings of this study revealed a significant increase in PRDX2 expression in ESCC tissues compared to normal tissues. Furthermore, the relationship between PRDX2 expression in ESCC tissues and various clinicopathological factors such as gender, age of onset, pathological differentiation, tumor size, distant metastasis, and clinical stage was investigated. Obviously, tumor pathological differentiation, size, and clinical stage significantly correlated with the expression of PRDX2, suggesting that PRDX2 could serve as a molecular biological index for the clinical stage of ESCC and the prediction of tumor pathological grade. Also, the changes in proliferation and apoptosis of ESCC cells were compared between the two groups *in vitro* (normal ESCC cells and ESCC cells with low expression of PRDX2). The results showed that the down-regulation of PRDX2 expression significantly decreased cell proliferation and increased cell apoptosis.

## CONCLUSION

Peroxiredoxin 2 is involved in regulating the occurrence and development of ESCC, and might be a potential biomarker and therapeutic target for ESCC. Although the data obtained show that there is a correlation between malignant tumor progression and the up-regulation of PRDX2, further studies would be required to fully elucidate the mechanism involved.

## DECLARATIONS

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**Ethical approval**

None provided.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Conflict of Interest**

No conflict of interest associated with this work.

**Contribution of Authors**

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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